In vivo roles of Fos-related antigen-2 (Fra-2) in diseased and normal tissue

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STATEMENT

The work presented in this thesis is the original work of the author unless stated otherwise in the text. Dr. Robyn Slattery and Ms Eloisa Pagler performed all the culturing work for the attempt to generate Fra-2 knockout mice and Dr. Donna Cohen and Ms Chenoa Barton performed the screening of the 600 clones by PCR. Ms Helen Taylor performed the microinjections to generate the transgenic mice over-expressing fra-2.

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ABSTRACT

The major components of the transcription factor AP-1 (Activator Protein 1) are encoded by the two families of genes related to the proto-oncogenes c-fos and c-jun. The fos-related antigen-2 (fra-2) gene is the most recently described member of the Fos family. This thesis describes a number of experimental approaches used to investigate the in vivo roles of Fra-2.

Firstly, the levels of fra-2 mRNA were analysed and compared in control and clinically determined inflammatory bowel disease (IBD) tissue sample extracts. The level of fra-2 mRNA expression was altered in 61% of the samples tested, raising the question of involvement of Fra-2 in IBD development. A mouse model of IBD was used to examine whether changes in fra-2 levels were a cause or an effect of IBD development. However, this model showed no significant changes in fra-2 levels throughout the progression of IBD. Thus, the relationship between Fra-2 and IBD could not be conclusively determined.

To identify other circumstances in which Fra-2 may function in vivo, a second, broader experimental approach was undertaken. An attempt to generate Fra-2 knockout mice and Swiss 3T3 fibroblasts lacking functional Fra-2 was made. For a number of reasons, these attempts were unsuccessful and were not pursued further.

A third approach was the generation of transgenic mice over-expressing fra-2. The major rationale for the generation of these mice was to determine the oncogenic potential of fra-2. The fra-2 transgenic mice showed over-expression of fra-2 in a number of tissues. However, no tumours were evident in any transgenic mice up to 2 years of age, although eye development was severely disrupted in these animals. Corneal abnormalities could be observed histologically as early as embryonic day 15.5 and eyelid fusion failed to occur. Adult eyes were
characterised by generalised anterior segment dysgenesis; some mice also had microphthalmia.

The abnormal eye phenotype was similar to that previously reported in both transgenic and knockout mice showing increased levels or lack of transforming growth factor α (TGFα), respectively. In vitro assays showed that expression of fra-2 increased following TGFα treatment of cultured 3T3 fibroblast cells, suggesting that AP-1 complexes containing Fra-2 contribute to TGFα signalling events. In addition, the effects of increased fra-2 levels on the transcription factors Mitf and Pax6, which are known to lead to similar eye abnormalities, were investigated. The results showed that Fra-2 does not regulate expression of Mitf, or vice versa, and that Mitf and Fra-2 do not directly interact with each other. However, the results did not rule out the possibility of an indirect interaction between Mitf and Fra-2. Fra-2 appears to negatively regulate the expression of Pax6.

Analysis of the fra-2 transgenic mice suggested two other regulatory mechanisms that may be important in controlling the amount of Fra-2 protein present in the cell. Northern blot analysis on the fra-2 transgenic mice showed that the presence of the transgene led to a reduction in the expression of the endogenous fra-2 gene in some circumstances. This suggested a negative feedback regulation by Fra-2 of its own promoter. The Northern blotting results also showed high levels of fra-2 mRNA in a number of tissues; however, Western blotting results did not show comparable high levels of Fra-2 protein in these tissues. This suggested some level of translational control on the expression of the Fra-2 protein. This translational control is not specific for the transgenic fra-2 RNA, but is also seen with the endogenous fra-2 mRNA. This is the first report of a mammalian AP-1 transcription factor family member being translationally controlled.

Finally, PCR-select differential screening protocols were used to identify potential target genes of Fra-2 in the eyes of the fra-2 transgenic mice and in Swiss 3T3
fibroblasts that had been transfected with fra-2. A number of potential target
genes of Fra-2 were identified, including γ-crystallin genes A, B, C and F,
cytochrome c oxidase subunit I, MHC heavy chain, cyclin D1, ST2/T1,
complement component C3. These results have highlighted areas where Fra-2
may be functionally important.

Taken together, the results of the various experimental approaches presented in
this thesis suggest that Fra-2 has a role to play, at least in some circumstances, in
switching the cellular signals from proliferation to differentiation.
PUBLICATIONS

Some of the work presented in this thesis has been published in Oncogene.

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Chapter 1

Introduction

Literature Review
1.1 Transcription

Transcription is the process by which an RNA molecule is made from a DNA template using an RNA polymerase enzyme. For eukaryotic transcription there are three types of transcription characterised by the use of three different RNA polymerases. Ribosomal RNA (rRNA) is transcribed by RNA polymerase I (reviewed in Moss and Stefanovsky, 1995; Roeder, 1996), messenger RNA (mRNA) is transcribed by RNA polymerase II (reviewed in Hampsey, 1998; Orphanides et al., 1996) and small RNA's such as transfer RNA (tRNA) are transcribed by RNA polymerase III (reviewed in Gabrielsen and Sentenac, 1991; Geiduschek et al., 1995). For the context of this thesis only transcription involving RNA polymerase II will be discussed.

Transcription can be divided into three phases, initiation, elongation and termination. Initiation involves the recruitment of the RNA pol II to the promoter by the formation of a pre-initiation complex (PIC) (reviewed in Hampsey, 1998; Orphanides et al., 1996). The elongation phase involves the movement of the core polymerase complex along the template DNA strand. This elongation complex is extremely stable and characterised by multiple and specific pausing events (reviewed in von Hippel, 1998). The termination phase occurs when the elongation transcription complex moves into one or more terminator sequences along the DNA. This involves the destabilisation of the transcriptional complex, usually with help from other regulatory factors, and the release of the nascent RNA strand (reviewed in von Hippel, 1998). This review will focus primarily on the events and molecules involved in transcriptional initiation.
1.2 RNA Polymerase II

RNA polymerase II (RNA pol II) is composed of 12 subunits (Woychik and Young, 1994) which are structurally conserved among eukaryotes (McKune et al., 1995). The largest RNA pol II subunit contains a carboxy-terminal repeat domain (CTD) which has the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This heptapeptide is highly conserved in eukaryotes but the number of tandem repeats varies between species (Hampsey, 1998). The CTD is thought to play a role in the conversion of RNA pol II from an initiation stage to an elongation stage, by alterations in its phosphorylation state. The CTD is extensively phosphorylated after the transition from initiation to elongation, the two forms of RNA pol II are called IIA and IIB for non-phosphorylated and hyper-phosphorylated respectively (Lu et al., 1991; O'Brien et al., 1994). There is also a form of RNA pol II lacking the CTD called IIB which is still able to initiate transcription in TATA-containing promoters (Akoulitchev et al., 1995; Buermeyer et al., 1995). Purified RNA pol II alone is not sufficient to initiate transcription (Roeder, 1976; Weil et al., 1979) but requires the addition of other protein factors (Matsui et al., 1980). RNA pol II transcription is a complex process that involves many components including the general transcription machinery along with other auxiliary factors which may be gene specific. The core promoter protein complex is in excess of $1 \times 10^6$ Da (Roeder, 1991).

1.3 Promoter Elements

Before transcription can occur the RNA pol II must recognise and bind to the promoter region of the gene it is about to transcribe. The most common promoter elements in eukaryotes are the TATA box (consensus TATAatAat), which is located 25-30 bases upstream of the transcriptional start site, and the pyrimidine rich initiator sequence (Inr) (consensus PyPyA_{+1}NT/APyPy) at the start site of transcription (reviewed in Smale, 1994). These two elements can occur together or
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separately in promoters. In TATA-less promoters, a downstream promoter element (DPE) approximately 30 bases downstream of the transcriptional start site appears to be bound by TFIID, in association with the Inr element (Burke and Kadonaga, 1996). These core promoter elements are recognised by components of the transcriptional machinery.

Constitutive gene expression has been shown to occur at Poly(dA-dT) elements which are bi-directional upstream elements that induce distinctive structures in the promoters. This is thought to impair nucleosome assembly or stability (Chen et al., 1987). There are also regulatory promoter elements which are gene-specific; these can be promoter-proximal elements, 50 to a few hundred bases upstream of the transcriptional start site, or promoter-distal elements located tens of thousands of bases away from the start site (Blackwood and Kadonaga, 1998; Mitchell and Tjian, 1989; Ptashne, 1988). These elements can be activators (enhancers) or repressors (silencers) (reviewed in Ogbourne and Antalis, 1998) of transcription and help in controlling the rate of transcriptional initiation. They are bound by specific transcription factors. A single gene contains numerous regulatory promoter elements. There are several common enhancer elements, including CCAAT box and GC boxes. The CCAAT box is located -80 to -100 bp upstream of the transcriptional start site and has the consensus sequence “GGCCCAATCA”. It can be bound by CCAAT-binding transcription factor (CBF), CCAAT-box-binding factor (CTF) and/or CCAAT/enhancer-binding protein (C/EBP). Multiple CCAAT boxes can occur in promoters of some genes, e.g. human topoisomerase IIα promoter (Maity and de Crombrugghe, 1998). The GC box element has the consensus “GGGCGG” and is located within the first 100 bp upstream of the start site. It is bound by the general transcription factor Sp1 (Gidoni et al., 1984).
1.4 General Transcription factors (GTFs)

The general transcription factors (GTFs) include transcription factor IIA (TFIIA), TFIIIB, TFIIID, TFIIIE, TFIIIF and TFIIH (reviewed in Hampsey, 1998; Orphanides et al., 1996). Many of these GTFs are multi-subunit complexes and thus the entire set of GTFs is composed of ~30 polypeptides. These GTFs bind promoter DNA in a specific order to assist in the recruitment of RNA pol II to the promoter, in a nucleoprotein complex termed the pre-initiation complex (PIC) (see figure 1.1 below) (reviewed in Hampsey, 1998; Orphanides et al., 1996).

The first step in the initiation of transcription, in TATA box-containing promoters, is the recognition of the TATA element by the TATA-binding protein (TBP). TBP is a component of TFIIID, which is a large multi-subunit complex composed of TBP and TBP-associated factors (TAFs) (Nakajima et al., 1988; Parker and Topol, 1984). The TBP is a universal transcription factor involved in transcription from all three eukaryotic RNA polymerases, RNA pol I, II and III (reviewed in Hernandez, 1993). There are multiple forms of TFIIID composed of different TAFs. These different forms are capable of responding to different transcriptional regulators (Brou et al., 1993; Brou et al., 1993; Jacq et al., 1994; Timmers et al., 1992; Timmers and Sharp, 1991). A core TFIIID complex is the basis of all TFIIID complexes; the addition of extra TAFs then produces the final TFIIID complex (reviewed in Chang and Jaehning, 1997). Some of the TFIIID TAFs have been shown to have sequence similarities to the histone proteins, H3, H4 and H2B (Hisatake et al., 1995; Hoffmann et al., 1996; Kokubo et al., 1994). They have also been shown to form a histone octamer-like structure (Hoffmann et al., 1996; Xie et al., 1996). The formation of this structure may assist in the stability of the PIC and may also allow protein-protein interactions between different TAFs (Xie et al., 1996).

The next GTF to bind the promoter is TFIIIB, which interacts directly with the TBP and is necessary before RNA pol II can bind (Buratowski et al., 1989). TFIIIB
is thought to help in stabilizing the PIC. Many gene-specific transcriptional regulators encourage the recruitment of TFIIB (Lin et al., 1991; Roberts et al., 1993). Following TFIIB binding RNA pol II can bind in association with TFIIF to the PIC.

TFIIF is comprised of two subunit, RAP30 and RAP74, which were identified by their interaction with RNA pol II (Sopta et al., 1985). They bind RNA pol II through RAP30. TFIIF prevents non-specific DNA binding of RNA pol II to non-promoter regions by delivering it to the TFIID-TFIIB complex (Killeen and Greenblatt, 1992). Both TFIIF subunits have been shown to bind promoter DNA (Coulombe et al., 1994; Robert et al., 1996) and it is thought that this promotes PIC stability (Conaway and Conaway, 1993; Greenblatt, 1991). TFIIF also functions in the elongation phase of transcription by suppressing the transient pausing of the RNA pol II (Bengal et al., 1991).

RNA pol II still requires the addition of two further transcription factors before transcriptional initiation can occur, namely TFIIE and TFIIH. TFIIE is a multi-subunit complex which interacts directly with the unphosphorylated form of RNA pol II (IIA) and both subunits of TFIIF. It assists in recruiting TFIIF and then stimulates the phosphorylation and ATP hydrolysis functions of TFIIF. One subunit of TFIIE contains a zinc ribbon motif (Peterson et al., 1991) which may allow it to bind single-stranded DNA together with TFIIB and help form or stabilize melted DNA at the promoter region (Orphanides et al., 1996).

The final GTF multi-subunit complex to bind and complete the PIC is TFIIH. This large complex has a number of enzymatic activities. It contains a DNA-dependent ATPase (Conaway and Conaway, 1989; Roy et al., 1994), ATP-dependent DNA helicase (Schaeffer et al., 1993) and CTD kinase (Lu et al., 1992). Transcriptional initiation by RNA pol II requires hydrolysis of an ATP β-γ phosphoanhydride bond (Bunick et al., 1982; Sawadogo and Roeder, 1984). The ATP-dependent DNA helicase, ERCC3, is the helicase requiring this energy. Initially TFIIH was
thought to be required for promoter clearance (Goodrich and Tjian, 1994; Guzder et al., 1994) but it now appears to be involved in promoter melting (Holstege et al., 1996). TFIIH not only plays a role in transcription but also in nucleotide excision repair (Drapkin et al., 1994). The CTD kinase was identified as cdk7 (Feaver et al., 1994; Roy et al., 1994; Serizawa et al., 1995; Shiekhattar et al., 1995), and this hyperphosphorylates the RNA pol II CTD. This is thought to occur at the progression to elongation phase of transcription.

Finally, TFIIA joins the PIC at any time after TFIID binding. It binds to the TBP (Buratowski et al., 1989), and assists in stabilizing the complex (Imbalzano et al., 1994). TFIIA is important in assisting the formation of an open promoter complex (Wang et al., 1992).

As well as binding to the promoter in a controlled order as described above, the GTFs may pre-assemble in an RNA pol II holoenzyme complex, which then binds to the gene promoter in one step (Koleske and Young, 1994).

1.5 General transcriptional regulators

The GTFs were identified as those factors required for "basal" transcription in vitro but in vivo transcription requires additional regulatory factors. These can be general or gene-specific regulators (reviewed in Hanna-Rose and Hansen, 1996).

Transcriptional regulators can function in various ways to control the level of transcription from a given promoter, including: (1) removal of repressor molecules from the promoter DNA including effects on chromatin structure;
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Figure 1.1: Schematic presentation of transcriptional pre-initiation complex

Transcription from class II genes is thought to require the coordinated binding of numerous general transcription factors into a pre-initiation complex. Transcription factors (TF) are labelled with their appropriate letter identification, the multisubunit complexes of TFIID and TFIIF are represented by boxes. The red oval represents the RNA polymerase II and the green strand represents the newly synthesising RNA strand. It is important to remember that DNA is usually folded into chromatin in cells, not free and linear, so therefore transcription also involves alterations in DNA conformation which are not depicted in this figure.
(2) recruitment of GTFs and RNA pol II to the promoter; (3) induction of conformational changes in the PIC; (4) induction of covalent modification of proteins in the PIC; and (5) stimulation of promoter clearance and elongation (reviewed in Orphanides et al., 1996). Many of the GTFs are direct targets of transcriptional regulators, including TFIIA, TFIIIB, TFIID, TFIIE (reviewed in Hampsey, 1998).

The general transcriptional regulators do not bind DNA in a sequence-specific manner, but can provide links between general transcriptional machinery and gene-specific regulators. They can also function by assisting in chromatin remodelling (reviewed in Hampsey, 1998). General regulators can be divided into activators and repressors of transcription. Common activators include TAFs, SRB/mediator, histone acetyltransferases (HAT) and other chromatin remodelling factors.

TAFs seem to regulate transcription by binding to gene-specific regulators but may only be essential for transcription in a subset of genes (Walker et al., 1997; Wang and Tjian, 1994). TAFs may also play other roles in regulation, for example human TAF$_{II}$250 has been shown to be a protein kinase that specifically phosphorylates TFIIF subunit RAP74 (Dikstein et al., 1996) and has a HAT function (Mizzen et al., 1996).

SRB/mediator is a multi-subunit complex which plays a general role in transcription by linking regulators to transcriptional machinery (reviewed in Hampsey, 1998). SRB/mediator associates with RNA pol II CTD (Nonet and Young, 1989) and has been shown to include a protein kinase (Liao et al., 1995) which assists in the phosphorylation of the CTD prior to transcriptional elongation.

HATs exist as complexes that possess histone recognition and acetylation properties (Grant et al., 1997). Chromatin can have repressive effects on
transcription and these HAT complexes may help overcome this by weakening histone-DNA interactions (Wolffe and Pruss, 1996). Other chromatin remodelling complexes have been identified, including SWI/SNF complexes, which promote nucleosome disruption or displacement in an ATP-dependent manner (reviewed in Hampsey, 1998).

Human upstream stimulatory activities (USA) were identified as general regulators of transcription, which act through interactions with the PIC. They can function as activators or repressors of transcription depending on the presence or absence of transcriptional activators respectively (Meisterernst et al., 1991).

General transcriptional repressors also act through interactions with general transcriptional machinery or through chromatin associations (reviewed in Hampsey, 1998). Many repressors act through effects on TBP binding. They can either inhibit TBP binding to DNA directly (MOT1, (Auble and Hahn, 1993) ) or indirectly (Not proteins, (Collart and Struhl, 1994) ) or they can block other factors from binding to TBP (Dr1-PRAP1/NC2, (Inostroza et al., 1992) ). Other transcriptional repressors function by effecting chromatin function, for example Spt4-Spt6 (Swanson and Winston, 1992) and histone deacetylase complexes (Kasten et al., 1997). There are also repressors that function by interacting with both the general transcriptional machinery and have effects on chromatin structure. For example Ssn6-Tup1 complex (Gavin and Simpson, 1997; Kuchin et al., 1995) and BURs (Prelich and Winston, 1993).
1.6 Sequence-specific Transcription factors

Many sequence-specific transcription factors are DNA-binding proteins which regulate gene expression. Eukaryotic transcription factors are generally arranged in a modular fashion with different functions in different domains and the DNA binding domain is only part of the complete protein. DNA-binding proteins are grouped into families according to common structural motifs (structural characteristics reviewed in Tan and Richmond, 1998). Some of these DNA-binding motifs include: helix-turn-helix motif (HTH), basic helix-loop-helix (bHLH), basic leucine zipper motif (bZIP), and zinc finger motif (reviewed in Pabo and Sauer, 1992).

There are few broad rules for site-specific DNA-recognition: (1) there are always contacts between the protein and DNA bases and backbone; (2) hydrogen bonds play important roles in recognition; (3) protein side chains make critical site-specific interactions; (4) the majority of base contacts occur in the major groove, usually with purines, particularly guanine; (5) interactions with the DNA backbone usually involve phosphodiester oxygens; and (6) multiple DNA-binding domains are usually found to be involved in site-specific recognition (Pabo and Sauer, 1992).

The most common structural motif used in DNA binding is the α-helical coiled coil, which contacts the DNA in the major groove (reviewed in Kohn et al., 1997). The overall shape and dimensions of the α-helix allow it to fit into the major groove, but surrounding regions of the protein help to determine the final position. The first DNA binding motif discovered was the HTH. It generally consists of a 20-residue sequence containing two α-helices connected by a glycine-hydrophobic turn of approximately 4-residues. The second helix is always the “recognition helix” which binds in the major groove and contributes to DNA base-specific
contacts. Examples of transcription factors containing the HTH motif are LexA, λ Cro and λ repressor proteins (Kohn et al., 1997; Pabo and Sauer, 1992).

There is two other DNA binding protein families related to HTH: the homeodomain and Ets domain. The homeodomain structural motif is used in many eukaryotic regulatory proteins and was initially found to be involved in *Drosophila* development. The homeodomain consists of approximately 60-residues including a HTH motif, arranged into 3 α-helices. The third α-helix corresponds to the second "recognition helix" in the HTH; however the α-helices tend to be longer in homeodomain proteins leading to different DNA interaction properties. Some homeodomain proteins have been shown to form protein dimers, e.g. Yeast α2 protein. Examples of homeodomain transcription factors are *Drosophila Engrailed* and *Antennapedia* proteins (Pabo and Sauer, 1992).

The Ets domain transcription factor family consists of a winged helix-turn-helix motif, with the turn comprising a 6-7 amino acid loop. The domain is characterised by three tryptophan residues separated by 17-21 amino acids. All known Ets-domain transcription factors bind to sites containing a central "GGA" sequence. The residues flanking these residues determine the specificity of individual family members (Sharrocks et al., 1997). Examples of Ets-domain transcription factors are Elk-1, Ets-1, Elf-1 and Spi-1.

The bHLH motif consists of two amphipathic α-helices connected by a loop of 5-24 amino acids. Proteins containing this motif commonly bind DNA as protein homo- or heterodimers. The basic region is involved in DNA binding and the HLH in dimerisation (Kohn et al., 1997; Pabo and Sauer, 1992). Examples of bHLH protein include MyoD and E47. There is a sub-class of bHLH proteins that also contain a leucine zipper domain adjacent to the bHLH, termed bHLH-ZIP proteins. The leucine zipper assists in dimerisation stability. It consists of a heptad repeat of leucines over 30-40 residues; the leucines are followed by hydrophobic
residues (especially valine and isoleucine) in the third position. The leucine zipper forms a coiled coil structure (Pabo and Sauer, 1992). Examples of bHLH-ZIP proteins are Max and USF (Kohn et al., 1997).

bZIP proteins also contain a leucine zipper and bind DNA as protein dimers. This motif consists of approximately 60-80 amino acids split into the two domains, a basic region that binds DNA and a leucine zipper that mediates dimerization. Examples of bZIP proteins include Fos, Jun, and GCN4. This class of transcription factors is discussed in more detail below.

There are also transcription factors that use structural motifs other than an α-helical structure to bind DNA. These include β-sheet motifs and zinc fingers. Proteins containing the β-sheet motif generally bind DNA as protein dimers through an anti-parallel β-sheet. An example is Escherichia coli (E. coli) IHF protein (Pabo and Sauer, 1992). The zinc finger motifs can be divided into several sub-families depending on their zinc-binding amino acid arrangements. This motif is extremely common in mammalian genes and occurs in nuclear and cytoplasmic proteins. Most nuclear proteins contain multiple zinc fingers while cytoplasmic proteins usually only contain one or two. This may reflect differences in binding specificity requirements. They have been shown to be involved in DNA binding and in protein-protein interactions (Mackay and Crossley, 1998). The classical motif contains ‘Cys-X_{2,4}-Cys-X_{12}-His-X_{3,5}-His’, (termed CCHH), which folds into a globular structure with zinc as the central core. The structure is formed from two anti-parallel β-sheet strands, containing the cysteine residues, connected by a turn and then followed by an α-helix containing the histidine residues (Kohn et al., 1997). Examples include TFIIIA and zif268. Other zinc finger motifs include CCCC (e.g., GATA-1 transcription factor), steroid receptors (e.g. glucocorticoid receptor), RING finger (e.g. GAL4) and LIM domain (e.g. LHX) (reviewed in Dawid et al., 1998). Unlike most zinc finger binding motifs, RING finger and LIM domains bind two zinc ions.
1.7 Regulation of transcription factors

In the majority of cells common sets of genes are constitutively expressed, they are often called “house-keeping” genes. These “house-keeping” genes provide the basic functions required by the cell for cellular metabolism. In contrast to the “house-keeping” genes there is a set of inducible genes whose expression is required in response to a specific stimulus or at a particular stage in the cell cycle, called “luxury genes”. The expression of “luxury genes” varies between cells. Each of these general gene categories consist of a vast range genes with different functional properties. Transcription factor genes represent only one sub-group of genes. There are both “house-keeping” and “luxury” transcription factor genes. However transcription factors can play an important role in regulating the expression of “luxury” genes. Thus the regulation of transcription factors themselves is very important. They can be controlled at a number of levels, from RNA synthesis to DNA binding (reviewed in Calkhoven and Geert, 1996).

Transcription factor function can be regulated by controlling either the amount of protein present in the nucleus or the activity of that protein. The amount of protein that is expressed can be regulated at several points, beginning with transcription of the gene encoding the transcription factor. This might involve control of other transcription factors that bind to the promoter of the transcription factor-encoding gene or in some cases by the transcription factor autoregulating its own promoter. For example, pituitary specific factor 1 (Pit-1) maintains expression from its own promoter after birth (Rhodes et al., 1993).

The rate of mRNA degradation is also an important level of regulation for some transcription factors and their transcripts often contain specific sequences that stimulate mRNA degradation. This is an effective form of control for transient protein expression after a specific cellular stimulus. For example the c-fos gene contains an AU-rich element (ARE) in its 3' untranslated region plus another
instability region in its coding region, making this mRNA extremely unstable (Veyrune et al., 1995).

Regulation of translation generally occurs at the initiation stage either through selection of the mRNA by the ribosomal complex and/or identification of the translation initiation codon. There are several initiation factors involved in these processes, of particular interest are eIF-4E and eIF-2 which can both be sequestered into inactive forms requiring phosphorylation for activation (Calkhoven and Geert, 1996). GCN4 translation is controlled by eIF-2 (Hinnebusch, 1994).

Finally, the amount of transcription factor available in the nucleus can be regulated by controlling its cellular localisation (reviewed in Vandromme et al., 1996). Proteins are made in the cytoplasm and need to be translocated back to the nucleus if they are to play a role in transcription. Proteins that are larger than 45 kDa require the presence of a nuclear localisation signal (NLS) for active transport into the nucleus. Phosphorylation plays a key role in this type of regulation (reviewed in Jans, 1995). A transcription factor may be retained in the cytoplasm by binding to an “anchor” protein forming an inactive complex. Phosphorylation of either component leads to accessibility of the NLS and translocation to the nucleus. A well characterised example of this is the transcription factor NF-κB p65 subunit bound by its inhibitor, IκB, thereby retaining it in the cytoplasm (Baeuerle and Baltimore, 1988; Baeuerle and Baltimore, 1988). The NLS can also be hidden by the conformation of a protein, as in the case of activating transcription factor-2 (ATF-2) and subsequent phosphorylation of the protein alters the conformation, revealing the NLS (Li and Green, 1996). Directly phosphorylating the NLS or residues close to it can inhibit nuclear transport of these proteins as is the case for Nuclear factor of activated T-cells (NF-AT) (Yeh et al., 1995). There are also proteins that contain cis-acting cytoplasmic-retention domains (CRD) which anchor the protein in the cytoplasm without effecting the
NLS, for example the Xenopus myogenic factor XMyoD (Vandromme et al., 1996).

Rates of nuclear transport can differ under changes in cellular conditions, for example during the cell cycle. This may be a result of changes to the nuclear import machinery rather than to the proteins themselves, by alterations in pore numbers or permeability (reviewed in Vandromme et al., 1996).

Transcription factors can also be controlled by regulating their activity, which can occur in several ways. One level of control involves alternative RNA splicing which can produce multiple mRNA forms. This can lead to different proteins with different transcriptional properties or affect the mRNA stability or translation efficiency (Calkhoven and Geert, 1996). For example, the CREM gene produces either an activating or a repressing isoform of the protein, through differential splicing of the mRNA.

Transcription factors can also be retained in inactive complexes, requiring ligand binding to induce a conformational change which allows the transcription factor to be activated, as is the case for many steroid receptors. For example, binding of ligand to the glucocorticoid receptor results in nuclear localisation (Picard et al., 1988).

Phosphorylation can also affect DNA binding and trans-activation functions of a transcription factor. For example, phosphorylation of cAMP responsive element (CRE) binding protein (CREB) leads to increased dimer formation; since dimer formation is required for DNA binding, this results in enhanced transcription (Yamamoto et al., 1988). The c-Jun transcription factor is phosphorylated in its N-terminal trans-activation domain following mitogenic stimulation and stress, resulting in increased trans-activation potential. c-Jun also contains phosphorylation sites in its DNA binding domain which inhibit DNA binding (Smeal et al., 1991).
DNA binding can also be blocked by interactions with other proteins. For example, NF-κB DNA binding is inhibited by interactions with glucocorticoid receptor (Scheinman et al., 1995). Other protein-protein interactions can assist in regulating transcription factors. Heterodimerisation can regulate transcription factors by competition for available partners, which often results in different regulatory outcomes. The small Maf proteins are examples of proteins that use this type of regulatory mechanism (Kataoka et al., 1995). Interactions between two different transcription factors can also change their individual transcriptional properties. For example, certain homeodomain proteins can interact with the serum response factor (SRF), changing its target sequence specificity (Grueneberg et al., 1995). Interactions with non-DNA binding factors can assist transcription factors in regulating transcription by linking or blocking access to the transcriptional machinery. Retinoblastoma protein (Rb) is recruited to promoters through E2F and blocks interactions of transcription factors (Weintraub et al., 1995).

Another level of control is through changes in redox (reduction/oxidation) potentials. This redox regulation of transcription factors occurs through highly conserved cysteine residues in the DNA binding domains of the proteins. A change in the redox potential can effect the activity of these transcription factors. For example, Activator protein-1 (AP-1) DNA binding is enhanced under reducing conditions and inhibited under oxidizing conditions (Sun and Oberley, 1996). There are also examples of a signal strength or duration of a stimulus affecting the activation of certain transcription factors. This is well documented in Drosophila development (St Johnston and Nüsslein-Volhard, 1992) but can also occur in other systems. Intracellular calcium ion concentration in B lymphocytes controls differential activation of pro-inflammatory transcriptional regulators like NF-κB and NF-AT (Dolmetsch et al., 1997).

Transcription factor activity is often strictly regulated and there may be multiple levels of control on the one factor. These control mechanisms can vary under different conditions. The NF-κB transcription factor is a good example of this. It
is regulated by detainment in the cytoplasm, heterodimerisation, inhibition of protein interaction, proteolytic cleavage of a precursor protein, phosphorylation and concentrations of a specific stimulus.

**1.8 Signal transduction**

Cells receive many extracellular signals, including growth factor stimulation, proliferation, differentiation, apoptosis, stress and developmental stimuli. The cell must translate these external signals into biological events within the cell, resulting in the transfer of information from the cell surface to the nucleus so that changes in gene expression can occur. This signalling cascade is referred to as the signal transduction pathway. It is extremely important for maintaining cellular control. Alterations in these pathways can lead to disease such as cancer (reviewed in Karin, 1992; Karin, 1994; Karin and Hunter, 1995). The general course of signal transduction pathways begins with a stimulus molecule, for example a growth factor, hormone or cytokine, binding to and activating its receptor at the cell surface. This leads to a change in the receptor, which initiates the signalling cascade within the cell.

There are 3 major types of pathway that a cell uses to transfer a signal from the membrane to the nucleus. The first of these involves a cascade of protein kinases. These signalling cascades generally rely on phosphorylation (or dephosphorylation) of tyrosine (reviewed in Neel and Tonks, 1997) or more commonly serine and threonine residues, to transfer information from one molecule to the next (Karin and Hunter, 1995). For example, the receptor may dimerise and be phosphorylated, as in the case of the epidermal growth factor receptor (EGFR). This receptor activation results in association with second messenger molecules inside the cell, like Grb2, which are themselves activated (often by phosphorylation) by the receptor (Batzer et al., 1994; Boulton et al., 1994). This type of association often occurs through specific protein kinase
domains like src-homology-2 (SH-2), src-homology-3 (SH-3) and pleckstrin homology (PH) domains (reviewed in Pawson, 1995).

The second messenger molecules transfer the signal along a sequential series of intermediate signalling molecules, using protein-protein interactions and phosphorylation as the main tools in this process. A common central player in eukaryotic signal transduction pathways is the GTPase family of proteins, such as Ras (reviewed in Malumbres and Pellicer, 1998; Vojtek and Der, 1998). Ras is active in its GTP-bound form and interacts with many downstream target proteins, thereby transferring the signal along the pathway. In many cases the signal is passed along a protein kinase cascade, for example the MAP kinase pathway (reviewed in Minden and Karin, 1997). Eventually, most signal transduction pathways reach the nucleus, at which point the signal is translocated from the cytoplasm into the nucleus. This may be through the translocation of an activated kinase, for example extracellular signal-regulated kinase 1 (ERK1) or ERK2 (reviewed in Minden and Karin, 1997), which then serves to phosphorylate and activate immediate-early transcription factors. This results in a reorganisation of gene expression necessary for the cellular changes to occur in response to the specific extracellular stimulus. Another mechanism by which the signal may be transferred into the nucleus is for the transcription factor to be activated in the cytoplasm (by direct phosphorylation or some other mechanism) and then translocated into the nucleus, ready to initiate the changes in gene expression required. An example of this is NF-κB (reviewed in Karin and Hunter, 1995).

The second major pathway involves a set of signal transduction pathways using the signal transducer and activators of transcription (STATs) transcription factors and Janus kinase (JAK) family of receptor-associated protein-tyrosine kinases (reviewed in Horvath and Darnell Jr, 1997; Karin and Hunter, 1995). This pathway is activated by a variety of cytokines and growth factors, e.g. interferon, and involves activation of the JAKs, following ligand receptor binding, which causes phosphorylation of the receptor and allows association with the STAT
proteins. The STAT proteins are phosphorylated on tyrosine residues, which enables them to dimerise. They then translocate to the nucleus where they bind to their cognate DNA enhancer element, thereby initiating transcription (Karin and Hunter, 1995).

The third type of pathway involves the nuclear receptor superfamily. Steroids, retinoids, thyroid hormones, vitamin D₃ and eicosanoids regulate transcription through nuclear receptors (reviewed in Calkhoven and Geert, 1996). They bind to specific intracellular receptors, which function as ligand activated transcription factors. The receptors contain a central DNA binding domain (DBD) which targets the receptor to specific DNA sequences known as hormone response elements (HRE). Most nuclear receptors function as dimers, hetero- or homodimers, and their HREs contain two copies of the degenerate core site, PuGGTCA (where Pu = purine). However, there are “orphan receptors” which have been shown to bind as monomers, e.g. NGFI-B (Wilson et al., 1993). These “orphan receptors” apparently lack ligand and have been suggested to function as constitutive factors or they may interact with novel ligands (Calkhoven and Geert, 1996).

Following binding to the HRE, the receptor responds to its specific hormone ligand through a conformational change in its ligand-binding domain (LBD). The LBD contains a highly conserved region necessary for transcriptional activation called the activation function-2 (AF-2). This region is thought to interact with co-activators to assist in the initiation of transcription (reviewed in Glass et al., 1997).

Signal transduction is not a simple process and is highly regulated. The changes that occur in a particular cell following an extracellular stimulus is dependent on the cell type, the cell-cycle status of the cell, the receptor activated and the downstream pathway used. The interactions of different signalling molecules can
lead to vastly different outcomes for the cell, ranging from cell death to cell proliferation or differentiation.

1.9 Transcription factor Activator protein-1 (AP-1)

AP-1 is a transcription factor complex which binds to the DNA consensus sequence “TGA$\gamma$/cTCA” (Lee et al., 1987). It was first identified as a DNA-binding activity in HeLa cell extracts that selectively bound to enhancer elements in the promoters of Simian virus 40 (SV40) and human metallothionein IIA genes (Lee et al., 1987). The AP-1 complex was found to be responsive to the phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and therefore the enhancer element was initially called TPA-responsive element (TRE) until it was noticed that other factors, like AP-2 (Imagawa et al., 1987) and serum response element (SRE) (Treisman, 1985), were also responsive to TPA. The enhancer element is now referred to as an AP-1 site. AP-1 is now known to be responsive to many other stimuli including Ultraviolet (UV) light, stress, serum, and growth factors (Angel and M., 1991).

The AP-1 transcription factor complex comprises a protein dimer of members of the Fos and Jun protein families (reviewed in Karin et al., 1997). The Fos family members include c-Fos (Curran and Teich, 1982), Fos-related antigen-1 (Fra-1) (Cohen and Curran, 1988), Fra-2 (Matsui et al., 1990; Nishina et al., 1990), FosB (Zerial et al., 1989) and its naturally occurring truncated version FosB2 (Nakabeppu and Nathans, 1991). The Jun family includes c-Jun (Maki et al., 1987), JunB (Ryder et al., 1988) and JunD (Hirai et al., 1989; Ryder et al., 1989). The Fos family of proteins can heterodimerise with any member of the Jun family (Cohen et al., 1989; Halazonetis et al., 1988; Rauscher III et al., 1988), while the Jun family members can also homo- and hetero-dimerise within their own family (Hirai et al., 1989; Nakabeppu et al., 1988). Jun-Jun homodimers have a lower DNA binding affinity than Jun-Fos heterodimers (Halazonetis et al., 1988;
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Rauscher III et al., 1988). The proteins interact through a leucine zipper binding motif (Landschulz et al., 1988). These interactions produce multiple AP-1 transcription factor complexes, with different DNA binding affinities (Ryseck and Bravo, 1991), which can regulate a vast number of genes.

The Fos and Jun families of proteins belong to the group of DNA binding proteins called bZIP proteins (reviewed in Kerppola and Curran, 1991; Lee, 1992). Many researchers have demonstrated that these proteins dimerise through their leucine zippers (Kouzarides and Ziff, 1988; Nicklin et al., 1993; Ryseck et al., 1990; Schuermann et al., 1989; Smeal et al., 1989; Turner and Tjian, 1989). Five leucine residues are spaced 7-residues apart and form an α-helical coiled coil structure such that the leucines all the face the same side of the helix with one leucine per two turns of the DNA (O'Shea et al., 1989; O'Shea et al., 1989). The leucine zippers from the two dimerizing proteins align in a parallel manner (O'Shea et al., 1989). This allows the basic regions of the proteins to bind the AP-1 site in either orientation (Glover and Harrison, 1995). Glover and Harrison determined the X-ray crystal structure of the Fos-Jun heterodimer bound to DNA describing it as a flexible fork gripping the DNA in the major groove. The coiled coil is asymmetric with the curved Jun chain wrapping around the straighter Fos chain (Glover and Harrison, 1995).

The other residues surrounding the leucines are important in stabilizing the dimer (Abate et al., 1990; Cohen and Curran, 1990). These other residues play important roles in dimerization through ionic interactions between the two proteins in the dimer. Fos proteins do not homodimerise, probably because of the electrostatic repulsions created by the net negative charge of the leucine zipper region. In contrast the Jun proteins leucine zipper regions have a slight positive charge and can form homodimers, although they are at least 1000-fold less stable than the Fos-Jun heterodimers (O'Shea et al., 1989; Smeal et al., 1989). The Jun homodimers are more dependent on hydrophobic interactions (Smeal et al., 1989).
The basic region, which immediately follows the leucine zipper, is involved in DNA binding and it also has an α-helical structure. The leucine zipper correctly aligns the protein dimer for DNA binding through the basic domain (Smeal et al., 1989). The basic region interacts with AP-1 enhancer element bases making conserved van der Waals and hydrogen bonds which results in high affinity binding of the AP-1 complex to the DNA (Glover and Harrison, 1995). There are several highly conserved residues in the basic region of bZIP proteins. Several basic residues, including a highly conserved arginine, surround a neutral core which includes an asparagine, Ala-Ala dipeptide and cysteine or serine residue. These residues align along the same face of the α-helix and bind the DNA (Kerppola and Curran, 1991).

AP-1 protein dimers have been shown to interact with three members of the general transcription machinery, TFIIE subunit 34, and both subunits of TFIIF, RAP30 and RAP74. These interactions occur through the basic region and basic-proximal region of the two proteins and are not dependent on the presence of DNA (Martin et al., 1996). High levels of AP-1 were shown to have an inhibitory effect on the rate of transcription probably because of a titration effect of these dimers on the general transcription factors, whereas low levels enhanced transcription (Martin et al., 1996). The c-Fos protein has also been shown to contain a TBP motif (TBM) which enables it to bind the TBP and activate transcription (Metz et al., 1994).

The AP-1 transcription factor complex is complicated further by the ability of individual AP-1 proteins to interact with other proteins. These combinations allow subsequent binding to more diverse AP-1-like sequences in promoter elements. A number of bZIP proteins have been shown to interact with members of the AP-1 family, e.g. the ATF family of transcription factors (also known as CREB), including ATF2, ATF3/LRF1, and B-ATF (De Cesare et al., 1995; Dorsey et al., 1995; Hai and Curran, 1991). ATF-Jun dimers bind CRE-like sequences which differ from AP-1 sites by an extra residue in the centre of the palindromic site. An
ATF-2-cJun heterodimer has been shown to regulate expression from the c-jun promoter (Van Dam et al., 1993). The Maf proteins, v-Maf and c-Maf, and the neural retina specific gene product Nrl also form heterodimers with Fos and Jun proteins. They bind to AP-1, CRE and asymmetric sites consisting of AP-1 and Maf half sites (Kerppola and Curran, 1994). The small Maf proteins, MafK, MafF, MafG and MafB form heterodimers with Fos but not Jun (Kataoka et al., 1994; Kataoka et al., 1995). Recently two new bZIP proteins that dimerise with Jun and bind to CRE and AP-1 sites were identified, namely Jun-dimerizing partner-1 and -2 (JDP1 and JDP2), which act as repressors of transcription (Aronheim et al., 1997).

Interactions of AP-1 components with other protein families have also been shown. For example, three members of the bHLH-ZIP protein family have been shown to interact specifically with members of the AP-1 family: Fos interacting protein (FIP) interacts with c-Fos (Blanar and Rutter, 1992); MyoD interacts with c-Jun (Bengal et al., 1992); and upstream stimulatory factor (USF) interacts with Fra-1 (Pognonec et al., 1997). The MyoD-c-Jun and USF-Fra-1 dimers have inhibitory effects while the FIP-Fos dimer has a stimulatory effect on transcription.

Several other direct interactions between transcription factor or co-activator proteins and members of the AP-1 complex have been identified. The glucocorticoid receptor specifically interacts with c-Fos forming a transcriptionally inactive complex (Kerppola et al., 1993; Lucibello et al., 1990). STAT3β, a short form of the STAT3 protein, interacts with the N-terminal domain of c-Jun (Schaefer et al., 1995). The CREB binding protein (CBP) can also directly interact with c-Fos and c-Jun to enhance transcription. CBP can bind TFIIB, so it may act as a bridging cofactor (Bannister and Kouzarides, 1995; Bannister et al., 1995). A final example is the interferon inducible protein p202, which has been shown to modulate the activity of c-Fos and c-Jun by binding to them. This inhibits their activity (Min et al., 1996).
The AP-1 dimeric protein complex can also interact with other proteins producing cooperative transcriptional regulators. AP-1 has been shown to be a component of the NF-AT complex (Castigli et al., 1993) and specific combinations, e.g. Fra-1/JunB in activated T cells, may play specific roles under certain conditions (Boise et al., 1993). The GATA proteins also directly associate with the AP-1 complex and have been shown to enhance transcription of some genes, e.g. Endothelin-1 gene (Kawana et al., 1995).

Finally AP-1, or components of AP-1, can functionally associate with other proteins to have an effect on transcriptional activity in the absence of direct physical interaction. A recent example of this is the promyelocytic leukemia (PML) protein that functionally associates with the C-terminal domain of c-Fos to enhance transcription of AP-1. However anti-PML does not immunoprecipitate either c-Fos or c-Jun in vitro, suggesting a lack of direct interaction between these proteins (Vallian et al., 1998).

The vast number of combinations of the AP-1 transcription factors and their interactions with other proteins allows for a complex array of regulatory proteins that control transcription of many different genes.

1.10 Jun Family

The Jun family of bZIP proteins, as mentioned earlier, includes c-Jun, JunB and JunD (reviewed in Rahmsdorf, 1996). c-Jun was the first member to be identified and is the cellular homologue of the viral oncogene v-Jun isolated from avian sarcoma virus 17 (Maki et al., 1987). The major difference between c-Jun and v-Jun is a 27 amino acid deletion in the N-terminal region of v-Jun (Vogt and Tjian, 1988). This region is called the delta (δ) domain and is involved in the transactivation function of c-Jun. It is regulated by phosphorylation (Smeal et al.,
1991). Deletion of the δ domain in v-Jun relieves the requirement for phosphorylation for transcriptional activation (Black et al., 1994).

The mouse Jun family members show significant homology at the amino acid level: 45% between c-Jun and JunB; 53% between c-Jun and JunD; and 45% between JunB and JunD (Hirai et al., 1989). The homology is particularly strong (75%) in the C-terminal region of the protein, which contains the DNA-binding and leucine zipper domains (Ryder et al., 1989). This suggests similar activities by this group of proteins. They can all dimerise with themselves and with members of the Fos family of proteins, as mentioned above. The C-terminal region of c-Jun also has significant homology to the DNA-binding domain of the yeast transcription factor GCN4 (Vogt et al., 1987).

The Jun proteins are induced by a variety of stimuli, including serum growth factors (Ryder et al., 1988), UV light, TPA and cAMP (Angel and M., 1991). The pattern of expression varies between the three proteins. For example, serum induction of the proteins results in an increase in c-jun (Lamph et al., 1988) and junB (Ryder et al., 1989) mRNA within 30 and 60 minutes respectively, but no significant induction in junD mRNA (Hirai et al., 1989).

The pattern of expression of the three jun mRNAs in adult and embryonic mouse tissue also varies. c-jun mRNA is relatively widely expressed in mouse embryos, with areas including skeletal muscle, central nervous system (CNS), developing limbs, lung, gut, kidney, skin and adrenal gland. Expression is detected at both 14.5 and 17.5 days postcoitus (pc) (Wilkinson et al., 1989). In the adult mouse c-jun is highly expressed in lung, ovary and heart, and very low to undetectable levels are detected in testis, spleen, liver and brain (Hirai et al., 1989). Expression of junB in the mouse fetus is observed at 17.5 days pc, and not at 14.5 days pc. The junB expression pattern is highly restricted with expression in developing skin, gut and oral cavity (Wilkinson et al., 1989). In adult tissue junB mRNA is
very highly expressed in testis and ovary and at significantly high levels in most other tissues (Hirai et al., 1989). In adult mice junD mRNA is expressed at high levels in the majority of tissues (Hirai et al., 1989).

c-Jun has been shown to efficiently transform chicken embryo fibroblasts (CEF). JunB can also cause transformation, but the results showed a milder effect with restricted growth in agar and lower plating efficiency. In contrast, JunD overexpression does not appear to exhibit any effects on cell growth (Castellazzi et al., 1991; Vandel et al., 1995). This reduced or lack of transformation by JunB and JunD respectively may be due to the inability of these proteins to accumulate high levels of homodimers. Mutational studies show all Jun protein homodimers can efficiently transform cells (Hartl and Vogt, 1992; Vandel et al., 1995). Transformation by the viral oncogene v-Jun also seems to occur predominantly through homodimer formation (Jurdic et al., 1995).

Studies to elucidate the functional roles c-Jun may play in a cell have involved investigations into the phenotypes resulting from the generation of transgenic and knockout mice. The c-Jun transgenic mice showed no phenotype (Grigoriadis et al., 1993), however v-Jun transgenic mice developed fibrosarcomas at wound sites (Schuh et al., 1990). Embryonic stem (ES) cells lacking functional c-Jun showed no growth differences in vitro compared to wild-type ES cells. They did show a decrease in the ability to form teratocarcinomas when injected into syngeneic mice indicating an important role for c-Jun in tumor formation in vivo (Hilberg and Wagner, 1992).

The c-Jun knockout mice studies showed the absolute requirement of c-Jun for mouse embryonic development. Embryos die at mid-late gestation (on average 11.5-13.5 days pc) (Hilberg et al., 1993; Johnson et al., 1993). Hilberg and colleagues found impaired hepatogenesis and generalized oedema especially in the brain of the Jun knockout embryos. In contrast Johnson et al. could see no developmental differences between wild-type and knockout embryos. They did
see an impaired growth rate of primary embryonic fibroblasts from 11.5 days pc knockout embryos. The heterozygous (+/-) fibroblasts also showed a decrease in growth, although not as much as the homozygous (-/-) knockout cells (Johnson et al., 1993).

To date, transgenic and knockout mouse studies involving JunB and JunD have not been reported.

1.11 Fos Family

The Fos family includes c-Fos, FosB, FosB2, Fra-1 and Fra-2. They are all members of the immediate-early class of genes, as they do not require de novo protein synthesis for their induction. c-Fos was first identified as the cellular homologue of the viral oncogene product v-Fos. v-Fos is carried by the Finkel-Biskis-Jinkins (FBJ) and the Finkel-Biskis-Reilly (FBR) murine osteosarcoma viruses (FBJ-MSV and FBR-MSV, respectively) (reviewed in Cohen and Curran, 1989). There are four regions deleted in the v-fos gene compared to the c-fos gene, three of these are intron sequences. The fourth deletion of 104 bp results in a frame shift, producing gene products that differ in the last 48/49 amino acids at the C-terminus (reviewed in Cohen and Curran, 1989).

The fos family of transcription factor genes all contain four exons and three introns, although the size of the introns and untranslated regions varies between the genes. The Fos family of proteins also show significant homology in their amino acid sequences, particularly in the basic and leucine zipper domains (Matsui et al., 1990; Suzuki et al., 1991). These proteins can all form heterodimers with the Jun family of proteins as discussed above.

The Fos family of transcription factors has been shown to transform cells, although their transformation abilities are variable. c-Fos efficiently transforms rat
208F fibroblast cells but as expected, v-Fos is a more potent transforming agent. FosB can also transform rat 208F fibroblasts with very high efficiency, at levels similar to v-Fos. The naturally truncated form of FosB, FosB2, has been shown to inhibit the transformation ability of these proteins (Yen et al., 1991). Fra-1 (Bergers et al., 1995) and Fra-2 (Foletta et al., 1994; Nishina et al., 1990) can both transform cells, but are weaker transforming agents than c-Fos.

Fos proteins are all responsive to serum stimulation and a variety of other stimuli (Angel and M., 1991; Cohen and Curran, 1988; Matsui et al., 1990; Nishina et al., 1990; Yen et al., 1991; Zerial et al., 1989). The pattern of expression differs among these proteins. In response to serum c-fos mRNA is induced rapidly with a peak within 15-30 minutes, which returns to basal levels after 1-2 hours (Angel and M., 1991). fosB and fosB2 mRNA levels peak within 60 minutes and return to basal after 2-3 hours (Yen et al., 1991; Zerial et al., 1989). The fra-1 and fra-2 genes are induced at much slower rates and mRNA levels remain high for longer periods of time in the cell. The induction of fra-1 mRNA occurs within 30-60 minutes and peaks at 90 minutes. After 4 hours the mRNA level still remains higher than basal fra-1 mRNA levels (Cohen and Curran, 1988). The induction of fra-2 mRNA by serum increased the levels within 20 minutes and peaked at 40 minutes. The levels remained high for approximately 5 hours and then slowly declined (Yoshida et al., 1991).

There are different expression patterns in adult and embryonic mouse tissues for each of the individual members of the fos gene family. Although there is overlap of expression in some tissues, which may result in functional redundancy, no member is expressed in all the same tissues as any other member. This suggests the possibility of some specific roles for each member.

c-Fos expression has been extensively studied by Northern analysis (Muller et al., 1982; Muller et al., 1983) and by employing a transgenic mouse strategy using a Fos-LacZ fusion construct (Schilling et al., 1991; Smeyne et al., 1992; Smeyne et
al., 1992; Smeyne et al., 1993). In the mouse embryo c-fos is expressed in growth regions of developing bone, including chondrocytes and osteoclasts, and in web-forming mesodermal cells (Dony and Gruss, 1987; Sandberg et al., 1988). High levels of c-fos mRNA have been detected in fetal mouse and human placenta and fetal liver (Muller et al., 1982; Muller et al., 1983). Newborn mice show c-fos expression in a broad range of tissues: high levels in bone and skin, moderate levels in intestine, kidney, spleen and liver; and low levels in brain, lung, testes and thymus (Muller et al., 1982). c-Fos is expressed in skin, the hair follicle, bone and several areas of the brain in the adult mouse (Smeyne et al., 1992). In adult humans c-fos expression levels had decreased in kidney, liver, lymph node and lung compared to fetal levels (Muller et al., 1983).

FosB is also expressed in developing bone but in different areas to c-Fos, for example in hypertrophied chondrogenic layer of long bones and bony and cartilaginous areas of epiphyseal growth plate. Embryonic FosB expression is also seen in mouse placenta, visceral yolk sac, epithelial layers of skin, whisker follicle, liver stroma, cells lining the arteries and bronchi of the lung, the CNS and peripheral ganglia. Interestingly, FosB was shown to be expressed in the skin covering the head and limbs but not the torso (Gruda et al., 1996; Redemann-Fibi et al., 1991). Later in gestation the expression is more restricted to outer differentiated regions of the brain, spinal cord and sensory organs (Redemann-Fibi et al., 1991). In adult tissues no fosB was detected by Northern analysis (Zerial et al., 1989).

Fra-1 expression appears to be very low and lack any tissue specificity in the mouse embryo (Carrasco and Bravo, 1995). In contrast Fra-2 is expressed at relatively high levels in a wide range of tissues during late organogenesis of the embryonic mouse. Fra-2 expression was detected in a number of epithelia, bone, CNS, skin, hair follicle, adrenal gland and kidney (Carrasco and Bravo, 1995). Northern analysis of adult mouse tissues identified fra-2 mRNA expression in a wide range of tissues (Foletta et al., 1994) (see below for more detail).
Transgenic and knockout mice have been used to study the functional roles of some of these *fos* family genes. Transgenic mice over-expressing c-Fos using metallothionein and H-2k\(^p\) class I major histocompatibility complex (MHC) promoters have been extensively analysed. In both cases, the *c-fos* 3' destabilizing sequences were replaced with a retroviral long terminal repeat (LTR). The development of osteosarcomas was the predominant phenotype, however the latency period varied from 2-10 months (Grigoriadis et al., 1993; Rüther et al., 1987; Rüther et al., 1989). Transgenic mice were also produced that did not develop osteosarcomas, but this was most likely due to low *c-fos* expression in the bones of these mice. These mice did show alterations in the thymus and spleen and an impaired immune system (Rüther et al., 1988). Double c-Fos/v-Jun transgenic mice showed a delay in early B cell development and impaired proliferation when stimulated with Interleukin-7 (IL-7) (Fujita et al., 1993). Concurrent over-expression of c-Fos and c-Jun enhanced the rate of osteosarcoma development (Van Dam et al., 1993).

ES and fibroblast cells lacking functional c-Fos expression showed no alterations in growth characteristics compared to wild-type cells (Brüsselbach et al., 1995; Field et al., 1992). Primary embryonic fibroblasts from knockout c-Fos mice also grow normally but did show reduced induction of some AP-1-dependent genes (Hu et al., 1994). Knockout c-Fos mice appear normal at birth but at approximately day 11 they begin to show signs of a mutant phenotype. They do not gain weight at the same rate, and always remain considerably smaller, than littermate controls. They develop osteopetrosis and lack tooth eruption due to a failure in bone resorption. They also have delayed sexual maturation, altered haematopoiesis and some behavioural alterations including hyperactivity (Johnson et al., 1992; Wang et al., 1992). T-cell development and function are unaffected in c-Fos knockout mice (Jain et al., 1994). The effects on haematopoiesis is probably due to the impaired bone marrow environment caused by osteopetrosis (Okada et al., 1994), since the osteopetrosis can be rescued by bone marrow transplantation (Grigoriadis et al., 1994). The results seen for the knockout mice correlate with
the expression pattern of c-Fos in embryonic and adult mice tissue, indicating an important role for c-Fos in bone development.

FosB transgenic mice showed no phenotype (Grigoriadis et al., 1993). In contrast, FosB2 transgenic mice whose expression was targeted to the thymus by the T-cell receptor β (TCR-β) promoter and CD2 enhancer showed the slow development of a phenotype over the first 3-7 months. The thymus became enlarged with an expanding medulla and reduced cortex. No tumors developed but mice were smaller and had decreased fertility (Carrozza et al., 1997). Lack of functional FosB protein had no affect on ES cells or mouse embryonic fibroblasts. The knockout mice appeared normal although an increase in seizures in older mice was reported (Gruda et al., 1996). Another group showed a defect in the nurturing behavior of FosB knockout mice (Brown et al., 1996).

Fra-1 knockout mice are not viable (Lallemand et al., 1997). To date, no studies with knockout mice have been reported for Fra-2. However, transgenic mice expressing an anti-sense Fra-2 construct have been generated and these display a decrease in fertility with age. They also had reduced splenocyte proliferation in response to α-CD3 stimulation (Foletta, 1995).

1.12 The regulation and roles of AP-1

The AP-1 transcription factor family plays many important signalling roles, hence their expression is highly regulated. The regulation occurs at many levels starting with strict transcriptional control. Many of the Fos and Jun family members contain regulatory AP-1 sites in the promoters of their own genes, suggesting they are themselves transcriptionally controlled by AP-1 complexes. These complexes may include their own proteins or other members of the protein family. For example, c-jun expression is regulated by c-Jun/ATF2 complex, although this complex does require post-translational modification to stimulate transcription
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(Van Dam et al., 1995). The c-Fos protein can down-regulate expression of its own promoter but also requires some post-translational modification (Ofir et al., 1990). Transcription from the fra-2 gene is maintained at basal levels by a Fra-2/c-Jun complex and stimulated by a c-Fos/c-Jun complex (Sonobe et al., 1995). Transcription of fosB is similarly regulated, with FosB and c-Fos acting as inhibitors of fosB transcription (Lazo et al., 1992). In contrast, fra-1 expression is strongly stimulated by c-Fos and FosB but is down-regulated by a Fra-1/c-Jun complex (Bergers et al., 1995).

Transcription of AP-1 members can also be controlled by the binding of other transcription factors to regulatory elements in their promoters. The c-jun promoter has been shown to contain binding sites for transcription factors Sp1, CTF, and MEF2 (Han et al., 1992). The MEF2 site mediates serum induction of the c-jun promoter (Han and Prywes, 1995). The c-jun gene also contains an estrogen response element-like motif within its coding region (Hyder et al., 1995). The junB promoter has been shown to contain an Ets domain, CRE-like site (Amato et al., 1996), IL-6 response element and a STAT binding site (Coffer et al., 1995).

The c-fos gene promoter contains several transcription factor response elements including the SRE, CRE, and the Sis-inducible enhancer (SIE). The SRE is bound by the serum response factor (SRF) in association with a ternary complex factor (TCF) (Treisman, 1992). A group of TCF proteins have been identified including Elk-1 and SAP-1 proteins (Dalton et al., 1993; Hipskind et al., 1991; Shaw et al., 1989). These factors are constitutively associated with the c-fos promoter and are activated upon phosphorylation of the TCF by mitogen-activated protein kinases (MAPK) (Chen et al., 1996; Davis, 1994). The CBP has been shown to mediate the transactivation of at least one of the phosphorylated TCF, Sap-1a (Janknecht and Nordheim, 1996). Other proteins have been shown to bind to the SRE in the c-fos promoter, for example YY1 (Natesan and Gilman, 1995), NF-IL-6, E12 (Metz and Ziff, 1991; Metz and Ziff, 1991), Phox1 (Grueneberg et al., 1992), SRE-ZBP (Attar and Gilman, 1992), and growth hormone (Meyer et al., 1993).
These have variable effects on the expression \textit{c-fos} and often function through affects on the SRF. For example, the YY1 structural transcription factor enhances binding of the SRF to the SRE by inducing bending of the DNA (Natesan and Gilman, 1995). The CRE site is bound by CREB, which mediates \textit{c-fos} induction via cAMP and Ca\textsuperscript{2+}-dependent signalling pathways (Sheng et al., 1991). The SIE element is recognised by the STAT proteins and can induce transcription in response to platelet-derived growth factor (PDGF) (Leaman et al., 1996).

Expression of the \textit{fra-1} gene has been shown to be regulated by multiple Tax1-responsive regions (TRR) which are bound by the Tax1 transcription factor from the human T-cell leukemia virus type 1 (Tsuchiya et al., 1993). The testis determining factor SRY has also been shown to bind and enhance transcription of \textit{fra-1} through an HMG-box response element (Cohen et al., 1994).

The \textit{fra-2} promoter contains many regulatory elements including SRE-like element, AP-1 sites, HMG box response element (HBRE), ATF/CREB element, sis-conditioned medium (SCM)-like element and multiple GC boxes (Foletta et al., 1994; Yoshida et al., 1993).

Post-transcriptional regulation of various AP-1 family members has been shown to occur through mRNA instability and alternative splicing. The \textit{c-fos} mRNA contains ARE in its 3' untranslated region and a mRNA instability element within its coding region (Chen et al., 1995; Veyrune et al., 1995). The ARE elements mediate mRNA decay by shortening the poly(A) tail. There are conflicting reports as to whether \textit{c-fos} mRNA degradation is coupled to translation. Veyrune \textit{et al.} showed that protein synthesis inhibition decreases the rate of \textit{c-fos} mRNA turnover. In contrast, Chen and colleagues show no requirement for translation for the \textit{c-fos} mRNA instability.

There are two alternatively spliced forms generated from the \textit{fosB} mRNA, \textit{fosB} and \textit{fosB2}. FosB2 lacks the C-terminal 101 amino acids containing the
transactivational domain found in FosB. FosB2 can form heterodimers with Jun proteins and inhibits transcription of genes containing AP-1 sites (Nakabeppu and Nathans, 1991; Yen et al., 1991). The junD mRNA produces two forms of JunD proteins by using alternative initiation codons 144 bp apart. Both forms are found in all mouse tissues tested but the ratio does vary, suggesting some regulatory control on their expression (Okazati et al., 1998).

Post-translational control has been shown for all the members of the AP-1 transcription complex. c-Fos has been shown to be controlled by regulating its nuclear localisation. c-Fos is retained in the cytoplasm by a factor which releases c-Fos upon cellular stimulation, allowing it to be translocated to the nucleus (Roux et al., 1990). Redox regulation of AP-1 DNA binding ability has been shown to occur through a highly conserved cysteine residue in the DNA binding domain of these proteins. Reduction induces DNA binding, while oxidation inhibits DNA binding. In vivo redox regulation has been shown to occur at both the transcriptional and translational levels (reviewed in Sun and Oberley, 1996).

Phosphorylation has been shown to be an important form of post-translational regulation for all members of the AP-1 transcription factor complex. All Fos and Jun proteins are phosphorylated and this can have enhancing or repressing affects on the activity of the proteins. The Jun family of proteins are all phosphorylated at C-terminal sites which inhibits their DNA binding activities (D'Arcangelo et al., 1995; Papavassiliou et al., 1995). The N-terminal domain of c-Jun is also phosphorylated on two serine residues (Binetruy et al., 1991; Smeal et al., 1991). This enhances the transactivation ability of c-Jun and is thought to stimulate the dephosphorylation of the C-terminus (Papavassiliou et al., 1995). The phosphorylation of the C-terminus of c-Jun involves the activity of ERK and casein kinase II (CKII) (Lin et al., 1992). The N-terminal phosphorylation involves the MAP kinase subgroup, c-Jun N-terminal kinases (JNK) (Derijard et al., 1994; Hibi et al., 1993).
The Fos family members are also all phosphorylated on their C-terminus. For c-Fos this increases its stability and enhances its transactivational activity (Chen et al., 1996). Two kinases have been shown to be involved in this phosphorylation, MAP kinase and 90 kDa-ribosomal S6 kinase (RSK) (Chen et al., 1993). Phosphorylation of the FosB protein is required for the transactivation and transformation ability of this protein. It does not seem to involve activity of a MAP kinase or JNK but may involve some other kinase activity (Skinner et al., 1997).

Phosphorylation of Fra-1 and Fra-2 at the C-terminus occurs outside the DNA binding domain but enhances DNA binding activity through conformational changes in the proteins. This involves the activity of a MAP kinase (Gruda et al., 1994). Fra-2 is extensively phosphorylated following stimulation (Yoshida et al., 1991) and this appears to involve the activity of the ERK2 MAP kinase (Murakami et al., 1997).

The rate of protein degradation is another form of regulation that controls the activity of AP-1 complexes. The c-Fos protein has a half-life of 10 minutes. It has been speculated that a PEST sequence in the C-terminal region of c-Fos targets the protein for degradation (Tsurumi et al., 1995). A ubiquitin-proteasome proteolytic pathway is thought to degrade c-Fos protein. The viral oncogene v-Fos is highly stable because it lacks the C-terminal degradation signal (Tsurumi et al., 1995). c-Jun appears to accelerate the degradation of c-Fos (Stancovski et al., 1995; Tsurumi et al., 1995). c-Jun is also degraded by the ubiquitin-proteasome proteolytic system and c-Fos is not required for assistance (Salvat et al., 1998).

Cytoplasmic degradation of c-Fos and c-Jun has also been shown to involve the cysteine proteases called calpains. All other Fos family members, except Fra-1, have also been shown to be sensitive to calpains (Carillo et al., 1994) suggesting the possibility of cytoplasmic degradation of these proteins by a similar mechanism.
Multiple protein-protein interactions have been shown to affect the function of AP-1 transcription factors. These interactions have been shown to involve general transcription factor components, other transcription factors and accessory proteins. Examples have been discussed in detail above.

AP-1 transcription factor complexes are responsive to numerous mitogenic stimuli and are known to have proliferative and differentiating effects on many different cells. This multi-family of transcription complexes plays both positive and negative regulatory roles. In many cases the composition of the AP-1 complex can be an important factor in determining the response initiated. It has been demonstrated that transcription factor AP-1 complex plays a role in a number of diverse cellular events such as the regulation of cytokine genes like IL-2 (Gonsky et al., 1998), some forms of apoptosis (Colotta et al., 1992; Preston et al., 1996; Smeyne et al., 1993), spermatogenesis (Cohen et al., 1993), transformation (Murakami et al., 1997; Okimoto et al., 1996; Suzuki et al., 1994) and keratinocyte differentiation (Gandarillas and Watt, 1995; Rutberg et al., 1996; Welter and Eckert, 1995).

1.13 A closer look at Fra-2

This literature review has presented an overview of transcription and general background on transcription factor AP-1. As this thesis describes investigations into the role Fra-2 may play in normal and diseased tissue, a detailed review of the information currently available on Fra-2 and its biological functions is given below.

Fra-2 is one of the most recent AP-1 transcription factor family proteins to be identified. The fra-2 gene was initially cloned from chicken (Nishina et al., 1990) and human (Matsui et al., 1990) by two independent groups at approximately the same time. The chicken fra-2 gene was recognised as a 46-kDa protein that was
immunoprecipitated by an anti-Fos peptide in serum stimulated CEF. The gene was cloned by initially using a mixed oligonucleotide probe, based on the amino acid sequence of the Fos peptide, to screen a Southern blot. A DNA fragment containing part of the fra-2 gene was then cloned into λ Charon 27 and used to screen a chicken genomic library (Nishina et al., 1990). The human fra-2 cDNA was isolated by screening human cDNA libraries at low stringency with c-fos DNA probes corresponding to the leucine zipper or C-terminal domains (Matsui et al., 1990). The cytogenetic location of the human fra-2 gene has since been assigned to chromosome 2 region p22-p23 (Molven et al., 1996). The mouse fra-2 gene has been cloned and characterised (Foletta et al., 1994) and the rat cDNA sequence has been reported (Baler and Klein, 1995).

The fra-2 gene contains four exons and three introns. The position of the three introns is highly conserved between species and within the fos gene family (Foletta et al., 1994). The introns and untranslated regions of the fra-2 mRNA are very large as compared to the other fos gene family members (Foletta et al., 1994; Nishina et al., 1990). The Fra-2 protein is highly conserved between species: 94% overall homology between human and mouse; 87.5% between mouse and chicken (Foletta et al., 1994); 98% between rat and mouse; 97% between rat and human; and 93% between rat and chicken (Baler and Klein, 1995).

The mouse fra-2 promoter has been shown to contain two transcriptional start sites 55 bp apart. The second start site is used predominantly but there appears to be some tissue specific expression from the upstream start site (Foletta et al., 1994). The chicken fra-2 gene contains a TATA-like sequence, GATAAAA (Yoshida et al., 1993), but the mouse gene lacks such a sequence at an equivalent location. The mouse fra-2 gene does contain a TATA-like sequence, AATAAAA, 7 bp upstream from the major transcriptional start site (site 2). However it is unknown whether this functions as a TATA box (Foletta et al., 1994). The mouse and chicken fra-2 gene promoters both contain multiple GC boxes, SCM element, SRE element, CRE element, HBRE elements and AP-1 sites (Foletta et al., 1994;
Yoshida et al., 1993). The HBRE element has been shown to be bound by the SRY protein and enhance transcription of fra-2 (Foletta, 1995). The chicken promoter contains two consensus AP-1 sites separated by 40 nucleotides (nt) but the mouse promoter contains only one consensus AP-1 site and another AP-1-like site 40 nt away (Foletta et al., 1994). This AP-1-like site seems to contain half a consensus AP-1 site and half a divergent sequence. Both fra-2 promoters have been shown to contain repeat element but the type of repeat element varies: the chicken promoter contains a 12 bp repeat (Yoshida et al., 1993); and the mouse promoter contains an AC repeat and several T clusters (Foletta et al., 1994).

Transcription from the human (Matsui et al., 1990), rat (Baler and Klein, 1995) and chicken (Yoshida et al., 1991) fra-2 genes have been shown to produce multiple mRNA transcripts. These different transcript were thought to be a result of alternative start sites of transcription, although this is not the case for the chicken fra-2 gene. In the chicken, the two mRNA transcripts have been shown to be due to extra polyadenylation signals in the 3’ untranslated region (Yoshida et al., 1993).

Fra-2 has been shown to be induced by a number of stimulatory agents including serum, the phorbol ester TPA, cAMP and calcium ionophore (Foletta, 1995; Matsui et al., 1990; Rezzonico et al., 1995; Sonobe et al., 1995). Stimulation by cAMP, probably through the CRE element, has been shown to correlate with inhibition of cell growth in interleukin-6-dependent cell lines (Rezzonico et al., 1995). The response to serum is mediated through the CRE element and AP-1 sites in the fra-2 promoter and not entirely by the SRE element (Foletta, 1995; Sonobe et al., 1995). Transient transfection experiments have shown that c-Fos/c-Jun heterodimers stimulate, while Fra-2/c-Jun complex repress, fra-2 transcription (Sonobe et al., 1995).

The kinetics of induction of the fra-2 mRNA can differ with alterations in stimuli or cell type. Serum induction of two fra-2 mRNA transcripts in CEF occurs within
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20 minutes and peaks after 40 minutes with a high level maintained for at least 280 minutes (Sonobe et al., 1995; Yoshida et al., 1991). In mouse BALB/c 3T3 cells maximum fra-2 mRNA expression occurs in 120 minutes and then rapidly declines (Foletta, 1995). Human U937 monocyctic leukemia cells stimulated with TPA induce the expression of three fra-2 mRNA transcripts within 120 minutes, with a peak at 240 minutes; expression is maintain until at least 360 minutes (Matsui et al., 1990). CEF stimulated with TPA showed peak fra-2 mRNA levels at 120 minutes (Yoshida et al., 1993). cAMP and calcium ionophore stimulation of CEF resulted in fra-2 mRNA expression within 60 minutes, returning to basal levels 8 hours later (Yoshida et al., 1993).

High expression of fra-2 mRNA has been detected in mouse placenta from E7.5 to E18.5 days. In the early mouse embryo fra-2 is only detected in maternally derived tissue. During late organogenesis (E13.5 to E18.5 days) fra-2 is detected in a wide range of tissues. Fra-2 expression is detected at high levels in a number of specialized epithelia including oral epithelia, tongue, forestomach, small intestine, skin, kidney and bladder. In the skin fra-2 is detected in association with the hair follicle and inner and outer root sheath. This staining is seen in embryonic and newborn mice. Both in situ hybridisation and immunohistochemistry identified developing bone as an area of Fra-2 expression. Highest expression is detected in the teeth, clavicle, ribs and nasal cavity. Both the cartilaginous and bony sides of the growth plate and chondrocytes are sites of expression. The central and peripheral neural systems show fra-2 expression. In the adrenal medulla the developing chromaffin tissue shows the highest expression, probably in the sympathetic neuroblasts. Expression of fra-2 mRNA in the kidney begins at approximately E12.5 days and is maintained through to adulthood. Fra-2 is detected in renal pelvis, ureteral buds, bifurcating ureters, C-shape tubules and proliferating and elongating tubules (Carrasco and Bravo, 1995).

Northern analysis of adult mouse tissues identified very high levels of fra-2 mRNA expression in ovary and high levels in stomach, small and large intestine.
Lower levels were observed in brain, heart, and lung and very low or undetectable levels were seen in thymus, liver, kidney, testis, spleen and pancreas. Reverse transcriptase polymerase chain reaction (RT-PCR) or primer extension studies did show fra-2 expression in spleen, kidney, testis and liver. (Foletta et al., 1994).

More recently Carrasco and Bravo used semi-quantitative PCR analysis to show a similar pattern of expression although there were some differences. They showed high levels of expression not only in those mentioned above but also in heart, lung and kidney. They detected moderate expression levels for liver, thymus and testis (Carrasco and Bravo, 1995). The differences are probably a reflection of the different techniques used in analysis.

Immunohistochemistry and in situ hybridisation analysis of adult tissues showed fra-2 mRNA and Fra-2 protein expression in similar regions as expected. Discrete cell populations expressed Fra-2: granulosa cells in the ovary; chief cells in the stomach; goblet cells in the intestines; and columnar epithelium of ducts of Bellini in the kidney (Foletta, 1995). Areas of the brain also show Fra-2 expression, with high levels detected in occipital cortex, pyriform cortex, caudate nucleus, subiculum, dentate gyrus and amygdala (Carrasco and Bravo, 1995; Foletta, 1995). Fra-2 is also detected in the basal and spinous layers of the lower epidermal layers of human skin. There may be a role for Fra-2 in keratinocyte differentiation (Welter and Eckert, 1995).

Fra-2 is a phosphoprotein. It is phosphorylated in response to various cellular stimuli. This alters the mobility of the Fra-2 protein from 40 kDa to 46 kDa on SDS-polyacrylamide gel electrophoresis (PAGE) gels (Yoshida et al., 1991). The phosphorylation of Fra-2 occurs at its C-terminus on serine and threonine residues. The MAP kinase ERK2 has been shown to be the major kinase responsible for this phosphorylation (Murakami et al., 1997). Both unphosphorylated and hyperphosphorylated Fra-2 can form stable heterodimers with c-Jun (Sonobe et al., 1995). Therefore Murakami and colleagues have
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postulated that phosphorylation may convert Fra-2 from an inefficient transcriptional activator into an active one (Murakami et al., 1997).

Fra-2 has been shown to heterodimerise with all members of the Jun family (Kovary and Bravo, 1992; Nishina et al., 1990; Suzuki et al., 1991). Fra-2/Jun heterodimer complexes have different effects on transcriptional activity at AP-1 binding sites. In combination with Fra-2, c-Jun and JunB repress activity and JunD enhances activity (Suzuki et al., 1991).

Weak transformation by mouse (Foletta et al., 1994) and chicken (Nishina et al., 1990) Fra-2 has been observed in CEF. In contrast, the human Fra-2 protein failed to transform an established rat fibroblast line (Wisdom and Verma, 1993). This difference may be due to the cell lines used rather than species differences. However, the chicken and human Fra-2 proteins lack a C-terminal transactivation domain (Wisdom and Verma, 1993) which may contribute to the lack of or weak transforming ability of this protein. Despite these weak transforming abilities, Fra-2 levels, along with other AP-1 members, have been shown to be affected in cells transformed by other factors. CEF transformed with v-src, c-Ha-ras, and activated c-raf all show an increase in the levels of Fra-2 and JunB as well as hyperphosphorylation of Fra-2 (Murakami et al., 1997; Suzuki et al., 1994). Elevations in the levels of Fra-2, Fra-1, Jun B and c-Jun were also seen in mouse 3T3 fibroblasts transformed with oncogenic Ha-Ras; however in this case Fra-2 showed the lowest level of induction. Fra-2 may be elevated as a result of the increased activity of Fra-1/c-Jun complexes (Mechta et al., 1997). In contrast, transformation by v-Rel, c-Rel and c-Rel delta in avian lymphoid cell line DT95 and CEF showed a repression in the levels of fra-2 mRNA (Kralova et al., 1998).

Fra-2 has been shown to be expressed in a number of tumor cell lines, with high expression seen in stomach carcinoma lines MKN-74 and KATO-III, kidney carcinoma line 253J, malignant fibrous histiocytoma line NMS10, vulva carcinoma line A431, and a neuroblastoma NB-3-nu line. Coexpression with Fra-
1 was commonly seen (Matsui et al., 1990). Over-expression of fra-2, fra-1, c-jun, junB and junD has also been observed in all renal cell carcinoma tissues and cell lines examined (Urakami et al., 1997). These results suggest a role for AP-1 and possibly Fra-2 in cellular transformation by a variety of agents. Furthermore, human myeloblastic leukemia and lipoma malignancies have been mapped to the same region of chromosome 2 where the fra-2 locus is situated. This raises the possibility that some rearrangements on chromosome 2 which result in disease might be due in part to alterations in the fra-2 gene (Molven et al., 1996).

A role for Fra-2, together with JunD, has been described in bone osteoblast differentiation. The bone-specific osteocalcin gene is specifically activated by this AP-1 dimer complex. Anti-sense oligonucleotides directed against fra-2 appeared to repress osteoblast differentiation. Different AP-1 complexes may be involved at other stages of osteoblast development (McCabe et al., 1996). This association with bone remodeling is interesting since c-Fos is known to play an important role in bone development, particularly in bone resorption.

Fra-2 has also been shown to play a role in spermatogenesis in the European red fox. Different patterns of expression were seen for various AP-1 family members, with the highest fra-2 expression detected at the onset and shutdown of peak spermatogenic activity. Expression was primarily observed in spermatocytes (Cohen et al., 1993).

Fra-2 may also have a role in the rat pineal gland. It is regulated on a circadian rhythm, with no expression detected during the day and significant levels of phosphorylated Fra-2 detectable at night. Norepinephrine is known to signal circadian changes in pineal function. It has also been shown to regulate Fra-2 expression in the same gland through the second messenger, cAMP (Baler and Klein, 1995).
There are likely to be many more circumstances in which Fra-2 contributes to transcriptional regulation. The wide expression pattern of Fra-2 and the changes to its level of expression during cellular transformation suggest it may be an important regulatory protein.

1.14 Aims of this thesis

The aim of the work presented in this thesis was to develop a clearer understanding of the biological function of Fra-2, to add to the general appreciation of the role of AP-1 complexes in transcriptional regulation. Several approaches were used to analyse the contribution of Fra-2 to cellular function in normal and diseased states. Firstly, studies involved analysis of fra-2 expression in human inflammatory bowel disease tissue samples and determine if Fra-2 does play any role in the development or progression of the disease. Further analysis using a mouse model system for inflammatory bowel disease was then undertaken. Secondly, fra-2 gene knockout constructs were generated and introduced into ES cells and cultured fibroblasts. This approach was designed to investigate some of the possible in vivo functions of Fra-2. Thirdly, transgenic mice over-expressing Fra-2 in a wide range of tissues were generated. The determination of tumour development in these transgenic mice helped evaluate whether fra-2 is an oncogene. Analysis of the phenotypes developed in these mice led to potential upstream and downstream target genes of Fra-2. Finally, further experiments investigating the potential downstream target genes of Fra-2 were performed.
Chapter 2

Role of Fra-2 in

Inflammatory Bowel Disease
2.1 Introduction

Inflammatory bowel disease (IBD) is an idiopathic disorder characterised by intestinal inflammation. There are at least two forms including ulcerative colitis and Chrohn’s disease. These two diseases vary in their clinical and pathological features (reviewed in Podolsky, 1991; Podolsky, 1991). Ulcerative colitis is generally characterised by the inflammation of the mucosal and superficial submucosal layers of the large intestine with lack of inflammation in the deeper layers. The disease occurs in an uninterrupted manner along the intestinal bowel. The histology of the disease includes: development of mucosal ulcerations; crypt microabscesses; depletion of mucin from goblet cells; the common appearance of polyp-like lesions; and inflammatory cellular infiltration of the lamina propria involving predominantly neutrophils and lymphocytes. The main clinical feature is the presence of bloody diarrhoea (Podolsky, 1991).

Chrohn’s disease is a more extensive form of IBD, which can involve the small and/or large intestines with inflammation of the deeper intestinal layers. The disease occurs in a segmental fashion. Extensive deep mucosal ulcerations, collagen deposits and inflammatory cellular infiltrates involving macrophages and lymphocytes are the histological features of this disease. Fistulas commonly extend the inflammation beyond the gastrointestinal tract, for example into the bladder, vagina, urethra, prostrate or skin. Clinical features include the presence of abdominal pain and diarrhea (Podolsky, 1991; Podolsky, 1991).

IBD is known to increase the risk of developing bowel cancer although the degree of risk has been controversial (Goldner, 1991; Podolsky, 1991). It is generally agreed that the risk of cancer increases after 10 years of disease, increasing with time. The presence of large numbers of polyp-like lesions in ulcerative colitis is often predictive of cancer. Chrohn’s disease is known to increase the risk of at least two rare carcinomas: adenocarcinoma of the small intestine and
cholangiocarcinoma (Podolsky, 1991). However, the risk of cancer may be less for Chrohn’s disease than for ulcerative colitis.

The pathogenesis of IBD is poorly understood although it is known to involve genetic factors, intestinal bacterial flora and the immune system. A number of IBD models have been developed (reviewed in Elson et al., 1995). The models vary from chemically inducible models (eg. administration of dextran sulfate sodium (DSS)) to gene targeting models (eg. IL-10 knockout mice). The DSS model reproducibly produces colonic inflammation. It is thought to initially affect the intestinal epithelial cells, which then leads to inflammation. Alteration in the expression of a number of cytokine genes (eg. IL-2, IL-4, and IL-6) has been shown. The DSS is found within the mucosal macrophages (Cooper et al., 1993; Okayasu et al., 1990).

Cytokine genes may play an important role in IBD, as an increase in the expression of many cytokine genes in IBD has been identified from mucosal biopsies. These include IL-4 and IL-10 in ulcerative colitis and IL-2, Interferon-\(\gamma\) (IFN-\(\gamma\)) and tumour necrosis factor \(\alpha\) (TNF-\(\alpha\)) in Chrohn’s disease (Mullin et al., 1996). Also many models of IBD through gene targeting involve cytokine genes, including IL-2 (Sadlack et al., 1993), IL-10 (Kühn et al., 1993) and TGF-\(\beta1\) (Kulkarni et al., 1993; Shull et al., 1992) knockout mice. Animals deficient for MHC class II, TCR-\(\beta\) chain, TCR-\(\alpha\) chain (Mombaerts et al., 1993; Takahashi et al., 1997) and G protein subunit Gr\(\alpha\)2 (Rudolph et al., 1995) also showed the development of IBD. These models all involve alterations in the immune system. It is also important to note that mice kept under germ-free conditions show milder IBD symptoms and slower disease progression, or lack of disease (Sadlack et al., 1993). This suggests the importance of the normal intestinal bacterial flora in the development or progression of the disease.
Transcription factor AP-1 has been shown to be involved in many aspects of the immune system (reviewed in Foletta et al., 1998). It has been shown to regulate expression of many cytokine genes including IL-2 (Jain et al., 1995; Jain et al., 1992), IL-3 (Cockerill et al., 1993; Mathey-Prevot et al., 1990; Park et al., 1993), IL-4 (Rooney et al., 1995), IL-5 (Lee et al., 1995), IL-9 (Zhu et al., 1996), GM-CSF (Cockerill et al., 1993; Wang et al., 1994), IFN-γ [Penix, 1993 #581; Cippetelli, 1995 #44] and TNF-α [Tsai, 1996 #277]. Four of these cytokines are potentially involved in IBD: IL-2, IL-4, IFN-γ and TNF-α. The promoters of these genes all contain elements that can be bound by AP-1 transcription factors. The IL-2, IL-4 and INF-γ promoters all contain AP-1 sites, which are directly bound by the AP-1 transcription factor. They also all contain other promoter elements which are bound by other factors whose binding is aided by AP-1, for example, NF-AT sites (reviewed in Rao, 1994), which are bound by NF-ATp transcription factor in cooperation with an AP-1 complex. Different members of the AP-1 family contribute to the regulation of these genes under different conditions. For example, Fra-1 and JunB are associated with NF-ATp in the IL-2 promoter in activated T cells (Boise et al., 1993) but all members of AP-1 have been shown to be expressed after T cell activation suggesting a dynamic regulatory role of AP-1 (Jain et al., 1992). TNF-α NF-AT sites are slightly divergent and are not associated with Fos proteins. They use an ATF2/Jun dimer bound to a 5' CRE site to assist their binding and consequent activation of the promoter (Tsai et al., 1996). Other transcription factor binding, including Oct proteins and NF-kB, can also be stabilized by AP-1 binding as seen in the IL-2 promoter (Jain et al., 1995; Jain et al., 1992).

AP-1 appears to play a very important role in the regulation of cytokine gene expression and cytokine genes also seem to be involved in IBD. Hence, AP-1 may play an important role in the development or progression of IBD by controlling the expression of these cytokine genes.
As mentioned in chapter 1, Fra-2 is expressed in both embryonic and adult mouse intestinal tissue (Carrasco and Bravo, 1995; Foletta, 1995; Foletta et al., 1994). In the adult mouse the goblet cells of the intestine show Fra-2 expression (Foletta, 1995). The goblet cells are involved in lubricating and protecting the lining of the intestine. This function of the cells is affected in IBD patients. The expression of Fra-2 has also been shown to be altered in a number of tumor cell lines, including a colon carcinoma cell line, Colo320DM (Matsui et al., 1990).

In this chapter, investigations into what roles, if any, Fra-2 may play in the development or progression of IBD was undertaken. The expression analysis of fra-2 in human IBD tissue samples was studied using RT-PCR to compare control and diseased tissue samples. An inducible murine DSS IBD model was then used for further analysis.
2.2 Methods
Solutions used in this chapter are listed in alphabetical order in appendix B. The oligonucleotide primers used in this chapter are described in appendix C.

2.2.1 IBD cDNA Samples

cDNA samples were kindly donated by Professor W Doe. Samples were prepared from biopsy tissue taken from patients with IBD. A tissue biopsy was taken from the inflamed area of the intestine, called diseased sample (B), and from an area of the intestine that appeared normal, called control sample (A). Total RNA and subsequently cDNA was prepared from the tissue samples. Eighteen pairs of samples were used in the analysis. The samples were identified by a number: 2, 6, 7.4, 7.6, 10, 12, 13, 14, 17, 23, 24, 31, 45, 50, 52, 82, 96 and 99.

2.2.2 Oligonucleotide primer preparation

Oligonucleotide primers were made by the JCSMR Biomolecular resource facility and supplied in ammonium hydroxide. Aliquots of the oligonucleotide primer (100-120 μl) were transferred to Eppendorf tubes and 10 X volume of butan-1-ol was added to each tube. The samples were vortexed and then spun at room temperature at 14,000 rpm for 1 minute in an Eppendorf microfuge (model 5415C). All the supernatant was carefully aspirated off and each pellet was resuspended in 20-30 μl of distilled water. The samples were pooled and spun again for 5 minutes (14,000 rpm) to remove insoluble debris. The supernatant was removed to a fresh Eppendorf tube. A 2 μl aliquot of each oligonucleotide primer was removed into 1 ml of distilled water for concentration determination by absorbance at 260 nm using the following calculation: primer concentration (pmol/μl) = (OD\textsubscript{260} x dilution factor)/E\textsubscript{260} x 1000. In most cases the dilution factor = 500. E\textsubscript{260} is the extinction coefficient of the oligonucleotide primer and is based
on the primer sequence, determined by using the following calculation: 

\[ E_{260} = 15.4 \times \#A's + 11.7 \times \#G's + 7.3 \times \#C's + 8.8 \times \#T's. \]

2.2.3 Semi-quantitative PCR analysis of IBD samples

A nested PCR approach was used to analyse the expression levels of fra-2 in the cDNA samples. The reactions were performed using oligonucleotide primers, Hfra2A and Hfra2B followed by Hfra2A and Hfra2C at 0.01 pmol/µl final concentration. The oligonucleotide primers Hfra2A, Hfra2B and Hfra2C were complimentary to the human fra-2 cDNA sequence identified by Nishina and colleagues (Nishina et al., 1990). β-Actin primer pairs ActinA and ActinB were used as internal controls. SuperTaq polymerase (P. H. Stehelin and CIE Ag, Basel) was employed at 0.2 units/ 100 µl reaction in 1 X SuperTaq PCR buffer to perform the PCR reactions. dNTPs (Pharmacia Biotech) were added to a final concentration of 0.2 mM. A 1 µl aliquot of the tissue cDNA sample (20 ng) was used in each PCR reaction. The reactions were covered with 50 µl of mineral oil (Sigma) to reduce evaporation. The fra-2 and actin primer pairs give expected PCR product sizes of approximately 650 bp and 600 bp respectively.

Optimum PCR conditions were determined for the analysis. The optimum number of PCR cycles was determined for both the first and second round reactions by removing aliquots after 10, 15, 20, and 25 cycles. The aliquots were then analysed on 0.8% agarose/ 1 X TAE/ 1 µg/ml ethidium bromide (Sigma) gels in 1 X loading dye. As a result of this analysis, 26 PCR cycles were performed for the first round reactions and 19 cycles for the second round. The PCR cycle was as follows: 30 seconds at 94°C; 30 seconds at 65°C; 2 minutes at 72°C, except for the first cycle which began with 5 minutes at 94°C instead of 30 seconds. A final step of 10 minutes at 72°C was performed to complete partial transcripts (MJ Research PTC-100 programmable thermal controller).
A 9 µl aliquot from each nested PCR reaction (plus 1 µl of 10 X loading dye) was analysed by electrophoresis, at 100 volts, on 0.8% agarose/1 X TAE/ 1 µg/ml ethidium bromide (Sigma) gels. A 10 µl aliquot of λ/HindIII plus φX174/HaeIII (New England Biolabs) DNA marker was loaded onto each gel for size comparisons. The gels were viewed on an UV transilluminator and photographed.

2.2.4 IBD mouse model

The mouse IBD experimental model developed by Okayasu et al. using dextran sulfate sodium continuous administration (Okayasu et al., 1990) was employed to study the potential role of fra-2 in development of IBD. Thirty-five male BALB/c mice aged between 7 and 8 weeks were divided into 7 groups of 5. Each group was put into a separate cage. The mice were all pre-weighed and marked for identification. Two cages of mice were marked as controls and were given water to drink. Mice in the remaining 5 cages were given 3% DSS (Sigma) to drink. The fluid bottles were weighed daily to determine the amount of fluid consumed by the animals. The bottles were then refilled with the appropriate filter sterilised fluid and reweighed. All mice were weighed daily. Two mice selected at random from the DSS fed cages were sacrificed every day up until day 6 and then on days 9 and 12. Mice were also taken from the control boxes at day 1 and day 12. The colons were removed from the sacrificed mice and rinsed in phosphate buffer saline (PBS). They were then quickly frozen in 95% hexane and stored at -70°C for later RNA extraction.
2.2.5 Total RNA preparation from mouse colon samples

Total RNA was prepared from the mouse colon samples using a modified method of Auffray and Rougeon (Auffray and Rougeon, 1980). The frozen mouse colon samples were homogenized in 6 ml of LiCl/Urea solution using a polytron PTA 7 probe (Kinematica, Switzerland) for 2 x 1 minute in sterile polyallomer centrifuge tubes (Beckman). The tubes were topped up with LiCl/Urea solution and incubated overnight at 4°C. The following day the samples were centrifuged at 25,000 rpm for 30 minutes at 4°C (SW41 rotor, Beckman). The supernatant was quickly decanted and the pellet was resuspended in 500 µl of SDS buffer. Vortexing and incubation at 65°C assisted in resuspension of the RNA pellet. An equal volume of TE equilibrated phenol:chloroform:isoamyl alcohol (PCI)(25:24:1) (Progen) was added and each sample vortexed for 1 minute. The samples were incubated at 65°C for 5 minutes and then spun at 14,000 rpm in an Eppendorf microfuge (model 5415 C) for 2 minutes. The upper aqueous phase was removed to a fresh tube. The extraction was repeated, without the incubation step, with equal volumes of PCI and then finally with chloroform:isoamyl alcohol (24:1). The final upper aqueous phase was ethanol precipitated overnight at -20°C with sodium acetate (final concentration of 0.3 M) and 2.5 volumes of 100% ice-cold ethanol. The RNA was pelleted by spinning at 14,000 rpm in an Eppendorf microfuge (model 5415 C) for 25 minutes at 4°C. The supernatant was decanted and the RNA pellet was washed in 70% ethanol then dried in a Savant SpeedVac concentrator (model SVC-100H) for approximately 10 minutes. The RNA was resuspended in 100 µl of TE buffer and the concentration was determined by absorbance at 260 nm using the following calculation: RNA concentration (mg/ml) = OD$_{260}$ x 40 x dilution factor. Total RNA samples were stored at -70°C.
2.2.6 RT-PCR reactions for mouse colon samples

Two micrograms of total RNA was denatured by heating at 90°C for 5 minutes. The denatured RNA was then reverse transcribed using 10 units of AMV reverse transcriptase (Promega), 500 ng of oligo(dT) primer (New England BioLabs) and 0.8 mM dNTP mix in 1 X AMV reverse transcriptase buffer (Promega) in a total reaction volume of 25 μl. Forty units of the RNase inhibitor, RNasin (Promega), was added to each reaction. The reactions were incubated at room temperature for 10 minutes, then 45 minutes at 42°C followed by 5 minutes at 95°C. The cDNAs generated from these reactions were stored at -20°C.

One third of each RT reaction was used in the nested PCR reactions employed to analyse the expression levels of fra-2 in the mouse colon cDNA samples. The reactions were performed using oligonucleotide primers, F2Ex1 and F2Ex3c followed by F2Ex1 and F2Ex3c.2, at 0.4-0.5 pmol/μl concentration. The oligonucleotide primers F2Ex1, F2Ex3c and F2Ex3c.2 were complimentary to the mouse fra-2 cDNA sequence identified by Foletta and colleagues (Foletta et al., 1994). Advanced Biotechnologies Taq DNA polymerase was employed at 0.025 units/50 μl reaction in 1 X PCR buffer IV (Advanced Biotechnologies) together with 1.5 mM magnesium chloride to perform the amplification. dNTPs were added to a final concentration of 0.2 mM. A PCR product size of approximately 380 bp was expected from these primer pairs.

PCR conditions were the same as those described in the semi-quantitative PCR section 2.2.3 above. A 9 μl aliquot from each nested PCR reaction was removed, 1 μl of 10 X loading dye was added and the amplification products were separated by electrophoresis, at 100 volts, on 1.5% agarose/1 X TAE/ 1 μg/ml ethidium bromide (Sigma) gels. A 10 μl aliquot of λ/HindIII plus φX174/HaeIII (New England BioLabs) DNA marker was loaded onto each gel for size comparisons. The gels were viewed on a UV transilluminator and photographed.
2.3 Results

2.3.1 fra-2 expression in human IBD tissue samples

Nested PCR reactions on the reverse-transcribed cDNAs prepared from human IBD tissue sample RNA were performed. The PCR results, comparing the control (A) and diseased (B) sample from each patient, are shown in figures 2.1 to 2.3. The amount of β-actin amplification product in control and diseased samples was very similar in most cases. If it is assumed that the levels of β-actin expression are the same in diseased and normal tissues, then this can be taken to indicate comparable initial cDNA concentrations used in the reactions such that semi-quantitative conclusions can be drawn from the fra-2 expression levels. There were two examples where the levels of β-actin amplification product varied between the control and diseased tissue samples, sample 7.6 (figure 2.1b) and sample 52 (figure 2.3b). The decrease or increase in the amplification product of β-actin in the diseased tissue samples respectively correlated with a similar difference in the fra-2 amplification product. Hence these samples may actually have similar levels of expression of fra-2 when comparing control and diseased tissue samples. Sample 50 was the only sample to show lack of fra-2 expression in both control and diseased tissue samples (see figure 2.3a). Taking these three samples (7.6, 50 and 52) into account, 39% of samples showed no significant difference in the expression patterns of fra-2 in IBD diseased or control tissue samples. The other samples in which no difference between fra-2 levels in diseased and control samples was observed were 2, 6, 23, and 24 (see figures 2.1a, 2.2b and c).

In contrast, 61% of cases showed changes in the levels of fra-2 expression between the control and diseased tissue samples. There were four cases which showed a decrease in the level of fra-2 expression in diseased tissue as compared to control: samples 12, 17, 96 and 99. In all cases there was very low
Figure 2.1: Nested RT-PCR analysis for fra-2 expression levels in human Inflammatory bowel disease control and diseased tissue samples 2, 6, 7.4, 7.6, 10 and 12

Nested RT-PCR was used to analyse cDNA samples for fra-2 expression levels with oligonucleotide primers Hfra2A and Hfra2B in a first round PCR amplification for 26 cycles, followed by a second round PCR amplification for 19 cycles with oligonucleotide primer Hfra2A and Hfra2C. The arrows indicate the 650 bp fra-2 product produced from this primer combination. Actin was used as control for equivalence in starting cDNA used. Actin oligonucleotide primers Actin A and Actin B were used in both rounds of PCR; this reaction gave a product of approximately 600 bp. The PCR products were run on 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gels. Water was used as a negative control (W) and M = DNA size markers (λ/HindIII plus φX174/HaeIII).

a) The results showed no significant difference in the expression levels of fra-2 in control (A) and diseased (B) tissue samples from inflammatory bowel disease human patients 2 and 6.

b) The results showed an increase in fra-2 expression in the diseased tissue (B) from sample 7.4 and a decrease in the expression in diseased tissue (B) from sample 7.6 as compared to control (A) samples. However, there is also a difference in the expression levels of actin in sample 7.6.

c) The results showed an increase in fra-2 expression in the diseased tissue (B) from sample 10 and a decrease in the expression levels in diseased tissue (B) from sample 12 as compared to control (A) samples.
Role of Fra-2 in IBD: Results
Figure 2.2: Nested RT-PCR analysis for fra-2 expression levels in human Inflammatory bowel disease control and diseased tissue samples 13, 14, 17, 23, 24 and 31

Nested RT-PCR was used to analyse cDNA samples for fra-2 and actin expression levels as described for figure 2.1. The arrows indicate the 650 bp fra-2 product produced from the PCR amplification. The PCR products were run on 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gels. Water was used as a negative control (W) and M = DNA size markers (λ/HindIII plus φX174/HaeIII).

a) The results show no increase in fra-2 expression in the diseased tissue (B) for both samples 13 and 14 compared to control tissue (A). Sample 14 also shows an unusual result with the presence of a faint extra band in the diseased tissue (B) sample.

b) The results show a decrease in fra-2 expression in the diseased tissue (B) from sample 17 and no significant difference in the expression levels of fra-2 in control (A) or diseased (B) tissue from sample 23.

c) The results show no significant difference in the expression levels of fra-2 in control (A) or diseased (B) tissue from sample 24. An increase in fra-2 expression in the diseased tissue (B) from sample 10 and an unusual result for sample 31 with the presence of an extra band in the diseased tissue (B) sample compared to the control (A) samples.
Role of Fra-2 in IBD: Results
Figure 2.3: Nested RT-PCR analysis for fra-2 expression levels in human inflammatory bowel disease control and diseased tissue samples 45, 50, 52, 82, 96 and 99

Nested RT-PCR was used to analyse cDNA samples for fra-2 and actin expression levels as described for figure 2.1. The arrows indicate the 650 bp fra-2 product produced from the PCR amplification. The PCR products were run on 0.8% agarose/1X TAE/1 µg/ml ethidium bromide gels. Water was used as a negative control (W) and M = DNA size markers (λ/HindIII plus φX174/HaeIII).

a) The results show an increase in fra-2 expression in the diseased tissue (B) from sample 45 and no detectable levels of fra-2 in control (A) and diseased (B) tissue from sample 50.

b) The results show an increase in fra-2 expression in the diseased (B) tissue for both samples 52 and 82 as compared to control (A) tissue. However, for sample 52 there also appears to be more actin expression in the diseased tissue.

c) The results show a decrease in fra-2 expression in the diseased tissue (B) for both samples 96 and 99 compared to control (A) tissue.
Role of Fra-2 in IBD: Results

(sample 17, figure 2.2b) or no (samples 12, 96 and 99, figures 2.1c and 2.3c) expression of fra-2 in diseased tissue. For samples 12 and 96, the level of fra-2 expression in the control sample was also very low.

Five samples showed an increase in the expression level of fra-2 in the diseased tissue. Two of these, 45 (figure 2.3a) and 82 (figure 2.3b) showed lack of fra-2 expression in the control tissue sample. Sample 45 also showed a low fra-2 expression level in the diseased tissue. Samples 7.4, 10 and 13 (figures 2.1b & c and 2.2a) all showed a significant increase in the level of fra-2 expression in the diseased tissue sample compared to the control. An unusual result was seen with samples 14 and 31 (figures 2.2a and c): there was an extra PCR product band present in the fra-2 expression analysis lane from the diseased tissue sample. This PCR product was slightly larger than the expected 650 bp fra-2 PCR product. It may represent an alternatively spliced form of fra-2 or an alteration in the fra-2 gene. If this band does truly represent a fra-2 product, then there is an increase in the total expression of fra-2 in the diseased tissue from these samples. Sample 14 does show an increase in the expression of fra-2 between the lower bands regardless of the extra band.

Histology analysis was performed on the IBD samples by Dr. Sanjiv Jain from the Canberra Hospital, Canberra, Australia. A summary of these histology results and the fra-2 expression changes for the IBD patients is shown in tables 2.1, 2.2 and 2.3. In this analysis histology scores of 1-4 were considered low, 5-8 moderate and 9-12 high, in terms of disease severity.

There was one sample, 52, which histologically did not show IBD (table 2.1). For another sample, 6, it was unclear whether there was true IBD present or not (table 2.1). Both of these samples showed no significant difference in the expression pattern of fra-2.
<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Disease</th>
<th>Disease histology analysis</th>
<th>Total score out of 12</th>
<th>fra-2 expression</th>
<th>Change in expression pattern</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>C</td>
<td>MI</td>
<td>NI</td>
</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>UC</td>
<td>2</td>
<td>3</td>
<td>3</td>
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<td>UC?</td>
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<td>1</td>
<td>3</td>
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<td>c. difficile</td>
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<td>0</td>
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<td>0</td>
</tr>
</tbody>
</table>

**Tables 2.1, 2.2 and 2.3:** Summary of IBD sample histology and fra-2 expression results (see next 2 pages)

* no histology on control tissue was performed for this sample.

# the level of actin expression varied between control and diseased samples similarly to the variation seen in the fra-2 expression levels.
Table 2.2: Samples showing an increase in fra-2 expression in diseased tissue

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Disease</th>
<th>Disease histology analysis</th>
<th>Total score out of 12</th>
<th>fra-2 expression</th>
<th>Change in expression pattern</th>
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</thead>
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<tr>
<td>31A</td>
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<td>0</td>
<td>0</td>
<td>moderate level</td>
</tr>
<tr>
<td>31B</td>
<td>CD</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>9 moderate level, extra band alteration in B</td>
</tr>
<tr>
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<td>0</td>
<td>1 none</td>
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<tr>
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<td>UC</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>11 low level</td>
</tr>
<tr>
<td>14A</td>
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<td>1</td>
<td>0</td>
<td>1 very low level</td>
</tr>
<tr>
<td>14B</td>
<td>CD</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>11 moderate level, extra band ++ and alteration in B</td>
</tr>
<tr>
<td>7.4A</td>
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<td>0</td>
<td>0 low-moderate level</td>
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<td>3</td>
<td>11 high level ++</td>
</tr>
<tr>
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<td>0</td>
<td>1 low level</td>
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<tr>
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<td>3</td>
<td>2</td>
<td>9 moderate level ++</td>
</tr>
<tr>
<td>13A</td>
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<td>1</td>
<td>0</td>
<td>1 moderate level</td>
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<tr>
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<td>3</td>
<td>9 high level ++</td>
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<tr>
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<td>1</td>
<td>3</td>
<td>10 high level +++</td>
</tr>
</tbody>
</table>

Tables 2.1, 2.2 and 2.3: Summary of IBD sample histology and fra-2 expression results (see next page)
Table 2.3: Samples showing a decrease in \textit{fra-2} expression in diseased tissue

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Disease</th>
<th>Disease histology analysis</th>
<th>Total score out of 12</th>
<th>\textit{fra-2} expression</th>
<th>Change in expression pattern</th>
</tr>
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<tbody>
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<td></td>
<td></td>
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<td>C</td>
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</tr>
<tr>
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<td>1</td>
<td>0</td>
</tr>
<tr>
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<td>CD</td>
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<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>12A</td>
<td>CD</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>12B</td>
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<td>3</td>
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<tr>
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<td>1</td>
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<td>2</td>
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<tr>
<td>99B</td>
<td>UC</td>
<td>1</td>
<td>2</td>
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</table>

Tables 2.1, 2.2 and 2.3: Summary of IBD sample histology and \textit{fra-2} expression results (This page and the previous two pages)

The histology of control and diseased tissue samples was analysed. Four different characteristics of the tissue were scored out of 3 in terms of the severity of disease (3 being the most severe) and the total score out of 12 recorded. All histology work was done by Dr Sanjiv Jain at the Canberra Hospital, Canberra, Australia. The "+" or "-" signifies either an increase or decrease in the \textit{fra-2} levels in the diseased sample compared to the control, respectively; the number of pluses or minuses indicates the degree of change. UC = ulcerative colitis, CD = Crohn’s disease, E = Epithelial cells, C = Crypts, MI = Mononucleocyte infiltration, NI = Neutrophil infiltration.
In the IBD samples in which fra-2 expression did not change between diseased and control tissue (2, 7.6, 23, and 24; table 2.1), there was either low or moderate level of fra-2 expression in both control and diseased tissue samples. Samples 7.6 and 24 both showed a moderate histology score of 7, while samples 2 and 23 had high scores of 11. However, the histology of the control tissue for sample 23 was not completed, so it is unclear what the disease status of this tissue was. Sample 50 differed in that it showed lack of fra-2 expression in both tissues and had the highest histology score 12 (table 2.1).

Samples 12, 17, 96 and 99 which had decreased fra-2 expression in the diseased tissue as compared to control all had moderate-high histology scores (7-10) (table 2.2). All the control tissues also showed a low histology score of 1. An increase or alteration in the expression pattern of fra-2 in the diseased tissue (table 2.3) correlated well with a high histology score, 9-11. It should be noted that the level of fra-2 amplification product in the control tissue samples varied between the different patients samples.

2.3.2 IBD mouse model fra-2 expression analysis

Considering over half of the human IBD samples examined above showed some alterations in the levels of fra-2 expression between control and diseased tissue, it was decided to further investigate any role fra-2 may potentially play in the development or progression of IBD. A DSS IBD mouse model (Okayasu et al., 1990) was employed for this purpose.

There were two main groups in the experiment, the control group which were fed water and the experimental group which were fed 3% DSS. There were 10 control group mice and 25 experimental group mice. To exclude the possibility of any differences seen between the two groups of animals being attributed to differences in fluid intake, the daily fluid consumption of each mouse box was recorded.
These figures were divided by the number of mice in the box to determine the average fluid intake for each animal (see table 2.4). There was no significant difference in the average amount of fluid consumed by either water or 3% DSS fed mice.

Mouse weights were also recorded daily to determine any changes in weight (see table 2.5). During the first 6 days of the experiment there was no significant difference in the weight changes between control animals on water and experimental animals on 3% DSS. In contrast, from day 7 mice fed 3% DSS generally lost more weight than animals fed water (see figure 2.4). The most probable cause for the differences in weight is the development of disease, since around day 6 the mice fed 3% DSS began to show signs of disease which got progressively worse with time. These symptoms included loose stools, diarrhoea and bleeding. A couple of the experimental animals (B5M1 and B5M3) died at day 13, the day after the experiment concluded, suggesting severe disease.

RT-PCR analysis on the RNA prepared from the experimental mice (on 3% DSS) colons showed no significant changes in the expression of fra-2 over time (see figure 2.5). There is an increase in the level of fra-2 amplification product over time but the increase is also seen in the controls. This suggests variation between levels of fra-2 in individual animals. Some of the variation may be due to PCR variation. The PCR analysis was repeated 4 times and each result showed no significant difference between the levels of fra-2 expression over time. Therefore this analysis was not pursued any further.
Role of Fra-2 in IBD: Results

Table 2.4: Amount of fluid consumed over time

The fluid bottles were weighed daily to determine the amount of fluid consumed by each box of animals. The amount of fluid consumed by a box was then divided by the number of mice in that box to get an estimate of the amount of fluid taken in by each mouse in that box.
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<th>Day 2</th>
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<th>Day 4</th>
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<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
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Table 2.5: Weight differences in animals over time (This page and continued next page)

Mice were weighed to determine weight changes; the negative values indicate a weight loss. Absence of a value (-) indicates that the animal was sacrificed for analysis at that time.
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Table 2.5: (Continued from previous page)
Figure 2.4: Changes in weight over time

The changes in animal weights from the IBD model experiment during the 12 days is graphically represented. Three mice from each of the two conditions, water control and 3% DSS fed, are shown. The solid lines represent mice fed water and the dashed lines represent mice fed 3% DSS.
Figure 2.5: Nested RT-PCR analysis for fra-2 expression levels in mouse colons from Inflammatory Bowel Disease model over time.

There was no significant change in the levels of fra-2 expression over time with the development of inflammatory bowel disease in this mouse model system. Mice were fed 3% DSS (d) to drink and then the colons were removed from 2 mice on days 1, 2, 3, 4, 5, 6, 7, 9 and 12 and RNA was prepared from the tissue. Control mice (c) were fed water and colons were removed from mice on days 1 and 12. RNA samples were reverse transcribed as described in method 2.2.6 and then analysed for fra-2 expression levels with oligonucleotide primers F2ex1 and F2Ex3 in a first round RT-PCR for 26 cycles, followed by a second round PCR amplification for 19 cycles with oligonucleotide primers F2Ex1 and F2Ex3.c. The arrow indicates a doublet fra-2 cDNA product of approximately 380 bp produced from this PCR amplification. The PCR products were run on a 1.5% agarose/TAE/1 μg/ml ethidium bromide gel. The numbers represent the days. Water was used as a negative control (W) and pCMV-fra2 as a positive control (P). M = DNA size markers (λ/HindIII plus φX174/HaeIII).
2.4 Discussion

2.4.1 Expression patterns of fra-2 in human IBD samples

There were 3 main groups identified from the analysis of the human IBD diseased and control tissue samples: those that lacked any change in the expression levels of fra-2; those that showed a decrease in fra-2 levels in the diseased tissue samples; and those that showed an increase or alteration in fra-2 levels in the diseased tissue samples. For the purpose of this discussion, the 2 samples that did not appear to have IBD by histological analysis were excluded.

An interesting aspect of the results was that the amount of fra-2 observed in the control samples varied between the samples in all 3 of the groups. This may have been due to the biopsy tissue being taken from different areas of the colon, ranging from the caecum and ileum right through to the rectum. The biopsy tissue was taken from the inflamed diseased area of the colon (ie. diseased sample), which differed between patients, and from another area of the colon which appeared to be non-inflamed (ie. control sample). Therefore some of the differences in fra-2 levels may be explained if a differential pattern of fra-2 expression occurs within the human colon, which appears to be the case from this work. In support of this, a recent report has shown that in the developing chicken gastrointestinal tract there is a differential pattern of AP-1 gene expression (Matsumoto et al., 1998). The exact pattern of fra-2 expression along the human colon is currently unknown. Hence, some of the differences between control and diseased tissue samples may purely be due to the different biopsy tissue sections taken for each sample. A larger sample size with tissue samples taken from similar regions of the colon for each patient may have been more beneficial for this analysis but was unfortunately unavailable at the time.

Secondly, changes in the functional activity of Fra-2 might be more apparent at some stages of the disease than at others, and if the samples were not all collected at the same stage of the disease this could explain why some of the samples showed changed levels of fra-2 mRNA and others did not. IBD causes recurring inflammation of the colon and severity (as measured by histology) is unrelated to the stage of the disease.
This suggests that some of the results that showed no change in fra-2 levels and were histologically severe (scores of 11) may have been at a stage in the development of the disease that did not require or involve Fra-2.

Fra-2 is known to be expressed in many epithelial cells (Carrasco and Bravo, 1995) but there appeared to be no correlation, in any of the groups, between the histology score for epithelial cells and the level of fra-2 detected (Table 2.1, 2.2 and 2.3, Disease histology analysis column “E”). There was also no correlation between fra-2 and any of the other individual regions of tissue scored for histology. Furthermore, no correlation was seen between the type of IBD, either ulcerative colitis or Crohn’s disease, and the level of fra-2 amplification product in diseased tissue samples.

In the analysis presented here, 69% of samples showed some change in the pattern of fra-2 amplification product between control and diseased tissue samples.

The group of samples which showed a decrease in the level of fra-2 in the diseased tissue sample compared to the control tissue sample (25% of the samples) all showed moderate to high histology scores (table 2.3). The severity of disease did not appear to correlate with the degree of change observed in the level of fra-2. However, 3 out of the 4 samples showed a lack of fra-2 in the diseased sample. Hence, the differences in the degree of change of fra-2 levels may be due to the different areas of the colon from where the control tissue sample section was taken, giving different control levels. Therefore, it may not be valid to compare the degrees of change amongst samples, but rather to purely assess the trend, ie. a decrease in fra-2 levels.

The group of samples which showed an increase or alteration in the level of fra-2 (44% of the samples) all showed high histology scores (table 2.2), but again the degree of change in fra-2 levels did not correlate with these scores, probably for the same reason as discussed above.

Taking these two groups together, suggests that some change in the functional activity of Fra-2 could be associated with molecular disturbances that occur in IBD. The question is: are the changes occurring because Fra-2 is involved in the development or
progression of IBD, or are they an effect of some other change? The approach undertaken in these studies was unable to answer this question. However, if fra-2 is involved in IBD such that either an increase or a decrease in fra-2 levels results in disease, then this suggests the possibility that a balance in Fra-2 levels is required for normal function. It should be remembered that Fra-2 must be part of a heterodimeric complex for it to bind to DNA and affect gene expression and it is likely that several Fos family members compete with each other at any one time for complex formation with appropriate partners. Thus an alteration in the level of the active Fra-2 containing complexes may be what is important rather than the levels of individual members. Therefore any changes in the expression of fra-2, either positively or negatively, would alter this balance.

The two samples, 14 and 31, which showed an extra band in the fra-2 analysis of diseased tissue, may suggest the presence of an alternatively spliced form of fra-2 in these samples. Alternatively spliced forms of fra-2 have been seen in other circumstances (Matsui et al., 1990; Sonobe et al., 1995; Yoshida et al., 1991). Individual functional roles for these alternate forms have not been reported. There are two forms of FosB, FosB and the shorter form FosB2. FosB2 has been shown to inhibit gene activation, possibly by competition for Jun partners (Nakabeppu and Nathans, 1991; Yen et al., 1991). The putative alternative form of fra-2 seen in these IBD samples could potentially play a similar role. Another possibility is that an alteration in one copy of the fra-2 gene, possibly an insertion event, has occurred. This may also affect the function of the resulting Fra-2 protein. Both of these samples, 14 and 31, were diagnosed with Crohn’s disease by histological analysis. Taking all the results together, 5 out of 7 cases of CD were accompanied by some kind of change in Fra-2. This suggests that if there is a correlation between CD and Fra-2, then there are several different ways in which the functional activity of Fra-2 can be altered which produce disease, ie. by simply increasing or decreasing the absolute amount of protein available to form complexes, or by changing the protein structure in some way.

The group of samples which showed no significant difference in the levels of fra-2 (31% of the samples) between control and diseased tissue samples showed both moderate and high histology scores. Hence, IBD was present at levels that were equally
as high or higher than those seen in both of the other groups of samples. Therefore, the lack of change in the pattern of fra-2 amplification product in these samples could not be attributed to lack of disease.

How can these results be reconciled with the previously discussed results, which suggested that alterations in fra-2 expression do correlate with disease?

Firstly, as stated above, the site from which the biopsy tissue was taken may have played a role in these results. As it was not possible to sample tissue from a single site both before and after onset of disease, it can not be stated with certainty the extent to which levels of Fra-2 expression might have changed at the site of inflammation.

Secondly, Fra-2 is regulated at many levels and mRNA expression is only one of these levels. For example, Fra-2 is a phosphoprotein and its phosphorylation status has been shown to play an important role in regulating its activity (Baler and Klein, 1995; Murakami et al., 1997). Thus post-translational controls may be disrupted in some cases where the mRNA levels are apparently unchanged, still producing a net effect on the function of Fra-2. Alterations in these other levels of control may include increased activity of a regulatory protein that keeps Fra-2 in an inactive state or sequestered in the cytoplasm, or an alteration in the redox potential which can either increase or decrease the DNA binding activity of the protein. Thus, mRNA levels may not always reflect the exact level of active protein in the cell. If appropriate material had been available, analysis of Fra-2 protein levels may have been informative on this point.

Thirdly, Fra-2 is only one step in a signalling cascade and if any step in this cascade is altered, the result may be development of IBD. In some cases, the alteration may occur at a step further along the signalling pathway from Fra-2 and in these cases the level of fra-2 would be unchanged. Thus, if the results presented here are suggestive of a correlation between Fra-2 levels and IBD, then it is probably more correct to conclude that it is the signalling pathway in which Fra-2 functions which is associated with this disease, rather than Fra-2 protein alone. In other words, alterations in Fra-2 function may be sufficient for development of disease, but they are not necessary. IBD is a complex condition and it is likely that disease results from disruption of more than one...
signalling pathway, as suggested by the vast number of IBD models (reviewed in Elson et al., 1995). Therefore, it may not be unreasonable to expect that Fra-2 may not be involved in the development or progression of all IBD cases.

The human IBD sample analysis suggested a possible role for Fra-2 in some cases of IBD, however, the evidence was not strong. A larger sample size may have helped in obtaining a stronger conclusion. There are a number of possibilities for the variety of results obtained in this analysis and they have been discussed above. There is also the question of a potential role for Fra-2 in the development of dysplasia or cancer in severe IBD cases. Fra-2 has been shown to be expressed in a colon carcinoma cell line (Matsui et al., 1990). Investigations into tumour tissue expression patterns from IBD patients who develop cancer would be useful in determining any role Fra-2 may play in this.

2.4.2 DSS IBD mouse model

The DSS mouse model of IBD did not show any correlation between fra-2 expression and development of IBD in this system. There are two possible explanations for this, the first of which relates to the DSS IBD model itself. Okayasu et al., suggested that the DSS mouse model would be a reliable model for studies on IBD pathogenesis (Okayasu et al., 1990). The model corresponds well to the clinical signs of human ulcerative colitis but does not resemble all the histopathologies of human IBD. No model exactly reproduces human IBD (Elson et al., 1995). Therefore the lack of change in fra-2 expression levels in this study does not rule out a role for Fra-2 in human IBD. The pathway leading to disease in this model may exclude events that might involve Fra-2 and/or AP-1.

Secondly, in this study RNA was prepared from the whole colon from each animal. Disease may not have been throughout the colon, but restricted to specific areas. These areas may have shown an alteration in the expression pattern of fra-2 but the result would have been masked by the expression of fra-2 in the rest of the colon. An
approach combining histological and *in situ* hybridisation analysis may have been informative in determining regions of diseased colon and patterns in fra-2 expression.

The AP-1 transcription factor has recently been shown to be up-regulated following CD2 activation in T cells from inflamed mucosal tissue from Crohn's disease patients. Non-inflamed mucosal tissue did not show this up-regulation of AP-1 (Gonsky et al., 1998). No information was obtained on the individual members comprising the AP-1 complex.

It has been suggested that activated T cells may play a central role in the development of IBD (Gonsky et al., 1998; Ma et al., 1995; Powrie, 1995). Interestingly, a key regulator of transcription during T cell activation, the NF-AT complex, has been shown to contain c-Fos, Fra-1, Fra-2 and Jun B (Boise et al., 1993; Jain et al., 1994). These AP-1 family members are probably present in the NF-AT complex at different times during T-cell stimulation. Therefore, a number of the AP-1 proteins may be required for correct signalling to occur. The regulation of T cell activation by AP-1 through many cytokine genes provides an interesting link between AP-1 and IBD, which needs further investigation. The analysis presented here provides preliminary evidence that changes in fra-2 levels occur in some cases of IBD.

In this chapter functional roles of Fra-2 were investigated in one disease, IBD. This disease was chosen for analysis for a number of reasons, including the role of AP-1 in cytokine gene expression, the expression of Fra-2 in the intestine and the availability of samples for analysis. This approach to determine functional roles for Fra-2 was largely unsuccessful and therefore a more direct approach to look at Fra-2 function in a wider range of tissues was employed. The following chapters discuss two direct approaches utilised to examine functional roles for Fra-2. They include the use of knockout and transgenic mouse strategies.
Chapter 3

Fra-2 Knockout Studies
3.1 Introduction

In the previous chapter analysis of the functional role of fra-2 in a specific system, the development and progression of IBD, was investigated. In this chapter a more direct approach was employed to investigate a broader range of possible functional roles for fra-2. As discussed in chapter 1, Fra-2 is known to be expressed at high levels in a range of embryonic and adult mouse tissues (Carrasco and Bravo, 1995; Foletta et al., 1994). fra-2 expression occurs in the mouse embryo during organogenesis suggesting roles for this gene in organ development. The pattern of fra-2 expression, as is the case with all other AP-1 family members, shows some overlapping expression with other AP-1 members and some areas specific for Fra-2 expression. For example, FosB has been shown to be expressed in many epithelia where Fra-2 is also known to be expressed (Gruda et al., 1996). In contrast, fra-2 is expressed in the differentiating developing kidney tubules where no other Fos family member has been shown to be expressed (Carrasco and Bravo, 1995).

All members of the AP-1 transcription factor family have been shown to be expressed in 3T3 fibroblasts (Gruda et al., 1994; Kovary and Bravo, 1991; Lallemand et al., 1997) and some have been shown to have an effect on cell cycle progression (Kovary and Bravo, 1991). Fra-2 is expressed at high levels in cycling NIH3T3 fibroblasts (Lallemand et al., 1997) and is rapidly induced in serum stimulated Swiss 3T3 fibroblasts (Gruda et al., 1994).

Despite many studies demonstrating a high level of fra-2 expression in a range of circumstances, there is still no clear picture of the precise role of Fra-2 in vivo. There are a number of strategies that can be employed to determine the functional role of a gene. One of these is gene targeting to produce ‘knockout’ cells and animals (reviewed in Melton, 1994). The first mammalian gene targeting experiment was reported in cell lines with the β-globin gene in 1985 (Smithies et al., 1985). The first targeted ES cells were made in 1987, in which a mutation in
an inactivate hypoxanthine phosphoribosyltransferase (HPRT) gene was corrected (Doetschman et al., 1987). These ES cells were later used in the generation of targeted mice (Thompson et al., 1989). A positive-negative selection protocol was reported in 1988, using neomycin resistance (NeoR) and thymidine kinase (TK) genes, which greatly enriched the number of cells in which the homologous recombination events occurred (Mansour et al., 1988).

Since then, many genes have been targeted through homologous recombination as a way to study different aspects of their function. Three AP-1 transcription factor family members, c-Jun, c-Fos and FosB, have been analysed by gene targeting studies. The results from these studies were discussed in chapter 1. Briefly, ES cells lacking functional c-Jun were normal (Hilberg and Wagner, 1992), primary mouse embryo fibroblasts (MEF) were growth defective but viable (Johnson et al., 1993) and knockout mice died in mid-late gestation (Hilberg et al., 1993; Johnson et al., 1993). Cells lacking functional c-Fos are normal (Brüsselbach et al., 1995; Field et al., 1992; Hu et al., 1994) but c-Fos knockout mice show bone and haematopoietic defects (Johnson et al., 1992; Wang et al., 1992). In contrast, FosB knockout cells and mice show no gross abnormal phenotype (Gruda et al., 1996).

An attempt to reduce the levels of functional Fra-2 in mice has been pursued previously. In this case, transgenic mice expressing anti-sense fra-2 RNA were made (Foletta, 1995). No significant changes in the level of Fra-2 protein were detected in any tissue and no gross abnormalities were observed. One line of transgenic animals, expressing high levels of the transgene, did show impaired splenic T cell proliferation in response to stimulus and male infertility after 6 months of age. The testes and the spleen both show very low levels of fra-2 expression, in contrast to the majority of mouse tissue, which show high fra-2 expression (Foletta et al., 1994). Therefore subtle changes in the levels of Fra-2 in these tissues may have had an effect on tissue function over time.
Another approach to produce mice with a reduced level or lack of functional Fra-2 protein would be by homologous recombination using a targeted Fra-2 construct. There are three potential outcomes from the generation of mice lacking functional Fra-2: the production of an embryonic lethal mutation, the lack of any phenotype or the development of an abnormal phenotype.

Considering that fra-2 is widely expressed and at high levels in a range of tissues (Carrasco and Bravo, 1995; Foletta et al., 1994) an embryonic lethal result is a serious possibility. The expression of fra-2 during organogenesis raises the possibility that lack of Fra-2 may disrupt the correct development of some tissues, e.g. kidney, skin, bone, intestine, CNS, adrenal gland, lung or bladder, which could potentially be fatal. The extensive expression pattern of fra-2 is similar to that seen for the jun family of genes (Hirai et al., 1989; Wilkinson et al., 1989). c-jun is the only member of this family for which knockout studies have been reported and the targeting of this gene proved to be an embryonic lethal mutation (Hilberg et al., 1993; Johnson et al., 1993). The similarities in expression patterns between fra-2 and c-jun raise the question of whether lack of functional Fra-2 would produce a similar result. However, an important caveat to such a comparison is that gene expression is not always a clear indication of gene function and it has been suggested that the unnecessary expression of some genes is more economical than the operation of control mechanisms to shut them down (Erickson, 1993). This is not likely to be the case with Fra-2, as there appears to be some level of control on the expression of fra-2 in the developing mouse embryo. The fra-2 gene is differentially expressed and the pattern changes during development. For example, in the skin fra-2 expression is high at day 13.5 p.c in the ventral skin covering the abdomen and decreased at day 17.5 p.c. At day 17.5 p.c there is also an increased in expression in the dorsal skin covering the tail and limbs (Carrasco and Bravo, 1995).

The embryonic lethal result can be bypassed by using another knockout approach, the cre-lox system, which can be used to establish the function of a gene product.
in particular tissues. This approach has been shown to be particularly useful for those genes which are essential for normal development, for example, the DNA polymerase β gene (Kühn et al., 1995). Thus, if the targeting of the fra-2 gene results in embryonic lethality, it may still be possible to determine functional roles for Fra-2 in particular tissues within the mouse. Another possibility is to target cells grown in culture, for example fibroblasts, to produce cells lacking functional expression of the gene. Targeted fibroblasts are often viable even when the whole animal is not, as was found to be the case with c-Jun (Johnson et al., 1993). Therefore, production of knockout tissue culture cells can often be informative as to some of the functional roles of that gene.

The second possibility is that the lack of functional Fra-2 may produce no gross phenotype, as was seen with FosB (Gruda et al., 1996). Lack of a discernible phenotype may indicate that the gene belongs to a group of genes that have overlapping functions and can therefore compensate for the loss of one of the members. For example, MyoD knockout mice showed increased myf-5 expression (Rudnicki et al., 1992). AP-1 members are known to have some overlapping functions, e.g. the expression of the stromelysin I and collagenase I genes have been shown to be regulated by both c-Fos (Hu et al., 1994) and FosB (Gruda et al., 1996). Indeed, inhibition of fibroblast cell cycle progression is more efficient with the use of antibodies directed against more than one member of the Fos family of proteins than with antibodies directed against individual members alone (Kovary and Bravo, 1991). Given the overlapping expression patterns of fra-2 and the other fos genes, the possibility that functional redundancy might obscure any phenotype in fra-2 knockout mice must be considered.

The third possible outcome from the targeting experiment is the presence of an abnormal phenotype in mice lacking functional Fra-2 protein. Presumably, this would reflect the alteration of expression of various genes that are normally targets for transcriptional regulation by Fra-2. Based on previous analyses of Fra-2, there are at least 3 tissues where lack of functional Fra-2 might be predicted to
result in development of an abnormal phenotype. As mentioned earlier, studies with transgenic mice expressing anti-sense fra-2 identified spleen and testes (Foletta, 1995) as two tissues in which loss of Fra-2 might not be compensated for by other AP-1 family members. The third potential target could be the developing kidney, as fra-2 was the only Fos family member identified to be expressed in the differentiating kidney tubules (Carrasco and Bravo, 1995).

Although anti-sense RNA strategies can be useful in reducing expression levels of the targeted gene, in the case of fra-2, given its high endogenous expression level, this approach did not prove to be very useful. Therefore, it was decided to employ a more direct approach to reducing the level of Fra-2 in the mouse, with the expectation that this would be more informative regarding the in vivo role of Fra-2. The successful development of Fra-2 knockout mice and cells should help to answer some of the following questions. Is Fra-2 essential for mouse development? What are some of the functional roles of Fra-2? What roles, if any, does Fra-2 play in the spleen, testes and developing kidney? This chapter describes attempts to use gene targeting to produce mice and cultured cells lacking functional Fra-2 protein.
3.2 Methods

Solutions used in this chapter are listed in alphabetical order in appendix B. The oligonucleotide primers used in this chapter are described in appendix C.

3.2.1 Phenol Extraction

An equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1) (PCI) was added to the DNA sample and the solution was thoroughly mixed by vortexing for 1 minute and then centrifuged for 2 minutes at 14,000 rpm (Eppendorf 5415 C). The upper aqueous phase was transferred to a fresh Eppendorf tube. To back-extract the DNA sample, 50 µl of TE buffer was added to the original tube and the sample was vortexed for 1 minute and then centrifuged for 2 minutes as above. The upper aqueous phase was transferred to the same Eppendorf tube as the first aqueous phase from the first extraction. The combined aqueous volume was extracted a second time with an equal volume of PCI by vortexing and centrifuging as above. The upper aqueous phase was transferred to a fresh Eppendorf tube and then extracted with an equal volume of Chloroform:Isoamyl alcohol (24:1) (CI) by vortexing and centrifuging as above. The upper aqueous phase was transferred to a fresh Eppendorf tube.

3.2.2 PCR Amplification

PCR reactions were performed as described in chapter 2 method 2.2.3 using either SuperTaq polymerase (P. H. Stehelin and CIE Ag, Basel) (see method 2.2.3), AB DNA Taq polymerase (Advanced Biotechnologies) (see method 2.2.6) or 0.5 units of QIAGEN Taq Polymerase in 1 X QIAGEN PCR buffer. The oligonucleotide primers were at a final concentration of 0.25 pmol/µl. Three different PCR programs were employed for the amplifications. Firstly, the genomic fragment program that began with 1 minute at 80°C and then cycled through 15 seconds at 94°C and 5 minutes at 68°C, extending by 10 seconds with each cycle. A final
step of 5 minutes at 72°C was performed to complete partially polymerised strands. Secondly, the NotI fragment program that cycled through: 30 seconds at 94°C; 30 seconds at 68°C; and 3 minutes at 72°C. The PCR reactions began with a hot-start at 80°C for 1 minute and concluded with a final step of 10 minutes at 72°C. Thirdly, the mouse detection program that included cycles of: 30 seconds at 95°C; 30 seconds at 62°C; and 2 minutes at 72°C. There were also initial steps of 1 minute at 80°C and 3 minutes at 94°C and a final step of 10 minutes at 72°C. Aliquots of each PCR product were checked by electrophoresis on a 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide (Sigma) gel.

The PCR fragments were purified by extraction with chloroform to remove the mineral oil and then by Phenol extraction (method 3.2.1). The PCR fragment samples were then run through a NICK spin column (Pharmacia) and a small aliquot of each purified fragment was checked on a 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gel.

### 3.2.3(i) Gel purification of PCR generated fragments

The PCR generated fragments were made blunt ended using T4 DNA polymerase in a reaction containing 1 X T4 DNA polymerase buffer, 0.1 mM of each dNTP and 15 units of T4 DNA polymerase (New England BioLabs). The reaction was incubated at 37°C for 30 minutes. The sample was phenol extracted (method 3.2.1) then loaded onto a preparative 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gel. The appropriate size band was excised from the gel and electroeluted using the BioRad electroeluter (model 422) in 1 X TAE/0.1 % SDS for 45 minutes at 10 mAmps per sample. The eluted DNA sample was transferred into an Eppendorf tube then phenol extracted and finally ethanol precipitated and resuspended in 50 µl of TE buffer. The concentration was determined by electrophoresis of 1 µl aliquots on a 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gel.
bromide gel in 1 X loading dye. The band intensities were compared to known quantities of λ/Hind III plus φX174/Hae III DNA marker bands (New England BioLabs).

3.2.3(ii) Gel Purification of DNA using GENOMED JetSorb protocol

DNA samples were electrophoresed on a preparative 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gel and the appropriate fragment was excised. The DNA was extracted from the gel slice using the JETSORB extraction kit (Genomed). Briefly, 300 μl of Buffer A1 and 10 μl of JETSORB suspension per 100 mg of gel slice were mixed with the gel slice. The reaction was vortexed, incubated at 50°C for 15 minutes and then centrifuged at 14,000 rpm for 30 seconds. The supernatant was carefully removed and the pellet was washed by resuspension in 300 μl of Buffer A1 followed by centrifugation as above. This washing step was repeated twice with 300 μl of Buffer A2. The JETSORB pellet was dried in a Savant SpeedVac and then the DNA was eluted in 20 μl of TE buffer by incubation at 50°C for 5 minutes followed by centrifugation at 14,000 rpm for 30 seconds. The supernatant, containing the DNA, was transferred to a fresh Eppendorf tube.

3.2.4 Ligation and Transformation

Purified DNA fragments were ligated into pGEM3Z plasmid (Promega), previously linearised with Sma I, at various vector to insert ratios: 1:0; 1:1; 1:2; and 2:1. The reactions typically contained 1 X T4 DNA ligase buffer, 1 mM ATP, 150 ng of pGEM3Z, 90-360 ng of the DNA fragment and 400 units of T4 DNA ligase (New England BioLabs). The reactions were incubated at 14°C overnight.
Two different transformation protocols were employed. Firstly, and most commonly, the ligation reactions were transformed into 100 μl of *E. coli* strain DH5α competent cells according to a modified method of Hanahan (Hananhan, 1983) The samples were incubated on ice for 40 minutes, heat-shocked for 1-2 minutes at 42°C and then chilled on ice briefly. A 1 ml aliquot of LB was added to each sample and the reactions were incubated at 37°C for 15 minutes. The cells were pelleted for 1 minute at 14,000 rpm (Eppendorf microfuge) and then resuspended in 110 μl of LB. Aliquots of 10 μl and 100 μl of each sample were spread on 100 mm LB agar/50 μg/ml ampicillin plates which had previously been coated with 0.1 M isopropyl-thiogalactosidase (X-gal) (Sigma) and 1 μg of 5-bromo-4-chlor-3-indolyl β-D-galactopyranoside (IPTG) (Sigma) diluted in dimethylformamide. This allowed for blue-white colour selection of recombinants. The plates were incubated overnight at 37°C in the dark.

Secondly, the ligation reactions were electroporated into SURE electro-competent cells (Stratagene) using a BioRad gene pulser (25 μF, 2.5 kV) and 0.2 cm gene pulser cuvettes (BioRad). After the electroporation pulse, 1 ml of SOC medium was added and the cells were transferred to a 15 ml Falcon tube and incubated at 37°C for 1 hour. The cells were pelleted, resuspended in 110 μl of LB and then 10 μl and 100 μl aliquots were spread onto LB agar plates containing 50 μg/ml ampicillin, 50 μg/ml kanamycin and 35 μg/ml tetracycline. The plates were incubated overnight at 37°C.

3.2.5 DNA linkers

Equimolar amounts of complimentary linker oligonucleotides were mixed together and phosphorylated using 20 units of T4 polynucleotide kinase (T4 PNK) (New England BioLabs) in 1 X T4 PNK buffer at 37°C for 1 hour. The oligonucleotides were then allowed to anneal by incubation at 85°C for 2 minutes,
followed by 15 minute incubations at 65°C, 37°C, 23°C and 0°C. The linkers were ligated to the DNA fragment using 400 units of T4 DNA ligase in 1 X ligation buffer at a molar ratio of 5:1 for 1 hour at room temperature then overnight at 16°C. The T4 DNA ligase was denatured at 65°C for 10 minutes and then the reaction was digested with an appropriate restriction enzyme to cut concatamers and produce sticky ends for the ligation of the linker-DNA fragment into the linearised plasmid. The sample was purified by phenol extraction and then ethanol precipitated. The linker-DNA fragment was further purified by electrophoresis on a 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gel followed by extraction using the JETSORB extraction kit (Genomed) as described above (method 3.2.3(ii)).

3.2.6 (i) Mini-preparation of plasmid DNA

Bacterial colonies were selected and grown overnight at 37°C with shaking in 3 ml of LB containing 50 μg/ml ampicillin. The plasmid DNA was prepared by the rapid boiling method (Holmes and Quigley, 1981). A 1.5 ml aliquot of each culture was centrifuged at 14,000 rpm for 5 minutes and the supernatant was removed. The bacterial pellet was thoroughly resuspended in 350 μl of STET buffer (#32) and 25 μl of freshly prepared lysozyme (10 mg/ml in STET buffer) was added. The tubes were boiled for 1 minute, centrifuged at 14,000 rpm for 10 minutes at room temperature and then 300 μl of the supernatant was removed into fresh tubes containing 300 μl of isopropanol. The tubes were shaken to mix the contents thoroughly, then incubated on dry-ice for 10 minutes. The DNA was pelleted at 14,000 rpm at 4°C for 10 minutes. The DNA pellet was washed in 70% ethanol, dried in a Savant SpeedVac and then resuspended in 30 μl of TE buffer.
3.2.6(ii) Plasmid DNA preparation by QIAGEN protocol

Bacteria containing plasmids of interest were grown overnight at 37°C in 6 ml of LB containing 50 µg/ml ampicillin. The plasmid DNA was prepared using the QIAGEN plasmid mini kit P20 column (QIAGEN) as described by the manufacturer. Briefly, 1.5 ml of culture was pelleted, thoroughly resuspended in 300 µl of buffer P1 and 300 µl of buffer P2 was added. The tube was mixed by inversion and then incubated at room temperature for 5 minutes. 300 µl of buffer P3 was added, the tube was mixed by inversion, incubated on ice for 10 minutes and then centrifuged at 10,000 rpm for 15 minutes. The supernatant was applied to a QIAGEN P20 column that had been previously equilibrated with QBT buffer. The column was washed with 4 x 1 ml aliquots of QC buffer and the plasmid DNA was eluted in 0.8 ml of QF buffer. The eluted DNA was precipitated with isopropanol, dried and resuspended in 15 µl of TE buffer. The concentration was determined by optical density at 260 nm using the following calculation:

\[
\text{DNA concentration (µg/ml)} = \text{OD}_{260} \times 50 \times \text{dilution factor.}
\]

A small aliquot of each purified plasmid was also removed for analysis on a 0.8% agarose/1 X TAE/1 µg/ml ethidium bromide gel.

3.2.6(iii) Plasmid DNA preparation by WIZARD protocol

Plasmid DNA was prepared using the WIZARD mini-preparation kit (Promega). Briefly, 1.5 ml of overnight culture was centrifuged for 5 minutes in an Eppendorf tube. The pellet was resuspended in 200 µl of cell resuspension solution, 200 µl of cell lysis solution was added and the tube was mixed by inversion. 200 µl of neutralisation solution was added and the tube was mixed by inversion and then centrifuged at 14,000 rpm for 5 minutes. The supernatant was loaded onto a WIZARD minicolumn, attached to a 3 ml syringe barrel, with 1 ml of WIZARD miniprep DNA purification resin. The column was washed with 2 ml of column
wash solution and then placed in an Eppendorf tube and centrifuged at 14,000 rpm for 20 seconds to remove the last traces of wash buffer. The column was transferred to a fresh Eppendorf tube and the DNA was eluted with 50 µl of TE buffer by centrifuging at 14,000 rpm for 20 seconds. The plasmid DNA concentrations were determined as described above by optical density at 260 nm.

3.2.7 Large scale plasmid DNA preparation

Overnight cultures of each plasmid-bearing bacteria, which had been grown in 400 ml of LB containing 50 µg/ml ampicillin, were centrifuged at 7,000 rpm for 10 minutes at 4°C in 250 ml GSA bottles (GSA rotor, Sorval centrifuge). The supernatants were removed, the pellets were resuspended in 30 ml of GTE buffer and then 0.15 g of lysozyme (Sigma) was added. The tubes were incubated for 10 minutes at room temperature. 60 ml of freshly prepared NaOH/1% SDS was added and the samples were mixed gently and then incubated for 5 minutes on ice. 30 ml of ice-cold 3M potassium acetate, pH 4.8, was added and a further incubation on ice for 15 minutes was performed. The samples were centrifuged as above and each supernatant was strained through gauze into a fresh GSA bottle to remove excess precipitate. The DNA was precipitated with 0.6 volumes of isopropanol and collected by centrifugation. The pellet was resuspended in TE buffer to a final volume of 8 ml, then 8.6 g of CsCl₂ (Boehringer Mannheim) was added. 4 mg of ethidium bromide (Sigma) was added. The samples were transferred to Oakridge tubes (Nalgene) and centrifuged at 9,000 rpm for 20 minutes (SS34 rotor, Sorval centrifuge). The supernatants were removed to fresh Oakridge tubes and centrifuged at 35,000 rpm for 48 hours at 20°C (Ti50 rotor). The DNA band was carefully removed from the sample and extracted with butanol to remove the ethidium bromide. The DNA samples were ethanol precipitated four times to remove the CsCl₂ and then resuspended in TE buffer. The DNA concentration was determined (see method 3.2.6(ii)).
3.2.8 Restriction endonuclease reactions

DNA was digested with restriction endonucleases in the appropriate 1 X NEB restriction digest buffer (New England BioLabs) for 1-6 hours at 37°C (except for Sma I digests which were done at 25°C). An excess of enzyme was added to each reaction to ensure complete digestion would occur. The minimum units required for each reaction were determined using the following calculation:

\[
\text{units of enzyme} = \frac{Q \times ND}{SD} \times \frac{1 \mu g \lambda/\text{unit} \times N\lambda/S\lambda}{1}
\]

Where, \(Q\) = quantity of DNA to be digested (\(\mu g\)); \(ND\) = Number of restriction sites in the DNA to be digested; \(SD\) = Size of the DNA to be digested (kb); \(N\lambda\) = Number of restriction sites in \(\lambda\); and \(S\lambda\) = Size of \(\lambda\) (approximately 50 kb).

Digest completion was usually confirmed by analysis of a small aliquot of the reaction on a 0.8% agarose/1 X TAE/1 \(\mu g/ml\) ethidium bromide gel. After complete digestion, the restriction enzyme was denatured at 65°C for 10 minutes. Then the digested plasmid DNA was either directly analysed on a 0.8% agarose/1 X TAE/1 \(\mu g/ml\) ethidium bromide gel or the ends were repaired with 10 units of Klenow (New England BioLabs) in 1 X Klenow buffer plus 0.1 mM dNTPs at 37°C for 30 minutes, and/or dephosphorylated at the ends with 10 units of calf intestinal alkaline phosphatase (CIAP) (Pharmacia) in 1 X One-Phor-All buffer at 37°C for 30 minutes. The CIAP enzyme was denatured at 75°C for 10 minutes and then loading dye was added to a final 1 X concentration and the samples were electrophoresed on a 0.8% agarose/1 X TAE/1 \(\mu g/ml\) ethidium bromide gel. The gels were viewed using a UV transilluminator and photographed.
3.2.9 Southern Blotting

Agarose gels were depurinated in 0.25 M HCl until the bromothymol blue from the loading dye turned bright yellow (approximately 7 minutes). The gel was rinsed in distilled water, then soaked in 2 changes of denaturing solution for 15 minutes and finally in 2 changes of neutralising solution for 15 minutes. The gel was then blotted overnight onto Hybond-C nitrocellulose (Amersham) by capillary transfer (Southern, 1975) in 20 X SSC. The following day the blot was dismantled and the nitrocellulose filter was rinsed in 2 X SSC. The filter was air dried for approximately 30 minutes and then baked in a vacuum oven (Napco, model 5831) at 80°C for 1-2 hours. Filters were stored at room temperature until they were used in hybridisation experiments.

3.2.10 PCR Probe generation

An exon 2 specific probe for fra-2 was generated by PCR using SuperTaq polymerase (P. H. Stehelin and CIE Ag, Basel) at 0.2 units/30 µl reaction in 1 X SuperTaq polymerase buffer. A 10 ng aliquot of the pGEMEx2 plasmid was used as the template with oligonucleotide primers F2Ex2 and F2Ex2c at 3.33 pmole/µl final concentration. The dNTPs were added at a final concentration of 5 µM except for dCTP, whose concentration was slightly lower at 4.5 µM. The probe was labelled by adding 50 µCi of $^{32}$P dCTP (Bresatec) to the reaction. The reaction was covered with 50 µl of mineral oil (Sigma) to stop evaporation and 16 cycles of PCR were performed. The probe PCR program cycle was as follows: 30 seconds at 94°C; 30 seconds at 60°C; 2 minutes at 72°C, except for the first cycle which began with 3 minutes at 94°C. A final step of 5 minutes at 72°C was performed to complete partially polymerised strands.
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Fifty microlitres of TE buffer was added to the PCR probe reaction which was then processed by extraction with 100 µl of Chloroform to remove the mineral oil followed by phenol extraction (method 3.2.1). The probe was further purified through a NICK (Pharmacia) column to remove unincorporated dNTPs. Two 2 µl aliquots were removed for scintillation counting in Beckman ready safe liquid scintillation cocktail in a Packard 1500 Tri-carb liquid scintillation analyser.

3.2.11 Oligonucleotide probe generation

End-labelling of oligonucleotide primers was performed with 100 pmole of primer, 80 µCi of γ-32P ATP (Bresatec) and 10 units of T4 PNK (New England BioLabs) in 1 X T4 PNK buffer at 37°C for 45 minutes. The enzyme was then denatured at 68°C for 10 minutes. The reaction was ethanol precipitated with ammonium acetate and the labelled primer was resuspended in 100 µl of TE buffer.

3.2.12 Hybridisation

The filter was re-wet in 2 X SSC and then pre-hybridised in 40 ml of 1 X pre-hybridisation solution for approximately 1 hour at 42°C. The probe was denatured at 95°C for 5 minutes and then added to 40 ml of 1 X hybridisation solution. The filter was hybridised overnight at 42°C and the following day the filter was rinsed briefly in 0.5 X SSC/0.1% SDS at room temperature twice, then washed twice in 0.5 X SSC/0.1% SDS at 55°C for 1 hour. The filter was placed in a radiography cassette with a piece of Kodak X-OMAT AR X-ray film. The film was exposed to the filter for 10 minutes then developed in a Kodak OMAT M20 processor.
3.2.13 Colony Hybridisation

A Hybond-C nitrocellulose filter (Amersham) was placed on a 100 mm LB agar plate containing 50 μg/ml ampicillin. White colonies from the original plate of transformants were transferred, using a sterile toothpick, onto the filter and onto a master LB agar/50 μg/ml ampicillin plate pre-coated with X-gal and IPTG as described above. The colonies were grown overnight at 37°C. The master plate was sealed with parafilm and stored at 4°C. The nitrocellulose filter was carefully removed from the other plate and denatured for 5 minutes by placing it on Whatman 3 MM paper previously wet in denaturing solution. The filter was then placed on Whatman 3 MM paper pre-wet with neutralising solution and finally onto Whatman 3 MM paper wet with 2 x SSC for 5 minutes each. The filters were air dried for 30 minutes and then baked at 80°C in a vacuum oven (Napco) for 1-2 hours to fix the DNA onto the filter.

The filters were wet in 2 X SSC and then incubated in 5 X SSC/0.5% SDS/1 mM EDTA, pH 8 at 50°C for 30 minutes. The filters were pre-hybridised in 40 ml of 1 X pre-hybridisation solution at 42°C for 1 hour, then hybridised in 40 ml of 1 X hybridisation solution containing 1-3 x 10^6 cpm/ml denatured PCR-generated probe (see method 3.2.10) at 42°C overnight. The filters were washed twice in 0.5 X SSC/0.1% SDS briefly at room temperature and then twice at 55°C for 1 hour. The filters were exposed to Kodak X-OMAT AR X-ray film for 3 hours then developed in a Kodak OMAT M20 processor.

Colony filters that were hybridised with an end-labelled oligonucleotide primer were treated as follows. The filters were pre-hybridised in 30 ml of end-labelling hybridisation buffer at 65°C for 1 hour. The labelled primer probe was added (8-9 x 10^5 cpm/ml final concentration) with another 10 ml of end-labelling hybridisation buffer and the filters were hybridised for 2 hours at 65°C. The filters were washed in 250 ml of 2 X SSC/0.1% SDS for 3 x 5 minutes. They were then
exposed to Kodak X-OMAT AR X-ray film in a radiography cassette at -70°C overnight.

3.2.14 Sequencing

The T7 sequencing protocol, which is based on the chain termination method (Sanger et al., 1977), was followed as per manufacturer instructions (Pharmacia). Briefly, 3 μg of plasmid DNA for each primer reaction was denatured in 0.4 M NaOH for 10 minutes at room temperature. The DNA was recovered by precipitation in 0.4 M NaOAc and 70% ethanol for 15 minutes on dry ice, followed by centrifugation at 4°C for 15 minutes at 14,000 rpm (Eppendorf, 5415C). The DNA pellet was dried in a Savant SpeedVac concentrator (model SVC-100H) for 5 minutes, and then resuspended in 10 μl of distilled water. Five picomoles of the appropriate primer was added and an annealing reaction was performed in 1 X annealing buffer at 65°C for 5 minutes followed by 37°C for 10 minutes and finally room temperature for at least 5 minutes. The annealed plasmid-DNA primer mix was then taken into a labelling reaction containing 1 X labelling mix-dATP, 10 μCi 33P dATP (Bresatec) and 3.2 units of T7 DNA polymerase diluted in T7 DNA polymerase dilution buffer. The reaction was incubated for 5 minutes at room temperature. The labelling mix was divided into four tubes (4.5 μl per tube) containing 2.5 μl of either A-short mix, G-short mix, C-short mix or T-short mix. The reactions were incubated at 37°C for 5 minutes and then 5 μl of stop solution was added. The reactions were stored at -20°C until required.

A denaturing 8% polyacrylamide sequencing gel was prepared using the BioRad Sequi-Gen nucleic acid sequencing cell apparatus and the gel was left to polymerise overnight. The gel was pre-run at 2000 volts (LBK Bromma 2197 power supply) until the temperature was 45-50°C (approximately 1 hour). The
sequencing reactions, previously prepared, were thawed and denatured at 80°C for 2 minutes. A 3 μl aliquot of each reaction was loaded onto the sequencing gel. The gel was run at 2000 volts for approximately 4 hours.

The sequencing gel was fixed in 10% acetic acid for 7 minutes, and then dried in a BioRad gel drier (model 383) at 50°C for 30-40 minutes and exposed to Kodak X-OMAT AR X-ray film in an autoradiograph cassette at -80°C for 1-3 days.

3.2.15 Erase-a-Base protocol

The Erase-a-Base system (Promega) is based on the procedure developed by Henikoff (Henikoff, 1984) using exonuclease III (Exo III). The manufacturer’s instructions were followed. The plasmid DNA, 15-20 μg, was digested with 30 units of Sph I (New England BioLabs) in 1 X NEB restriction buffer #2 at 37°C for 1-2 hours to generate a 3’ overhang; this protected the primer binding site from Exo III digestion. The plasmids were then digested with 100 units of Sal I (New England BioLabs) in 1 X NEB restriction buffer #3 at 37°C for 1-2 hours to produce a 5’ overhang which was susceptible to Exo III digestion. The samples were phenol extracted (method 3.2.1) and then ethanol precipitated with 100 mM NaCl. The digested plasmids were resuspended in 60 μl of 1 X Exo III buffer (Promega) and warmed to 37°C.

Exo III deletions were performed at a rate of 450 bp/minute at 37°C with 500 units of Exo III. 2.5 μl samples were removed every 30 seconds from the deletion reaction into tubes containing 2.25 units of S1 nuclease in 1 X S1 nuclease buffer previously chilled on ice. The 20 samples collected were incubated at 37°C for 30 minutes and then 1 μl of stop solution was added followed by incubation at 70°C for 10 minutes. The extent of deletion was determined by removing 2 μl aliquots from each sample to analyse by electrophoresis on a 0.8% agarose/1 X TAE/1
µg/ml ethidium bromide gel. The remainder of each sample was incubated at 37°C and the DNA ends were filled in using 3.5 units of Klenow DNA polymerase in 1 X Klenow buffer for 3 minutes. 0.125 mM of each dNTP was added and the reactions were incubated for a further 5 minutes. The deleted plasmid samples were re-ligated in 40 µl of freshly prepared ligase mix at room temperature for 1 hour. A 10 µl aliquot of each sample was transformed into E. coli DH5α competent cells as described above (method 3.2.4). The samples were plated onto LB agar/50 µg/ml ampicillin plates and incubated overnight at 37°C. Four colonies were picked from each deletion time point and screened by the ‘cracking’ method (Bames, 1977). The bacterial colonies were first spotted onto a master LB agar/50 µg/ml ampicillin plate and then added to 30 µl of 1 X cracking buffer and vortexed well. The samples were incubated for 5 minutes at room temperature and then 10 µl aliquots were analysed on 0.8% agarose/1 X TAE/µg/ml ethidium bromide gels. Ten progressively deleted plasmids for each orientation of the insert were selected for sequencing.

3.2.16 Transfection of ES cells for Gene Targeting

Forty micrograms of plasmid DNA was digested with an appropriate restriction enzyme to linearise the DNA. Completion of digestion was assessed by analysis on a 0.8% agarose/1 X TAE/1 µg/ml ethidium bromide gel. Once digestion was complete the enzyme was denatured at 65°C for 30 minutes. The DNA sample was purified by phenol extraction (method 3.2.1) and the DNA was recovered by ethanol precipitation. Dr. Robyn Slattery and Ms Eloisa Pagler performed the transfection of ES cells. The E14.1 ES cells were cultured on MEF feeders. A minimum of 10^7 cultured ES cells were transfected with 20 µg of linearised pGfra2neo plasmid by electroporation (0.24 Kvolts, 500 µF, 0.4 cm BioRad cuvette, BioRad gene pulser). The cells were allowed to recover for 24 hours before 350 µg/ml of Geneticin (G-418 sulfate) (GibcoBRL) was added for
selection of transfected cells. The transfected ES cells were cultured for at least 7
days at 37°C/5% CO₂. Colonies were selected and plated into individual wells of
48-well plates and cultured for 3 days. 80% of each cell population that had grown
was frozen down for later use and the remainder was cultured for a further 5 days
in ES media. DNA was prepared from the clones as follows. Cells were scraped
from the wells and collected by brief centrifugation. The cells were then
resuspended in 500 µl of lysis buffer containing 100 µg/ml of proteinase K
(Promega) and incubated overnight at 56°C. The samples were centrifuged at
6,000 rpm for 10 minutes and each supernatant was removed to a fresh Eppendorf
tube containing 500 µl of isopropanol to precipitate the DNA. The DNA threads
were collected using a glass pipette hook and allowed to air dry for 5 minutes. The
DNA samples were resuspended in 100 µl of TE buffer and dissolved at 65°C for
1-2 hours. The DNA concentration was determined by optical density at 260 nm
(see method 3.2.6(ii)). The DNA was analysed by PCR (refer to method 2.2.6) to
screen for homologous recombination using oligonucleotide primers: F2Ex3c;
F2Ex3c.2; NeoP6 and F2SD3.

3.2.17 Generation of targeted Swiss 3T3 fibroblasts

Swiss 3T3 cells were cultured in Dulbecco’s modification of Eagle’s medium
(DMEM) (Sigma) containing 10% fetal calf serum (FCS) until confluent. The
cells were trypsinized and washed off the culture flask in DMEM containing 10%
FCS. A minimum of 1-2 x 10⁷ cells were resuspended in 300 µl of DMEM
containing 10% FCS. The cells were transfected with 30-40 µg of linearised
plasmid DNA, which had been previously resuspended in 100 µl of PBS. Cells
were transfected by electroporation (500 µF, 270 volts, 0.4 cm BioRad cuvette,
BioRad Gene Pulser with a capacitance extender attached). The electroporated
cells were split into three 100 mm dishes and cultured in DMEM containing 10%
FCS. The cells were allowed to recover for 24 hours at 37°C/5% CO₂ and then the
media was replaced with fresh DMEM plus 10% FCS and the cells were cultured for a further 24 hours at 37°C/5% CO₂. The cells were then washed in 2 x 5 ml of PBS and grown under the appropriate selection in 10 ml of DMEM containing 10% FCS. The cells were cultured under selection at 37°C/5% CO₂ for 7 days. For selection, the media for pGfra2neo transfected cells contained 350 µg/ml of G-418 sulfate (GibcoBRL), and the media for pGf2HTK transfected cells contained 200 µg/ml Hygromycin B (GibcoBRL). If positive-negative selection was performed, the cells were washed in PBS as above and then cultured in media containing 200 µg/ml Hygromycin B (GibcoBRL) and 2 µM Ganoclovir (Cymeven, Syntax) at 37°C/5% CO₂ for a further 7-10 days.

Clones were selected from the plates of transfected cells and cultured in 48 well plates (CoStar) for 3-5 days. The cell populations were then expanded for a further 5 days in 60 mm dishes. DNA was prepared from the cells and analysed by PCR (see method 3.2.16 above)

3.2.18 Genomic DNA preparation

Genomic DNA was prepared from cell cultures using the WIZARD genomic DNA preparation kit (Promega). Briefly, the cells were trypsinised and then washed off the culture plates in 5 ml of PBS. The cells were collected by centrifugation at 1500 rpm for 5 minutes and then resuspended by pipetting in 600 µl of Nuclei Lysis Solution. 3 µl of RNase solution was added, the tube was mixed by inversion and then incubated at 37°C for approximately 30 minutes and then room temperature for a further 5 minutes. 200 µl of Protein Precipitation Solution was added and the tube was vortexed vigorously for 20 seconds. The protein was removed by centrifugation at 14,000 rpm for 3 minutes; the supernatant was transferred to a clean Eppendorf tube. The DNA was precipitated by addition of an equal volume of isopropanol. The DNA was collected using a
glass pipette hook, rinsed in 70% ethanol and then air dried for 5-10 minutes. The DNA was resuspended in 100 μl of DNA Rehydration Solution at 65°C for several hours.
3.3 Results

3.3.1 The generation of pGCBEx2 plasmid

The generation of Fra-2 knockout mice was to be accomplished using C57Bl6 mouse ES cells that had lost one copy of the endogenous fra-2 gene by homologous recombination. A knockout construct involving fra-2 exon2 was designed to make these ES cells. The pGEMEx2 plasmid (made by Victoria Foletta) carries a genomic mouse fra-2 fragment containing exon 2. However, the genomic DNA in this plasmid is from an unknown strain of mouse and the idea was to target C57Bl6 ES cells using an isogenic targeting construct. Therefore, it was necessary to obtain a genomic fragment containing fra-2 exon 2 from C57Bl6 DNA. This would be done by PCR. Thus, the pGEMEx2 plasmid was partially sequenced using the Pharmacia T7 sequencing protocol. Oligonucleotide primers SP6 and the universal M13 primer, which are complimentary to plasmid vector sequences, were employed to sequence both ends of the plasmid insert. The sequence information obtained was used to design four oligonucleotide primers corresponding to the ends of the genomic fra-2 fragment present in the plasmid pGEMEx2. Two oligonucleotide primers were designed for each end of the insert. F2int1.1 and F2int1.2 were obtained from the SP6 primer sequencing reactions and correspond to sequences in intron 1 of the fra-2 gene. F2int2.3 and F2int2.4 were obtained from the M13 universal primer sequencing reactions and correspond to sequences in intron 2. The oligonucleotide primers were prepared as described in method 2.2.2.

The oligonucleotide primers, F2int1.1, F2int1.2, F2int2.3 and F2int2.4 were used in various combinations in nested PCR reactions using SuperTaq DNA polymerase and a C57Bl6 genomic DNA template (see table below).
**Reaction** | **First round PCR** | **Second round PCR** | **PCR fragment produced**
--- | --- | --- | ---
1 | F2int1.1 | F2int1.1 | CBEx2.1.4
2 | F2int1.1 | F2int1.2 | CBEx2.2.3
3 | F2int1.1 | F2int1.2 | CBEx2.2.4
4 | F2int1.2 | F2int2.3 | CBEx2.2.4

Different amounts of C57Bl6 genomic DNA were used in the reactions (100 ng, 250 ng or 500 ng) to see which would give the best amplification product. Control reactions using pGEMEx2 plasmid DNA (2 ng and 10 ng) were also performed. Figure 3.1a shows a schematic presentation of the PCR strategy.

A Southern blot was done to confirm the presence of fra-2 exon 2 in the PCR fragments produced. The results of this are shown in figure 3.1b and c, and clearly demonstrate the presence of fra-2 exon 2 in all the PCR fragments generated. Thus the PCR reactions were successful. Therefore, the PCR fragments were blunt-end ligated into the Sma I site of pGEM3Z. The fragment Ex2.2.3 (see table above) was used to generate the plasmids pGCBEx2a, which contained the insert in one orientation, and pGCBEx2b, which contained the insert in the opposite orientation.

### 3.3.2 Sequencing of pGCBEx2

The two pGCBEx2 plasmids were sequenced using the Erase-a-Base sequencing system and the T7 sequencing kit with the SP6 oligonucleotide primer, as previously described in methods 3.2.14 and 3.2.15. The sequenced sub-clones were aligned using the SeqED program version 1.0. Approximately 88% of the sequence of the 3.3 kb C57Bl6 genomic fra-2 fragment was obtained from the sequentially deleted templates generated by the Erase-a-Base protocol. Gaps in the sequence information were filled in from supplementary sequencing reactions.
Fra-2 Knockout studies: Results

**Figure 3.1**: Generation and Southern blot of the genomic C57Bl6 fra-2 PCR fragments

a) Schematic representation of pGEMEx2 plasmid. The pGEMEx2 plasmid, containing a genomic fra-2 DNA fragment covering exon 2, was sequenced at each end using M13 or SP6 (open head arrows) oligonucleotide primers. Oligonucleotide primers F2int1.1, F2int1.2, F2int2.3 and F2int2.4 (solid arrowheads) were designed based on the sequence information obtained.

b) Nested PCR amplifications were carried out using C57Bl6 genomic DNA as template and various combinations of the oligonucleotide primer F2int1.1, F2int1.2, F2int2.3 and F2int2.4. Thirty PCR cycles of the genomic fragment program were performed for the first round reactions and 25 cycles for the second round (see method 3.2.2) using the SuperTaq DNA polymerase. A 2 μl aliquot of each PCR product was run on a 0.8% agarose/TAE/1 μg/ml ethidium bromide gel. The gel was visualised on a UV transilluminator and photographed. The letter codes depict which primer pairs were used to generate the fragments in the second round of PCR amplification: A = F2int1.1 and F2int2.4 primers, B = F2int1.2 and F2int2.3 primers, C = F2int1.2 and F2int2.4. The numbers 1, 2 and 3 identify the amount of C57Bl6 genomic DNA template used in the PCR reactions, 100 ng, 250 ng or 500 ng respectively. pGEMEx2 (PC) was used as a positive control and Water (WA and WC) was used as a negative control in the PCR reactions. M = DNA size markers (λ/HindIII plus φX174/HaeIII).

c) The gel was blotted to nitrocellulose in 20 X SSC (see method 3.2.9) and the filter was hybridised overnight at 42°C with a PCR generated fra-2 exon 2 specific probe prepared using primers F2Ex2 and F2Ex2c and SuperTaq DNA polymerase (see method 3.2.10). Following washing in 0.5 X SSC/0.1% SDS at 55°C, the filter was exposed to film for 10 minutes.
(a) Fractionation studies: Results

(b) Gel analysis showing a 3.3 kb band

(c) Gel analysis showing a 3.3 kb band
that employed insert-specific primers. The complete sequence of the 3.3 kb *fra-2* genomic fragment is shown in figure 3.2.

### 3.3.3 Insertion of Not I restriction site

The pGCBEx2b plasmid was digested with a range of restriction enzymes to check the presence of unique sites and the absence of sites within the insert. This was to identify appropriate sites for insertion of the Neo\(^R\) and the TK genes. A unique site within exon 2 was required for Neo\(^R\) gene insertion and a 3' or 5' site was required for the TK gene insertion. Restriction enzymes *BspMII, Apa I, Acc I, Avr II, BamHI, Xba I, Not I, Sma I, Nco I, Sfi I, Cla I, Nhe I* and *EcoRV* were tested. Two restriction enzymes were identified, *Apa I* and *Avr II*, that cut only once in the 3' end of the insert fragment (see figure 3.2) and restriction sites for *Not I, Sma I, Nco I, Cla I, Nhe I* and *EcoRV* were all absent. A closer look at the sequence revealed a sequence within exon 2 which was very close to a *Not I* site (see figure 3.2); the alteration of two nucleotides, TG to GC, would produce a unique *Not I* restriction site at that position.

A PCR strategy was employed to change these two nucleotides on both strands of the DNA (figure 3.3). The strategy employed the use of two new oligonucleotide primers corresponding to sequences within exon 2, which incorporated the new *Not I* site: *Ex2NotI5' and Ex2NotI3'* (figure 3.3a). The *Not I* site is identified in bold with the two altered nucleotides in lower case. These primers were paired with the F2int1.2 and F2int2.3 oligonucleotide primers as shown in figure 3.3b to produce two overlapping fragments from the pGCBEx2b plasmid by PCR. Fragment 1 was generated using F2int1.2 and Ex2NotI3' primers and fragment 2 was generated using primers Ex2NotI5' and F2int2.3. The PCR amplifications were performed using the *NotI* fragment program as described in method 3.2.2. The two PCR fragments were purified and a second PCR step was performed
Figure 3.2: Sequence of pGCBEx2b plasmid insert

A plasmid, pGEMEx2, containing a mouse fra-2 genomic fragment of unknown origin covering exon 2, was partially sequenced using SP6 and M13 oligonucleotide primers. From this sequence information, specific oligonucleotide primers were designed. These primers were used to produce a corresponding fragment from C57Bl6 mouse DNA by nested PCR. The fragment, CBEx2.2.3, was cloned into pGEM3Z and then sequenced using Promega’s Erase-a-base system and the Pharmacia T7 sequencing protocol (see methods 3.2.14 and 3.2.15). The sequence of this fragment is presented in the figure. The sequence in lower case at the beginning of the sequence is not present in the final fragment used to generate the pGCBEx2 plasmid. It indicates the primer F2int1.1 used in the first round of PCR amplification. The underlined sequence indicates exon 2 and the sequence shown in yellow indicates the sequence that was targeted to produce a Not I site. The two nucleotides to be changed, to generate the Not I site, are shown in their unaltered form in lower case. The Apa I and Avr II restriction sites used to insert the TK gene and linearise the construct for transfection are also indicated.
2321 TCTCCTTTTC AAATGTGAAT CTGGTGAAAC CTTCCTGTTA ACTGTGGAGG CCTTCACCCC AACTCCTGTC TGTTTCCACT
2401 CCGGACCCCA GTGTCCACTC TATCCTAGTA CCAGTACCAA TAAACATGGC TTCATGTGCA TAGTGAGGGA CCCAGACCCC
2481 ACAGACCCCA GTATGCCCGG AGTTACTGGG CTGACAAAGA GCTGGAGGCT CCCCCCTCAG CAACCTTGCT CCCCAGAAGA
2561 CAACATGGGA CCTCATGGCC AATAATCCAT GCTGACATAG GCCCCAGTGA A GGGCCC CCA AGGGCTATGG GTAGTAGACA
2641 CTGCAGTCCA GTTGCACTGG TGTTGAGTGC TACAGGCCC CTTCTGGGCT TCCCCCTGTC ACAGTTACAG GTGATGATGC
2721 AGTGCACTCA CTGCGGCTTTT TGGTTGTACT TCCCAAGGTA AGGCAATGAC GTTTGATCA AAATATCAAT TTGCTGAGAC
2801 GTCAGATCGT TCTAGCATAT ACATGATCCT GAGTGAGCCA GGCCCCCTGG GGTGTGGGAG ACATTGAAGG GCAGCTGGTG
2881 TCAGCCTTTT TCTCTGTGGG GTCTGACCTCA CACTACATGC AGAATTTAT ACCTGAGGAG GCCAGTTGAG ACTGTATCTC
2961 TAAACTCTTC CCCCTGAGCC AGCTCATAGA TAAAGAATGC CAGACACAAGC GAGAAGGCC CAGAGTTACA CAGGTTTAGT
3041 AGCAGAGACT GGGATCCACC ATATTGACCT TCAGTTGAGT GGTGTGGATG CCACATCACC TAGATGTGCT TCAAGCCAG
3121 TCATCTGAC ACATCCTCTT GTGCGAGGGA AAGAGAGAGA AGACAGAGG ACCACTAACC ATATTAGAAT CTCTGCCTAG
3201 CCAGCCACAT TCTAGAGCCT TGACTCTTTG TGCTCTCCG CCTAGG TCTT GTCTGTGACT GTTCCACTG CCACCTCTGT
3281 TTCTACCCTA AGGCTTCCCA CAAGAGTCTG CTGATGGTGC CT CATG

Apa I

2561 CAACATGGGA CCTCATGGCC AATAATCCAT GCTGACATAG GCCCCAGTGA AGGGCCC AGGGCTATGG GTAGTAGACA
2641 CTGCAGTCCA GTTGCACTGG TGTTGAGTGC TACAGGCCC CTTCTGGGCT TCCCCCTGTC ACAGTTACAG GTGATGATGC
2721 AGTGCACTCA CTGCGGCTTTT TGGTTGTACT TCCCAAGGTA AGGCAATGAC GTTTGATCA AAATATCAAT TTGCTGAGAC
2801 GTCAGATCGT TCTAGCATAT ACATGATCCT GAGTGAGCCA GGCCCCCTGG GGTGTGGGAG ACATTGAAGG GCAGCTGGTG
2881 TCAGCCTTTT TCTCTGTGGG GTCTGACCTCA CACTACATGC AGAATTTAT ACCTGAGGAG GCCAGTTGAG ACTGTATCTC
2961 TAAACTCTTC CCCCTGAGCC AGCTCATAGA TAAAGAATGC CAGACACAAGC GAGAAGGCC CAGAGTTACA CAGGTTTAGT
3041 AGCAGAGACT GGGATCCACC ATATTGACCT TCAGTTGAGT GGTGTGGATG CCACATCACC TAGATGTGCT TCAAGCCAG
3121 TCATCTGAC ACATCCTCTT GTGCGAGGGA AAGAGAGAGA AGACAGAGG ACCACTAACC ATATTAGAAT CTCTGCCTAG
3201 CCAGCCACAT TCTAGAGCCT TGACTCTTTG TGCTCTCCG CCTAGG TCTT GTCTGTGACT GTTCCACTG CCACCTCTGT
3281 TTCTACCCTA AGGCTTCCCA CAAGAGTCTG CTGATGGTGC CT CATG

Avr II

2561 CAACATGGGA CCTCATGGCC AATAATCCAT GCTGACATAG GCCCCAGTGA AGGGCCC AGGGCTATGG GTAGTAGACA
2641 CTGCAGTCCA GTTGCACTGG TGTTGAGTGC TACAGGCCC CTTCTGGGCT TCCCCCTGTC ACAGTTACAG GTGATGATGC
2721 AGTGCACTCA CTGCGGCTTTT TGGTTGTACT TCCCAAGGTA AGGCAATGAC GTTTGATCA AAATATCAAT TTGCTGAGAC
2801 GTCAGATCGT TCTAGCATAT ACATGATCCT GAGTGAGCCA GGCCCCCTGG GGTGTGGGAG ACATTGAAGG GCAGCTGGTG
2881 TCAGCCTTTT TCTCTGTGGG GTCTGACCTCA CACTACATGC AGAATTTAT ACCTGAGGAG GCCAGTTGAG ACTGTATCTC
2961 TAAACTCTTC CCCCTGAGCC AGCTCATAGA TAAAGAATGC CAGACACAAGC GAGAAGGCC CAGAGTTACA CAGGTTTAGT
3041 AGCAGAGACT GGGATCCACC ATATTGACCT TCAGTTGAGT GGTGTGGATG CCACATCACC TAGATGTGCT TCAAGCCAG
3121 TCATCTGAC ACATCCTCTT GTGCGAGGGA AAGAGAGAGA AGACAGAGG ACCACTAACC ATATTAGAAT CTCTGCCTAG
3201 CCAGCCACAT TCTAGAGCCT TGACTCTTTG TGCTCTCCG CCTAGG TCTT GTCTGTGACT GTTCCACTG CCACCTCTGT
3281 TTCTACCCTA AGGCTTCCCA CAAGAGTCTG CTGATGGTGC CT CATG

F2int2.4

2561 CAACATGGGA CCTCATGGCC AATAATCCAT GCTGACATAG GCCCCAGTGA AGGGCCC AGGGCTATGG GTAGTAGACA
2641 CTGCAGTCCA GTTGCACTGG TGTTGAGTGC TACAGGCCC CTTCTGGGCT TCCCCCTGTC ACAGTTACAG GTGATGATGC
2721 AGTGCACTCA CTGCGGCTTTT TGGTTGTACT TCCCAAGGTA AGGCAATGAC GTTTGATCA AAATATCAAT TTGCTGAGAC
2801 GTCAGATCGT TCTAGCATAT ACATGATCCT GAGTGAGCCA GGCCCCCTGG GGTGTGGGAG ACATTGAAGG GCAGCTGGTG
2881 TCAGCCTTTT TCTCTGTGGG GTCTGACCTCA CACTACATGC AGAATTTAT ACCTGAGGAG GCCAGTTGAG ACTGTATCTC
2961 TAAACTCTTC CCCCTGAGCC AGCTCATAGA TAAAGAATGC CAGACACAAGC GAGAAGGCC CAGAGTTACA CAGGTTTAGT
3041 AGCAGAGACT GGGATCCACC ATATTGACCT TCAGTTGAGT GGTGTGGATG CCACATCACC TAGATGTGCT TCAAGCCAG
3121 TCATCTGAC ACATCCTCTT GTGCGAGGGA AAGAGAGAGA AGACAGAGG ACCACTAACC ATATTAGAAT CTCTGCCTAG
3201 CCAGCCACAT TCTAGAGCCT TGACTCTTTG TGCTCTCCG CCTAGG TCTT GTCTGTGACT GTTCCACTG CCACCTCTGT
3281 TTCTACCCTA AGGCTTCCCA CAAGAGTCTG CTGATGGTGC CT CATG

F2int2.4 (cont)

3201 CCAGCCACAT TCTAGAGCCT TGACTCTTTG TGCTCTCCG CCTAGG TCTT GTCTGTGACT GTTCCACTG CCACCTCTGT
3321 TTCTACCCTA AGGCTTCCCA CAAGAGTCTG CTGATGGTGC CT CATG
The pGCBEx2b sequence (see figure 3.2) was engineered to produce a NotI restriction site within exon 2 using a PCR strategy.

a) The sequence from nucleotides 1601 to 1760 of the altered pGCBEx2b plasmid. Oligonucleotide primers, Ex2NotI3' and Ex2NotI5' (as indicated on sequence), were used to change nucleotides TG to GC (shown in lower case bold) to complete a NotI restriction site (shown in bold).

b) The PCR strategy employed to engineer the NotI site within exon 2. The PCR amplifications were performed using AB Taq polymerase and the NotI fragment program as described in method 3.2.2. Initially two fragments containing the generated NotI restriction site were created by pairing the oligonucleotide primers F2int1.2 with Ex2NotI3' to produce fragment 1 and Ex2NotI5' with F2int2.3 to produce fragment 2 (as indicated). The two fragments produced were then used as templates together in a second PCR reaction using oligonucleotide primers F2int1.2 and F2int2.4 (as indicated). This produced the final C57Bl6 genomic fra-2 fragment containing exon 2 with a NotI restriction site, called Ex2NotI. This fragment was blunt end ligated into the SmaI restriction site of pGEM3Z. The final plasmid was called pGE2NotI.
Fra-2 Knockout studies: Results

First PCR Step

Second PCR Step
using approximately 10 ng of each fragment together with oligonucleotide primers F2int1.2 and F2int2.4. The PCR conditions were as described above for the generation of the two fragments. The PCR fragment that was produced was purified and a small aliquot was removed for digestion with Not I restriction enzyme to confirm the presence of the Not I site (data not shown).

The Ex2NotI PCR fragment was repaired and ligated into the Sma I site of pGEM3Z. A positive clone was selected by colony hybridisation (see method 3.2.13) and confirmed by restriction digests with EcoR1 and Not I restriction enzymes. This plasmid was called pGEx2NotI.

3.3.4 Generation of the gene targeting constructs

pGEx2NotI plasmid DNA was prepared using a QIAGEN plasmid mini kit P20 column (method 3.2.6(ii)). The plasmid DNA was digested with the Not I restriction enzyme and then dephosphorylated using CIAP. The linearised plasmid was gel purified (method 3.2.3) and recovered by using the JETSORB extraction kit (method 3.2.3(ii)).

The Neomycin resistance (Neo\textsuperscript{R}) gene cassette, in which the Neo\textsuperscript{R} gene was linked to the PGK promoter and the PGK Poly-adenylation site, was excised from the pBSK-PGK-Neo plasmid (obtained from Dr. Klaus Matthaei) by restriction digestion with EcoRI and HindIII. The DNA fragment containing the cassette was dephosphorylated and gel purified by extraction from the gel slice using the JETSORB extraction kit (method 3.2.3(ii)).

As the Neo\textsuperscript{R} gene cassette fragment possessed EcoRI and HindIII sticky ends, an oligonucleotide linker was designed which would allow the cassette fragment to be ligated into the Not I site of pGEx2NotI. Two partially complementary
oligonucleotides were synthesised and were annealed as described in method 3.2.5. The resulting linker, E1NotH3;

\[
\begin{align*}
5' & \text{AATTCGCGGCCGACATCTA} & \cdots & 3' \\
3' & \cdots & \text{GCGCCGGCGTCTAGATTCG} & 5'
\end{align*}
\]

was ligated to the Neo\textsuperscript{R} gene fragment overnight and then digested with the Not I restriction enzyme. The linker- Neo\textsuperscript{R} fragment was purified and sticky end ligated to the linearised pGEX2NotI plasmid. A colony hybridisation screen (see method 3.2.13) of 330 clones with an end-labelled primer, NeoP1, which is specific for the neomycin resistance gene, revealed many positive clones. Twenty positive clones were selected and plasmid DNA was prepared using the plasmid mini-preparation protocol described previously (method 3.2.6(i)). The presence of the Neo\textsuperscript{R} gene within the pGEX2NotI plasmid was confirmed by restriction enzyme digests with Sal I, Not I, BamHI and Nco I plus Apa I. The resulting plasmid was called pGfra2neo (figure 3.4a).

The TK gene cassette, in which the TK gene was linked to a PGK promoter and a PGK Polyadenylation site, was excised from the pBSK-PGK-TK plasmid (obtained from Dr. Klaus Matthaei) by restriction digestion with EcoRI and HindIII. The DNA ends were repaired. Then the TK fragment was purified from a preparative gel slice using the JETSONSORB extraction kit (method 3.2.3(ii)).

An aliquot of the pGfra2neo plasmid was linearised with Apa I. The DNA ends were repaired and dephosphorylated. The TK gene was then inserted into this unique 3' Apa I site of the pGfra2neo plasmid. The ligation reaction was transformed by electroporation (method 3.2.4) in SURE cells. DNA was prepared using the plasmid mini-preparation protocol (see method 3.2.6(i)) and the clones were screened by restriction digest analysis with Apa I, Sac I, Sma I and Pst I restriction enzymes. A positive clone was identified and called pGf2NTKap. A schematic presentation of this plasmid is shown in figure 3.4b.
Fra-2 Knockout studies: Results

Figure 3.4: Diagram of the gene targeting constructs

a) The neomycin resistance gene was inserted into the NotI site in pGEx2NotI using linkers, to generate the plasmid pGfra2neo. The arrows indicate the location and direction of oligonucleotide primers, NeoP6 (solid arrowhead) and F2SD3 (open arrowhead) (product size = 1.6 kb), used in PCR amplification to identify knockout cells.

b) The thymidine kinase gene was inserted into the ApaI site of the pGfra2neo to generate pGf2NeoTK plasmid. The arrows indicate the location and direction of oligonucleotide primers, NeoP6 (solid arrowhead) and F2SD3 (open arrowhead) (product size = 4.4 kb in the construct or 1.6 kb following homologous recombination), used in PCR amplification to identify knockout cells.

c) The neomycin resistance gene was excised from the pGf2NeoTK plasmid and the hygromycin gene was inserted in its place. The arrows indicate the location and direction of oligonucleotide primers, HygroM1 (solid arrowhead) and F2SD3 (open arrowhead) (product size = 5.5 kb in the construct or 2.7 kb following homologous recombination), used in PCR amplification to identify knockout cells.

Bracketed restriction sites denotes sites lost during construct generation.
Fra-2 Knockout studies: Results

(a) pGfra2neo

(b) pGf2NeoTK

(c) pGf2HTK

Exon 2 of fra-2
Neomycin resistance gene cassette
Thymidine Kinase gene cassette
Hygromycin resistance gene cassette
A second knockout construct containing a different selection marker was
designed, based on this original knockout construct. This was to be used in cell
line experiments to try to reduce the expression of functional Fra-2 protein. The
selection marker gene hygromycin was selected. The hygromycin resistance gene
was excised from the p3’ SS plasmid (Stratagene) by digestion with HindIII and
AlwII (New England BioLabs) and the DNA ends were repaired. The hygromycin
resistance gene fragment was purified from a preparative gel slice using the
JETSORB extraction kit (Genomed).

An aliquot of the pGf2NTKap plasmid was digested with Not I to remove the
NeoR gene. The DNA ends were repaired and then dephosphorylated. The
digested plasmid and the Hygromycin resistance gene were ligated and
transformed into E. coli DH5α competent cells (see method 3.2.4). A colony
hybridisation screen of 230 clones with a HygroM1 end-labelled primer probe
identified positive clones (see method 3.2.11). The resulting plasmid was called
pGf2HTK. A schematic presentation of this plasmid is shown in figure 3.4c.

DNA was prepared for the plasmids, pGfra2neo, pGf2NTKap and pGf2HTK,
using the WIZARD mini-preparation protocol (method 3.2.6(iii)). There was a
problem in obtaining large amounts of pGf2NTKap and pGf2HTK. These
plasmids are both large and contain the TK gene, which appeared to show some
inhibitory effect on bacterial cell growth.

3.3.5 PCR analysis

PCR reactions were performed to confirm the PCR product sizes obtained from 9
different primer pair combinations for later use in knockout clone identification.
The oligonucleotide primers used were F2Ex2, F2Ex3c, F2Ex3c.2, NeoP6 and
F2SD3. Figure 3.5a shows a schematic representation of the endogenous fra-2
gene, the targeting vector and the expected targeting result in one fra-2 allele. The
positions of the primers are indicated and the primer pair combinations for each reaction are shown in figure 3.5b.

The reactions were performed on three different templates. Firstly, 100 ng of C57Bl6 genomic DNA, as this was the mouse strain the constructs were designed for use in. Secondly, 100 ng of 129 genomic DNA, because a change in circumstances meant the ES cells used would now be from the 129 strain of mice. Thirdly, 50 pg of the pGfra2neo plasmid, to confirm the product sizes obtained in the presence of the Neo\(^R\) gene.

The results from these PCR reactions are shown in figure 3.5c. The product bands in the 129 genomic DNA template reactions were fainter when the primer combination included either F2Ex3c or F2Ex3c.2 as one of the primers, as compared to those obtained from the reaction with the C57Bl6 genomic DNA template. This is most likely due to some strain sequence variation between the primers and the 129 genomic DNA. The primers were designed for the C57Bl6 DNA. However, since all the primer combinations did produce some product of the expected size with each template, it was decide to pursue the use of the constructs in the generation of Fra-2 knockout mice using the 129 ES cells (E14.1).

### 3.3.6 Attempt to generate of Fra-2 knockout mice

The pGfra2neo construct was used in an attempt to produce ES cells with one copy of the endogenous fra-2 gene functionally knocked out. This plasmid was used because bacteria bearing the other plasmids containing the TK gene did not grow very well and therefore there were problems in producing enough DNA for the experiment. Dr. Robyn Slattery and Ms Eloisa Pagler did all the culturing...
Figure 3.5: PCR amplification reactions to confirm primer pair product sizes
(following 2 pages)

PCR amplification reactions were performed with 9 different primer pair
combinations on mouse 129 genomic DNA, C57Bl6 mouse genomic DNA and
pGfra2neo plasmid DNA to check the product sizes produced by each pair for
later screening of knockout clones. 30 PCR amplification cycles of the mouse
detection program (see method 3.2.2) were performed using the QIAGEN Taq
polymerase.

a) Schematic representation of the endogenous *fra-2* gene, the pGfra2neo
targeting construct and the resulting *fra-2* targeted allele after homologous
recombination. The oligonucleotide primers used in the PCR amplification
reactions are indicated.

b) The table shows the primers used in each reaction and the predicted PCR
products for each combination with each of the DNA templates. The genomic
DNA refers to the predicted results for both 129 and C57Bl6 genomic DNA. The
targeted genomic DNA indicates the predicted results if the *fra-2* gene is targeted
by homologous recombination.

c) The PCR products were analysed on 0.8% agarose/1 X TAE/1 µg/ml ethidium
bromide gels and then visualised under UV light and photographed.
(i) 129 genomic DNA, (ii) C57Bl6 genomic DNA and (iii) pGfra2neo targeting
construct DNA.
Fra-2 knockout studies: Results

(a)

(i) Endogenous fra2 gene

(ii) pGfra2neo targeting construct

(iii) fra2 allele targeted by homologous recombination

(b)

<table>
<thead>
<tr>
<th>Reaction Number</th>
<th>Primer 1</th>
<th>Primer 2</th>
<th>Product size with template (kb):</th>
</tr>
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<tbody>
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<td></td>
<td>pgftra2neo</td>
<td>genomic DNA</td>
<td>targeted genomic DNA</td>
</tr>
<tr>
<td>1</td>
<td>F2Ex2</td>
<td>F2Ex3c</td>
<td>no product</td>
</tr>
<tr>
<td>2</td>
<td>F2Ex2</td>
<td>F2Ex3c.2</td>
<td>no product</td>
</tr>
<tr>
<td>3</td>
<td>F2Ex2</td>
<td>F2SD3</td>
<td>3.68</td>
</tr>
<tr>
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<td>F2Ex3c</td>
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</tr>
<tr>
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<td>F2Ex3c.2</td>
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<td>F2Ex3c.2</td>
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</tr>
<tr>
<td>9</td>
<td>NeoP6</td>
<td>F2SD3</td>
<td>1.61</td>
</tr>
</tbody>
</table>
(c)

(i) 129 DNA

(ii) C57Bl6 DNA

(iii) pGfra2neo DNA
work with the 129 ES cells (E14.1). The pGfra2neo plasmid, previously linearised with Nde I restriction enzyme, was electroporated into the ES cells which were then cultured under G-418 (GibcoBRL) selection (see method 3.2.16). Positively selected clones were picked and screened by PCR for the presence of a recombination event involving the insertion of the Neo$^R$ gene within exon 2 of one allele of the endogenous fra-2 gene. Thirty cycles of the PCR mouse detection program were performed using the AB Taq DNA polymerase. Dr. Donna Cohen and Ms Chenoa Barton did the screening of 600 clones by PCR (data not shown). There were no positive clones identified. It was therefore decided not to pursue this approach in the 129 ES cells.

### 3.3.7 Attempt to generate knockout Swiss 3T3 fibroblasts

An attempt was made to produce Swiss 3T3 fibroblast cells that lacked either one or two functional copies of the fra-2 gene. The pGfra2neo and pGf2HTK plasmids were used. Initially homologous recombination in one copy of the fra-2 gene was attempted with each plasmid separately. The plasmids were linearised with Nde I or Avr II restriction enzymes respectively. The linearised plasmids were electroporated into the cells and grown under the appropriate selection (see method 3.2.17). The cells were left to grow for 7 days and then the pGf2HTK-transfected cells were doubly selected for the presence of the hygromycin gene and the loss of the TK gene. The cells were grown for a further 7-10 days. The 144 selected pGfra2neo transfected clones and 28 doubly selected pGf2HTK transfected clones were picked for PCR screening. DNA was prepared from the cells and screened by PCR with oligonucleotide primers F2Ex3c and NeoP6. A product of 3.6 kb should only be produced using this primer combination if the Neo$^R$ gene is inserted within an endogenous fra-2 gene. This is because the NeoP6 primer corresponds to a sequence in the Neo$^R$ gene and the F2Ex3c primer corresponds to a sequence in exon 3 of the fra-2 gene. This exon 3 sequence is not present within the pGfra2neo construct.
The screening identified one potential positive clone, clone #36 (see figure 3.6), from the pGfra2neo transfection. However, the amplification product observed with clone #36 appears to be approximately 2.5 kb (figure 3.6). Given that this PCR product was smaller than expected, further PCR analysis was undertaken with the 9 different primer pair combinations used in the preliminary PCR analysis (figure 3.4), to gain more information about the nature of clone #36. The PCR amplifications were performed on non-transfected Swiss 3T3 fibroblast DNA, clone #36 DNA and the pGfra2neo plasmid (see figure 3.7b). The PCR reactions were repeated 3 times with the same result produced each time. The predicted and actual PCR product sizes obtained from each reaction with each template are shown in figure 3.7a. The results obtained with pGfra2neo were as predicted, except for the presence of a 0.8 kb band in reaction 4 and a 0.9 kb band in reaction 7. These unexpected amplification products were obtained previously with these primer-template combinations (see figure 3.5c), and are probably a result of non-specific priming events.

However, the results obtained with the Swiss 3T3 fibroblast DNA and clone #36 DNA were not all as predicted. The Swiss 3T3 fibroblast DNA PCR amplification produced both predicted and unpredicted results. Reactions 3, 5, 6 and 7-9 all produced the predicted size products (or lack of amplification product). In contrast, reactions 1, 2, and 4 unexpectedly lacked amplification product except for low molecular weight products, which were probably due to a primer dimer effect. These latter reactions all utilised the primers F2Ex3c or F2Ex3c.2 that have been shown previously to produce product (refer to figure 3.5). The lack of amplification product may indicate some sequence variation between the BALB/c DNA, the mouse strain of the Swiss 3T3 fibroblasts, and the C57BL/6 DNA, the mouse strain that the primers were designed on. These differences could make the
Figure 3.6: PCR analysis of G418-resistant Swiss 3T3 fibroblast clones

100 ng of genomic DNA from G418-selected Swiss 3T3 fibroblast clones #16 to #50 were analysed by PCR amplification using AB Taq DNA polymerase and oligonucleotide primers NeoP6 and F2Ex3c (predicted product size = 3.6 kb) for 30 cycles of the PCR knockout program (see method 3.2.2). Clone # 36 gave an amplification product smaller than the predicted size, approximately 2.5 kb (as indicated by arrow). PCR amplification products were analysed on a 0.8% agarose/TAE/1 μg/ml ethidium bromide gel. M = DNA size markers (λ/HindIII plus φX174/HaeIII).
PCR amplifications were performed to confirm the targeted status of the fra-2 allele in clone #36 (compared to figure 3.5a). Nine different primer pair combinations were used with control Swiss 3T3 fibroblast genomic DNA, clone #36 genomic DNA and pGfra2neo plasmid DNA as templates. The PCRs were performed using AB Taq DNA polymerase and 30 cycles of the mouse detection PCR program (method 3.2.2). Water was used as a control template (data not shown).

a) The table shows the primers used in each PCR amplification and the predicted and actual PCR products obtained for each reaction with each template.

b) The PCR products were analysed on 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gels and then visualised using a UV transilluminator and photographed. (i) control Swiss 3T3 fibroblast genomic DNA, (ii) clone #36 genomic DNA and (iii) pGfra2neo plasmid DNA. There were very low molecular weight products observed for reactions 1, 2, 4 and 7 (arrows).
<table>
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<td>Non-transfected Swiss 3T3 cells</td>
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<tr>
<td></td>
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</tr>
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<tr>
<td>9</td>
<td>NeoP6</td>
<td>F2SD3</td>
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</tr>
</tbody>
</table>
(b)  

(i) control Swiss 3T3 fibroblast genomic DNA  

(ii) Clone #36 genomic DNA  

(iii) pGfra2neo DNA
primers less specific for the site they were designed for. Reaction 5 also utilised the primer F2Ex3c.2 and did produce the predicted 3.6 kb amplification product, however, the intensity of this product was fainter than the intensity of the products obtained in reactions 3 and 6. This also may indicate a reduction in the specificity of the primers for the DNA template.

The clone #36 DNA PCR amplifications also produced predicted and unpredicted results. Reactions 3, 5, 6 and 9 all produced amplification products of the predicted size, however, reactions 3, 6 & 9 also produced additional fainter unpredicted bands. Reaction 3 produced two unpredicted fainter bands at 1.2 kb and 2.3 kb and reactions 6 and 9 produced three additional unpredicted fainter bands at 1.2 kb, 1.4 kb and 1.6 kb. The predicted 3.7 kb amplification product for reaction 3 and the band in reaction 5 are also fainter than the other predicted bands seen in reactions 3, 6 and 9. Reactions 1, 2, 4 and 7 all unexpectedly lacked amplification product, except for the low molecular weight bands, which are probably due to a primer dimer effect as seen with the Swiss 3T3 fibroblast DNA. Reaction 8 produced a faint 1.8 kb amplification product which is smaller than the predicted 3.6 kb product. The lack of predicted amplification products and the presence of some faint predicted bands suggests a reduction in the specificity of some of the primers, particularly F2Ex3c and F2Ex3c.2, for the BALB/c DNA.

The presence of an amplification product in reaction 8 should only occur if the Neo\textsuperscript{R} gene is inserted within exon 2 of one of the alleles of the endogenous fra-2 gene. The reason for this is that the sequence that corresponds to the F2Ex3c.2 primer is only present in the endogenous fra-2 gene and the sequence that corresponds to the NeoP6 primer is only present in the targeting vector. Therefore, although the product size is incorrect for reaction 8, it still does suggest that a homologous recombination event has taken place. The presence of the predicted 3.7 kb and 3.6 kb bands in reactions 3 and 5 respectively also indicates that an homologous recombination event has occurred. Taken together, these results suggest that there may have been some additional alterations to the targeting site
when the recombination event occurred which would explain some of the unpredicted results. The smaller than expected bands may suggest some deletions within the copy of the targeted fra-2 allele. The multiple bands obtained in some of the reactions with clone #36 DNA could potentially be explained by the presence of more than one copy the pGfra2neo construct inserted within the genome.

A Southern blot was performed to confirm the presence of the Neo\textsuperscript{R} gene within one copy of the endogenous fra-2 gene. DNA was prepared from a culture of clone #36 cells and untransfected Swiss 3T3 fibroblasts using the WIZARD genomic DNA preparation protocol. An aliquot of each DNA sample was digested with Avr II or EcoRI restriction enzymes and Southern blot analysis was performed on the digested DNA (method 3.2.9). The result from this Southern blot is shown in figure 3.8. Firstly, it shows the presence of higher molecular weight bands in both the EcoRI (identified by E1) and the Avr II (identified by A1) digests for the clone #36 DNA compared to the Swiss 3T3 fibroblast DNA. The intensity of the band in the clone #36 Avr II digest is much stronger than the bands in the control Swiss 3T3 fibroblast DNA Avr II digest and there is also an extra 1 kb band of strong intensity observed in the clone #36 EcoRI digest. The higher molecular weight bands in the clone #36 digests suggest that the Neo\textsuperscript{R} gene had been inserted within exon 2 of one copy of the endogenous fra-2 gene. However, the increased band intensities suggest multiple insertions of the pGfra2neo construct within the genome, although, it is unclear where the multiple insertions have occurred. Therefore, although it seems likely that one copy of the endogenous fra-2 gene has been altered by homologous recombination, the effects of these multiple plasmid copies at unknown sites within the genome would make any results obtained from work with this cell line inconclusive as to the cause. Thus it was decided that the clone #36 cell line would not be a reliable tool in determining the effects of altered fra-2 expression in the cells. This knockout approach was not pursued further.
Fra-2 Knockout studies: Results

**Figure 3.8**: Southern blot analysis of *fra-2* single copy knockout clone #36

a) 15 µg aliquots of control Swiss 3T3 fibroblast genomic DNA (C) and clone #36 genomic DNA (36) were digested with either *Avr* II or *EcoRI* restriction enzyme and then loaded on to a 0.8% agarose/TAE/1 µg/ml ethidium bromide gel. M = DNA size markers (λ/HindIII plus φX174/HaeIII). The gel was viewed under UV light and photographed.

b) The gel in (a) was blotted overnight onto nitrocellulose in 20 X SSC. The filter was hybridised at 42°C overnight with a PCR generated *fra-2* exon 2 probe prepared using primers F2Ex2 and F2Ex2c and AB Taq DNA polymerase (see method 3.2.10). The following day the filter was washed in 0.2 X SSC/0.1% SDS and then exposed to film for 48 hours. The results for the *Avr* II digestion show the presence of a higher molecular weight species (indicated by arrow A1) in the clone #36 genomic DNA sample. This product also has a stronger signal intensity to that seen in the control Swiss 3T3 fibroblast genomic DNA sample. For the *EcoRI* digestion two extra bands are present, one at approximately 6 kb (indicated by arrow E1) and one very strong signal at approximately 1 kb (indicated by arrow E2).
Fra-2 Knockout studies: Discussion

(a) Table:

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<tr>
<th></th>
<th>M</th>
<th>C/AvrII</th>
<th>36/AvrII</th>
<th>C/EcoRI</th>
<th>36/EcoRI</th>
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</tbody>
</table>

(b) Diagram:

- Corresponding kb values: 23.1, 4.4, 2.2, 1.35
- Marked regions: A1, E1, E2
3.4 Discussion

3.3.1 Fra-2 knockout mice and cells

The attempts to make knockout mice and cells using the generated targeting constructs were unsuccessful in producing any targeted ES cells or any reliable Swiss 3T3 cells lacking a functional fra-2 gene. The Swiss 3T3 fibroblast clone #36 may have lacked one functional copy of the fra-2 gene, but the results obtained in the PCR and Southern blot analysis showed two potential problems. Firstly, it seems possible that there was an insertion of more than one copy of the pGfra2neo targeting vector within the genome, at least one of which was at an unknown site. This was a major problem, as any results obtained from experiments with these cells would have been inconclusive as to the cause of the problem. The function of another gene may have been affected by the insertion of the other copies of the pGfra2neo construct.

Secondly, a deletion event may have occurred, which may have involved either a copy of the pGfra2neo construct or even the targeted endogenous fra-2 itself. The latter possibility may not have been such a large concern, as the aim of the approach was to interrupt the normal expression of the fra-2 gene. This may have been achieved but by a slightly different method than anticipated. This phenomenon of unpredicted locus alteration has been described before using replacement vectors (Hasty et al., 1991). However, to be able to use these cells as a reliable tool in analysis of Fra-2 function, sequencing of the fra-2 locus would be required. It was decided not to pursue this analysis further because of time constraints and other problems with the construct design.
3.4.2 Construct design

A major reason why the homologous recombination strategy was not pursued further was problems with the targeting vectors. These problems were probably major factors contributing to the lack of success in obtaining ES cells with a targeted fra-2 allele. Firstly, the DNA used in the targeting constructs was not isogenic with that of the ES cells. This was initially taken into consideration when the constructs were designed, and C57Bl6 DNA was obtained specifically for the purposes of targeting C57Bl6 ES cells. However, after the constructs were generated, the ES cell strain was changed in the Gene Targeting Facility, JCSMR, because of more efficient results obtained with 129 ES cells. The targeting of genes has been shown to be 20-fold less efficient when using non-isogenic DNA constructs as compared to isogenic DNA constructs (te Riele et al., 1992) due to small differences in DNA sequence. Comparison of 129 and BALB/c mouse DNA reveals an average of one sequence mismatch every 160 nucleotides (te Riele et al., 1992). Strain variation in the DNA sequences of C57Bl6 and BALB/c mouse DNA was evident by the lack of, or faint, products in many of the PCR reactions, especially those using primers F2Ex3c and F2Ex3c.2 (see figures 3.7a and b). The reason some reactions produced an amplification product and others did not was probably due to the different primer combinations.

The second important point in targeting vector construction is the size of homologous sequence between the targeting vector and the target gene. In the vector constructs generated here, the length of homology is approximately 1.68 kb at the 5’ end of the construct and either 1.1 kb (pGf2NTKap and pGf2HTK) or 1.55 kb (pGfra2neo) at the 3’ end. This gives total homology regions of 2.78 kb or 3.23 kb respectively. Efficient targeting has been reported with as little as 472 bp homology at one end of the vector (Hasty et al., 1991). However, the total length of homology in the targeting vector can have significant impact on the efficiency of homologous recombination. A 200-fold increase in targeting efficiency was reported with an increase from 1.3 kb to 6.8 kb of total homology (Hasty et al.,
These authors suggest the concept of a critical length of homology that is required for efficient targeting. The length of homology used in the constructs for c-Fos (Field et al., 1992; Johnson et al., 1989; Wang et al., 1992), FosB (Gruda et al., 1996) and c-Jun (Hilberg and Wagner, 1992; Johnson et al., 1993) knockout studies were considerably larger than that used here. The targeting vector used to generate knockout Fra-2 mice and cells had homology lengths that have been shown to be adequate for homologous recombination, but may have low efficiencies. Also, the sequence mismatches from the use of non-isogenic DNA would presumably have reduced the length of homology. Therefore, producing targeting constructs with very low targeting efficiencies is likely to have been one of the major factors contributing to the lack of success.

Another consideration in construct design is the use of a replacement or insertion construct. Some studies have shown that insertion constructs are more efficient in targeting experiments (Hasty et al., 1994; Hasty et al., 1991), however, the particular gene being targeted appears to influence the results obtained with each type of construct. There may be DNA sequence or chromatin structural influences on targeting events (Hasty et al., 1994). These may help explain the different targeting efficiencies observed with different gene constructs. Many knockout animals have been generated using replacement constructs showing this is a reliable method; for example, the AP-1 gene family knockouts (Field et al., 1992; Gruda et al., 1996; Hilberg and Wagner, 1992; Johnson et al., 1989; Johnson et al., 1993; Wang et al., 1992).

There are a number of other factors that can influence gene targeting efficiency, for example, sufficient marker gene expression and DNA transfection efficiency (te Riele et al., 1992). The selectable markers, neomycin and hygromycin, appeared to work sufficiently in these constructs. However, the presence of the TK gene in some of the vectors did seem to effect the growth of plasmid in bacterial cells. One explanation maybe that there was some low-level expression of the TK gene in the bacterial cells, inhibiting their growth. The TK gene is
inducible with gancyclovir but maybe in these SURE cells (Stratagene) and under the control of the PGK promoter there is a low level of constitutive expression. Because of the problems with generating enough plasmid DNA for transfection, the positive-negative selection protocol was tried only in the Swiss 3T3 fibroblast knockout experiments but not in the generation of ES cells and this reduced the likelihood of obtaining correctly targeted ES cells.

Any future Fra-2 knockout constructs would be designed differently with these above considerations in mind. A lack of time restricted the ability to generate another targeting construct. Therefore this work was not pursued further, instead the time was spent on the analysis of transgenic mice over-expressing Fra-2 (see next chapter).
Chapter 4

fra-2 Transgenic mice
4.1 Introduction

The previous chapter discussed the use of a gene targeting strategy to investigate fra-2 gene function. This approach was unsuccessful and therefore another approach was employed to determine functional roles for Fra-2, the generation and studies of transgenic mice over-expressing Fra-2. The first transgenic mice were produced nearly 20 years ago (reviewed in Gordon, 1993). Since then, they have been used extensively to study many different genes. They have provided useful information on gene regulation, gene function, oncogenic potential and many have become useful models for human diseases.

Transgenic mice over-expressing other AP-1 members have been reported. The results from these studies were discussed in chapter 1; briefly: c-Jun transgenic mice were normal (Grigoriadis et al., 1993); v-Jun transgenic mice developed fibrosarcomas (Schuh et al., 1990); c-Fos transgenic mice developed osteosarcomas and immune system deficiencies (Grigoriadis et al., 1993; Rüther et al., 1987; Rüther et al., 1989); c-Jun/c-Fos double transgenic mice showed an increased rate of osteosarcoma development and alterations in early B cell proliferation and development (Fujita et al., 1993); FosB transgenic mice showed no phenotype (Grigoriadis et al., 1993); FosB2 transgenic mice, with over-expression targeted to the thymus, showed effects on growth, fertility and enlargement of the thymus (Carrozza et al., 1997). These results highlight the range of circumstances in which AP-1 family members play a role.

As discussed in chapter 1, Fra-2 can weakly transform CEF and does not transform rat fibroblast lines (Foletta et al., 1994; Nishina et al., 1990; Wisdom and Verma, 1993). To date, no transforming virus has been identified that carries the fra-2 gene but a number of human cancer cell lines have significantly elevated levels of fra-2 mRNA (Matsui et al., 1990). fra-2 expression levels have also been shown to be altered in cells transformed by other oncogenes. Fibroblasts transformed with v-yes v-fps, c-Ha-ras, oncogenic Ha-ras, activated c-raf and v-
src show an increase in fra-2 phosphorylation and expression levels (Mechta et al., 1997; Murakami et al., 1997; Suzuki et al., 1994). It has been suggested that phosphorylation converts Fra-2 from an inefficient to an efficient transcriptional activator (Murakami et al., 1997). In contrast, cellular transformation by v-Rel, c-Rel and c-Rel delta showed a decrease in fra-2 expression levels (Kralova et al., 1998). These results suggest Fra-2 may be involved in cellular transformation caused by the over-expression of these oncogenes but there is little evidence to show fra-2 is itself an oncogene.

To investigate the oncogenic potential of the fra-2 gene, and to get some insight into functional roles of Fra-2, transgenic mice over-expressing Fra-2 in a wide range of tissues were generated. The human cytomegalovirus immediate early gene enhancer/promoter was used to generate the mice. This promoter has been shown to have high transcriptional activity in many tissues (Schmidt et al., 1990) and has been used previously in the generation of transgenic animals (Furth et al., 1991).

Some of the work presented in this chapter has been published in Oncogene, 1998 (McHenry et al., 1998).
4.2 Methods

Solutions used in this chapter are listed in alphabetical order in appendix A. The oligonucleotide primers used in this chapter are described in appendix B.

4.2.1 Generation of transgenic mice over-expressing Fra-2

Ms Helen Taylor of the Gene Targeting Facility (JCSMR) performed the zygote injections. The purified linearised pCMV-mfra2 DNA was microinjected into the pronuclei of fertilized mouse oocytes. The zygotes were derived from C57Bl6 F1 mice fertilized by (C57Bl6 X SJL1) males. Injected zygotes were incubated at 37°C/5% CO₂ for 3-4 hours and were then implanted into pseudo-pregnant C57Bl6 X BALB/c foster mothers.

4.2.2 Identification of transgenic mice

Crude genomic DNA was prepared from mouse-tails by removing approximately 0.5-1 cm from the tail of each mouse at weaning (approximately 3 weeks of age). The tail fragment was placed in 500 µl of lysis buffer containing 100 µg/ml proteinase K (Promega) and incubated overnight at 56°C. The DNA was prepared as described in method 3.2.16. Litters were screened by PCR analysis on tail DNA, using primers F2Ex3c and CMV5’, which only produced a product in the presence of the transgene. Initially PCR reactions were performed with SuperTaq DNA polymerase (P.H. Stehelin and CIE Ag, Basel) (see method 2.2.3) and later with AB Taq DNA polymerase (Advanced Biotechnologies) (method 2.2.6). The reactions contained 0.25 pmol/ml of each oligonucleotide primer and 0.2 mM of each dNTP in the appropriate 1 X DNA polymerase buffer (plus 1.5 mM magnesium chloride when AB DNA polymerase was used). Thirty cycles of the PCR mouse detection program was used as described in method 3.2.2. A 9 µl aliquot of each PCR reaction was analysed on a 0.8% agarose/1 X TAE/1 µg/ml
ethidium bromide gel with 1 μl of 10 X loading dye. In all cases water was included as a negative control and 10 pg of pCMV-mfra2 was used as a positive control.

4.2.3 Genomic DNA preparation

The spleen was removed from a mouse and immediately frozen in a dry ice-ethanol bath and stored at -70°C. Genomic DNA was prepared from each spleen using a modified method of Blin and Stafford (Blin and Stafford, 1976). The spleens were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powdered tissue was slowly added to 20 ml of TEN9 buffer containing 100 μg/ml RNase A (Sigma). The solution was swirled to submerge the material and then transferred to a 50 ml Falcon tube. The tube was mixed on a rocking platform at room temperature for 30 minutes. 1 ml of 20% SDS was added and the sample was mixed by inversion and then rocked for a further 10 minutes. 10 mg of proteinase K was added to each sample and the tube was mixed by inversion and then incubated overnight at 55°C with gentle shaking. The samples were extracted with an equal volume of TE equilibrated phenol, pH 8.0 (Progen) by gentle shaking for 4 hours at room temperature. The samples were centrifuged at 3000 rpm (Beckman GS-6R centrifuge) for 10 minutes and the lower phenol phase was removed by aspiration. The phenol extraction was then repeated. The DNA was precipitated with 1.4 M ammonium acetate and 100% ethanol and the DNA was recovered using a glass pipette drawn out into a hook. The DNA was rinsed in 70% ethanol and then air dried. Finally, the DNA was resuspended in 1 ml of TE buffer by shaking overnight at 65°C.
4.2.4 RNA preparation

Tissue samples were collected from mice and immediately frozen in 95% hexane, chilled in a dry ice-ethanol bath. Samples were either used immediately or stored at -70°C until required. The QIAGEN RNeasy kit was employed for isolation of total RNA. Briefly, tissues were homogenized in 1 ml of lysis buffer RLT using a small Polytron PTA 7 probe (Kinematica, Switzerland) and the RNA extraction procedure was performed according to the manufacturer’s instructions. Briefly, the lysate was centrifuged for 3 minutes (14,000 rpm, Eppendorf 5415 C microfuge) and the cleared supernatant was removed to a fresh tube and mixed with an equal volume of 70% ethanol. The entire mixture was gradually applied to an RNeasy spin column in 700 µl aliquots and centrifuged for 15 seconds at 8,000 x g (10,000 rpm, Eppendorf 5415 C microfuge). The column was washed in 700 µl of Wash buffer RW1 and then 500 µl of Wash buffer RPE by centrifuging as above. A final wash step in 500 µl of Wash buffer RPE and centrifugation at 14,000 rpm for 2 minutes was performed. The RNA was eluted with 50 µl of DEPC-treated water and centrifugation at 10,000 rpm (Eppendorf) for 1 minute. The RNA concentrations were determined by absorbance at 260 nm (see method 2.2.5).

3.2.5 Nick translated probe preparation

Nick translated probes were prepared using 75 ng of cDNA template, 50 µCi of $^{32}$P-dCTP and 5 µl of nick translation enzyme mix (Amersham) in 1 X nick translation buffer. The reaction was incubated at 15°C for 2 hours then stopped by the addition of 2 µl of 0.5 M EDTA and purified through a NICK column (Pharmacia) to remove unincorporated label. 2 x 1 µl aliquots of the purified probe were counted in a scintillation counter to check the efficiency of the labelling reaction and to determine the specific activity of the probe.
3.2.6 Northern blotting

For Northern blotting analysis, 5 µg of each total RNA sample was dried down in a Savant SpeedVac concentrator and resuspended in 9 µl of 1 X RNA sample buffer. The samples were then denatured at 65°C for 10 minutes, 1 µl of 10 X loading dye was added and the samples were electrophoresed under denaturing conditions on 0.8% agarose/1 X MOPS/2.2 M formaldehyde gels in 1 X RNA gel running buffer. The gels were stained in 1 X MOPS containing 10 µg/ml ethidium bromide for 30 minutes and then destained in 1 X MOPS for 2 x 15 minutes. The gels were visualized on a UV transilluminator and photographed. The gels were then blotted overnight onto Hybond-C nitrocellulose (Amersham) in 20 X SSC as described in Chapter 3 method 3.2.9. The filters were hybridised overnight (see method 3.2.12) with approximately $9 \times 10^5$ cpm/ml of a nick translated $^{32}$P-dCTP-labeled fra-2 cDNA probe. After washing in 1 X SSC/0.1% SDS at 55°C the filters were exposed to Kodak X-OMAT AR film overnight at -70°C.

4.2.7 Antibodies

The αF2-FL polyclonal antibody was raised against a recombinant GST Fra-2 fusion protein (McHenry et al., 1998). The recombinant protein, which incorporated the full length Fra-2 protein, was used to produce primary and secondary antibody responses in a rabbit. A third injection of the recombinant protein was administered to increase the antibody yield. The animal was sacrificed and bled out and the antibodies were purified using a standard IgG purification protocol. This partially purified antibody preparation was used in all experiments described below. The Fra-2 L-15 affinity-purified antibody was commercially produced by Santa Cruz Biotechnology. It was raised against a peptide corresponding to the amino acids 288-302 of the human Fra-2 protein sequence (Matsui et al., 1990).
4.2.8 Protein preparation

Tissue samples were collected from mice and immediately frozen in 95% hexane chilled in a dry ice-ethanol bath. Samples were used immediately or stored at -70°C until required. Protein extracts were prepared using the method of Schreiber et al. (Schreiber et al., 1989). The tissues were homogenised in 10 ml of PBS for 2 x 1 minute using a small Polytron PTA 7 probe (Kinematica, Switzerland) and then centrifuged at 1,500 x g (Beckman GS-6R centrifuge) for 5 minutes. The pellet was resuspended in 1 ml of PBS, transferred to an Eppendorf tube and centrifuged at 14,000 rpm (Eppendorf, model 5415 C microfuge) for 15 seconds. The pellet was resuspended in 400 µl of freshly prepared chilled buffer A and the cell suspensions were allowed to swell for 15 minutes on ice. 25 µl of 10% NP40 solution (Sigma) was added and the samples were vortexed vigorously. The samples were centrifuged at 14,000 rpm (Eppendorf, model 5415 C microfuge) for 30 seconds. The supernatants containing the cytoplasmic protein extracts were transferred to Oakridge tubes (Nalgene) and spun at 100,000 x g (Ti50 rotor) for 1 hour to remove particulate debris. The remaining nuclear pellet was resuspended in 50 µl of chilled buffer C and rocked at 4°C for 15 minutes. The samples were centrifuged at 14,000 rpm (Eppendorf, model 5415 C microfuge) for 5 minutes and the supernatant containing the nuclear protein extract was mixed with the cytoplasmic protein extract to give the total protein extract. For determination of protein concentration, a 10 µl aliquot of protein extract was mixed with 1 ml of 1:5 diluted (in water) BioRad protein assay dye. Optical density was measured at 595 nm and the unknown samples were compared to a protein standard curve. The standard curve was prepared using an Immunoglobulin G (IgG) protein standard (1.41 µg/ml, BioRad). The protein extracts were stored at -70°C until required.
4.2.9 Western blotting

For Western blotting, 10 μg of each total protein extract was adjusted to 1 X SDS gel loading buffer and heated at 100°C for 3 minutes. Samples were electrophoresed on a 10% SDS-polyacrylamide gels in 1 X Laemmlid buffer. 1 μg of ECL protein molecular weight markers (Amersham) was included on the gels. The gels were electroblotted overnight onto Hybond-C nitrocellulose in 1 X transfer buffer using a BioRad mini trans-blot transfer cell apparatus at 25 mAmmps (Burnette, 1981; Towbin et al., 1979). The filters were air dried and used immediately or stored at 4°C until required.

Filters were pre-blocked for 1 hour with 5% BSA in PBS containing 0.15% Tween-20 (Sigma). The filters were rinsed three times in 1% BSA/0.15% Tween-20 in PBS (PBS-T) and then incubated with 1:500 diluted αF2-FL polyclonal antibody or Fra-2 L-15 antibody (Santa Cruz Biotechnologies) in PBS-T for 1 hour. The filters were then washed for 4 x 5 minutes in PBS-T. The filters were then incubated in a second antibody (donkey anti-rabbit Immunoglobulin G antibody conjugated to horseradish peroxidase, Amersham) diluted 1:10,000 in PBS-T for 1 hour. The filters were washed twice in PBS-T for 5 minutes and then incubated with streptavidin-conjugated horseradish peroxidase antibody (Amersham) diluted 1:1500 in PBS-T for 30-60 minutes for detection of the ECL protein markers. The filters were washed for 4 x 5 minutes in PBS-T. The bound antibodies were detected using the ECL Western blotting detection system (Amersham). Equal volumes of the two detection solutions were mixed together and overlaid onto the filters. The filters were incubated for 1 minute and then wrapped in plastic-wrap and placed in a film cassette with Kodak X-OMAT AR film for 30 seconds and then again for 3-30 minutes.
4.2.10 Histology

Tissues were removed from mice and fixed in 10% neutral buffered formalin or Michel’s fixative for at least 24 hours, or Bouin’s solution for 6-14 hours or Carnoy’s solution for 6-8 hours. The tissues that were fixed in Bouin’s solution were then washed repeatedly in 50% ethanol until all the yellow from the picric acid was removed. The tissues were then stored in 70% ethanol until they were embedded. The tissues fixed in Carnoy’s solution were stored in 100% ethanol until they were embedded. All tissues were embedded in paraffin wax, except those fixed in Michel’s solution (see below), and 4 µm thick sections were cut and mounted onto microscope slides. Tissues used for immunofluorescence were mounted onto microscope slides that had been precoated in 2% 3’aminopropyltriethoxy-silane (in acetone). Those tissues fixed in Michel’s solution were washed in 3 changes of Michel’s wash buffer and then frozen in OTC mountant. Cryostat sections were cut and mounted on silanised microscope slides. Tissues for histological analysis were stained with hematoxylin and eosin (H&E) stain. The embedding, freezing, sectioning and staining was performed by either Ms Sandra Veness or Ms Anne Prins from the Histology facility, JCSMR.

4.2.11 Immunofluorescence

Fra-2 protein, both endogenous and transgenic, was detected by immunofluorescence staining (Cohen et al., 1993). The tissue sections were dewaxed in 2 changes of xylene for 2 minutes and then rehydrated. Rehydration was through 2 x 1 minute incubations in 100% ethanol, 90% ethanol, 70% ethanol and then water. The slides were rinsed in PBS and then blocked overnight at 4°C with 5% BSA/PBS. All subsequent steps were performed at room temperature. The slides were rinsed in 3 changes of PBS and then incubated with primary antibody diluted in 1% BSA/PBS for 1 hour in a humid chamber. The primary antibodies used were either 1:50 diluted Fra-2 L-15 affinity purified antibody
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(Santa Cruz Biotechnologies) or 1:40 diluted αF2-FL polyclonal antibody. The slides were washed in 3 changes of PBS and then incubated with sheep anti-rabbit Immunoglobulin F(ab')2 fluorescein isothiocyanate (FITC) conjugated antibody diluted 1:50 in 1% BSA/PBS for 1 hour in a dark humid chamber. The slides were washed in PBS as above, dried briefly and coverslips were mounted using 90% glycerol/PBS mounting fluid. The tissue sections were viewed and photographed using a BioRad MRC600 laser scanning confocal microscope.

4.2.12 Testes DNA content experiment

One testis was removed from each animal and placed in 4 ml of PBS on ice. The testis was mashed through a mesh grind and then washed in 2 x 4 ml of PBS followed by centrifuging at 300 x g (1000 rpm, Beckman GS 6R) for 5 minutes at 4°C. The cells were resuspended in 4 ml of cold PBS and then counted. 1-2 x 10^6 cells were resuspended in 1 ml of PBS and fixed by adding 3 ml of cold 95% ethanol and then mixing well by inversion. The cells were fixed for 24-48 hours at 4°C and then washed 3 times in 2 ml of PBS. The cells were pelleted and stained in 1 ml of propidium idodide/RNase (PI/RNase) solution in the dark at room temperature for 30 minutes. The propidium idodide was a kind gift from Dr. Paul Waring (JCSMR). The cells were analysed on the FACSCAN (Becton Dickinson) at 585 nm ± 42 and the results were processed using CellQuest software.
4.3 Results

4.3.1 Plasmid construction and generation of transgenic mice

The pCMVβ vector (Stratagene) was digested with Not I restriction enzyme to remove the β-galactosidase gene. The digest reactions were then religated using T4 DNA ligase and transformed into E. coli DH5α competent cells as described previously (method 3.2.4). Not I restriction digests were performed on DNA prepared from the religated plasmids to identify the plasmids that no longer contained the β-galactosidase gene. This plasmid was called pCMV. pCMV DNA was prepared using a QIAGEN p20 column (see method 3.2.6(ii)). The pCMV DNA was digested with Not I and the DNA overhang of the vector was filled in for blunt end cloning (see method 3.2.8). The linearised plasmid was purified by phenol extractions (see method 3.2.1), followed by the use of a NICK spin column (Pharmacia) and finally ethanol precipitation.

A 1 kb mouse fra-2 cDNA fragment was generated by restriction digestion of pGEMZfra-2 (Foletta et al., 1994) with HindIII and EcoRI at 37°C. The cohesive ends of the DNA fragments were filled-in and the reaction was run on a preparative 0.8% agarose/1 X TAE/1 μg/ml ethidium bromide gel in 1 X loading dye. The band containing the fra-2 cDNA fragment was excised from the gel and purified by electroelution (see method 3.2.3(i)).

The fra-2 cDNA fragment was blunt end ligated into the filled-in Not I site of the linearised pCMV vector and the ligation mixture was used to transform E. coli DH5α competent cells as described in method 3.2.4. Transformants were selected for analysis; plasmid DNA was prepared by the mini-preparation method (see method 3.2.6(i)) and small aliquots were digested with EcoRI and Kpn I to identify a plasmid in which the fra-2 insert had been inserted in the correct orientation with respect to the CMV promoter. The resulting 4.8 kb plasmid was
called pCMV-mfra2 (see figure 4.1). High quality plasmid DNA was prepared using the large scale plasmid preparation protocol (method 3.2.7) and an aliquot of the DNA was linearised with HindIII and gel purified by electroelution (see method 3.2.3(i)). The DNA was dissolved in 1 X injection buffer at 2 ng/μl concentration and microinjected into zygotes. The zygotes were then re-implanted into pseudo-pregnant females (refer to method 4.2.1). A total of 62 mice were examined by PCR of tail DNA using the oligonucleotide primers, CMV5’ and F2Ex3c (see method 4.2.2), for transgenic status. Three founder animals (N0) were identified, female 6492, male 6526 and male 6536 referred to as transgenic lines 1, 2 and 3 respectively. The results from the tail PCR analysis for these animals is shown in figure 4.2. Breeding programs with C57Bl6 mice were established for each line so that offspring could be analysed for expression of the transgene.

### 4.3.2 Identification of an eye abnormality in transgenic mice from line 1

Although none of the founder transgenic animals showed any physical manifestations of the presence of the transgene, some of the transgenic N1 offspring in transgenic line 1 possessed an obvious eye defect. In the first N1 litter for line 1, there were 5 transgenic animals, including 4 males (A to D) and one female (E). Three of the animals, namely A, B and D, possessed small, recessed eyes with visible corneal opacities and a mucous discharge. Severity of the defect varied, with A being the most severely affected and D the least affected. The animals C and E did not appear to have an eye defect; however, transgenic offspring from these animals did possess the eye abnormality. Later post-mortem analysis of animal E showed the presence of corneal opacities in the eyes of this animal. Unfortunately, post-mortem analysis was not possible for mouse C, but it may also have had a mild eye phenotype. As the five N1 transgenic animals varied in terms of the penetrance of the phenotype, these animals were treated as five
Figure 4.1: Schematic representation of the pCMV-mfra2 plasmid construct

The pCMV-mfra2 plasmid construct was used to generate transgenic mice. The fra-2 cDNA is 1044 bp in length, of which 981 bp comprises the complete fra-2 protein coding sequence. The remaining 63 bp are either fra-2 untranslated sequences or small amounts of pGEM3Z polylinker sequences (the insert was excised from the pGEM3Z-based construct pGEMZfra-2). The arrows indicate the location and direction of the oligonucleotide primers, CMV5' and F2Ex3c, used in PCR amplifications to identify transgenic mice (product size = 1225 bp). The bracketed restriction sites denote sites lost during cloning. The HindIII restriction site in the vector was used to linearise the plasmid for microinjection.
PCR amplification was employed to detect mice in which successful integration of the pCMV-mfra2 transgene had occurred. 100 ng of crude genomic DNA from each mouse and oligonucleotide primers, CMV5' and F2Ex3c, were used in the PCR. A 1.2 kb amplification product (arrow) was diagnostic for the presence of the transgene. Following 30 cycles of the PCR mouse detection program (see method 3.2.2), the samples were electrophoresed on a 0.8% agarose/TAE buffer/1 μg/ml ethidium bromide gel.

a) Mouse 6492 identified as transgenic, called line 1.

b) Mice 2526 and 6536 identified as transgenic, called lines 2 and 3 respectively. Water (W) was used as a negative control and 5 pg of pCMV-mfra2 was used as a positive control (P). M = DNA size markers (λ/HindIII plus φX174/HaeIII).

**Figure 4.2:** Identification of founder mice carrying the transgene, pCMV-mfra2
distinct sub-lines, namely A to E according to the initial N₁ parent animal. All transgenic offspring in all of the five sub-lines of transgenic line 1 subsequently showed the presence of the eye defect, although the severity did vary between animals, and even between eyes of the same animal. The transgenic mice were outcrossed with BALB/c mice and these offspring also showed the presence of the eye abnormality phenotype indicating it was not a strain-specific phenotype. All other breeding was done with C57Bl6 mice.

No transgenic mice in lines 2 and 3 (bred to 5 generations) displayed any observable phenotype.

4.3.3 Southern blot analysis of transgenic mouse DNA

Genomic DNA was isolated from spleen tissue taken from offspring from transgenic lines 2 and 3 and sub-lines A, B, C, and E from line 1. An aliquot of each genomic DNA sample was digested with Bgl II restriction enzyme (this enzyme does not cut anywhere within the pCMV-mfra2 plasmid), electrophoresed and blotted onto Hybond-C nitrocellulose. The filter was used in a Southern blot with a PCR generated ³²P-dCTP-labeled fra-2 probe (see method 3.2.10) prepared using oligonucleotide primers F2Ex1 and Fra2.3P and pCMV-mfra2 as the template. This probe would detect both the endogenous and transgenic copies of fra-2.

Figure 4.3 shows the results of this analysis. The photograph of the ethidium bromide stained gel (figure 4.3a) shows some unequal lane loadings. While lanes for samples 1B, 1C and 1E contained approximately equivalent amounts of DNA, the sample 2 lane contained slightly more DNA and the lanes for samples 1A and 3 contained considerably more DNA. This point is important when interpreting the results of the Southern blot shown in Figure 4.3b, and the degree of difference
Figure 4.3: Southern blot analysis of the pCMV-mfra2 transgene

a) 5 μg (as determined by sample OD) aliquots of genomic DNA isolated from spleen from transgenic mouse lines 2 and 3 and sub-lines 1A, 1B, 1C and 1E were digested with BglII restriction enzyme and electrophoresed on a 0.8% agarose/TAE/1 μg/ml ethidium bromide gel. The gel was visualised under UV light and photographed. M = DNA size markers (λ/HindIII plus φX174/HaeIII).

b) The gel was blotted to nitrocellulose overnight in 20 X SSC as described in section 3.2.4. A $^{32}$P-labelled fra-2 PCR generated probe was prepared using oligonucleotide primers F2Ex1 and Fra2.3P and 10 ng of the pCMVmfra-2 as the template (see method 3.2.10). The nitrocellulose filter was hybridised at 42°C with approximately 2 x 10$^6$ cpm/ml of the PCR generated probe. Following washing at 55°C in 1 X SSC/0.1% SDS, the filter was exposed to film for 8 days. Arrows at 7 kb, 5 kb and 0.4 kb indicate the bands corresponding to the endogenous fra-2 gene. T1, T2a, T2b, T3a and T3b identify the bands containing the fra-2 transgene for each of the transgenic lines 1, 2 and 3 respectively. A, B, C and E identify the sub-lines from line 1, and 2 & 3 indicate transgene line 2 and line 3 respectively.
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(a)

(b)
Transgenic mice: Results

in lane loadings is made clearer when the intensity of the endogenous fra-2 bands (at 7 kb, 5 kb and 0.4 kb) are compared between the lanes.

In addition to the endogenous bands, the transgenic line 1 sub-lines (A, B, C and E) all show the presence of a positive transgene signal (T1). It is not clear whether this signal represents a single band of high intensity or a collection of lower intensity bands. However, comparing the relative intensities of this transgene signal and the endogenous fra-2 bands suggests multiple copies of the transgene. In this regard, it should be noted that the signal intensity for the endogenous fra-2 bands might be lower than expected for a single copy gene, as the regions of homology between the probe and the gene fragments are disrupted by introns and the presence of internal Bgl II sites. Thus, with the particular probe that was used, which covered exons 1 to 4, a single copy of the pCMV-mfra2 transgene would be expected to have a higher intensity hybridisation signal than a single copy of the endogenous fra-2 gene. Therefore, simple quantitative comparisons between the hybridisation signal of the transgene and endogenous gene are not likely to be a good indicator of relative copy number. Given the size of the T1 band (approximately 15 kb) and the fact that the Bgl II enzyme used for the digestion does not cut within the transgene, there could be no more than 2 or 3 tandem copies of the pCMV-mfra2 transgene at the T1 site (assuming T1 is a single band). The T1 hybridisation signal is much higher for sub-line 1A than for the 1B, 1C and 1E samples, however, it should be remembered that this lane contained considerably more DNA, as discussed above. Sub-line 1D Southern blot analysis is not shown here because at the time of this experiment insufficient animals were available from this sub-line to allow an animal to be sacrificed for this analysis. However, later analysis did show a similar single positive transgene signal for sub-line 1D (data not shown). Therefore it is likely that there is no significant difference, in terms of site of integration and transgene copy number, between the line 1 sub-lines.
The transgenic line 2 sample showed two positive transgene signals (T2a and T2b) in addition to the endogenous fra-2 bands, suggesting at least two sites of integration of the transgene. These signals each represented a single band as determined by analysis of Southern blot films with lower exposure times (data not shown). The size of the bands (approximately 9 kb and 8 kb respectively) would allow no more than a single copy of the transgene within each band. Despite the higher amount of DNA loaded onto the lane for sample 2 compared to samples 1B, 1C and 1E, the combined signal intensity for T2a and T2b was less than that of T1, suggesting a lower number of copies in transgenic line 2 than in transgenic line 1.

Transgenic line 3 also showed two positive transgene signals in addition to the endogenous fra-2 bands: T3a (a smear >23 kb) and T3b (approximately 10 kb). This result suggests at least 2 sites of integration of the transgene in this line. The T3b signal is a single copy band, as determined by analysis of Southern blot films with lower exposure times (data not shown), and could only contain 1 or 2 copies of the transgene. However, it is unclear whether the T3a hybridisation signal represents a single band with a very high intensity or multiple bands. The very strong intensity of the T3a hybridisation signal also suggests a much higher transgene copy number for line 3 than either lines 1 or 2, even when lane loadings are taken into consideration.

4.3.4 Determination of transgene copy number

A PCR strategy was used to determine the number of transgene copies inserted into the genome of each transgenic line. The number of copies of the transgene in the first five N1 transgenic offspring (A to E) obtained from line 1 was also analysed. A known amount of pCMV-mfra2 plasmid DNA was also subjected to PCR amplification (see method 3.2.2) for use as a standard. The intensity of the PCR amplification product band obtained for each transgenic line was compared
to the intensity of the PCR amplification product obtained with 50 fg of plasmid DNA and used in a calculation to estimate the number of copies of the transgene per cell. Figure 4.4 shows the results obtained from this analysis. The intensity of the PCR amplification product band obtained for transgenic line 1 was approximately 3-6 fold higher than the intensity of the PCR amplification product obtained with 50 fg of plasmid. For line 2 it was approximately 80 fold higher and for line 3 it was approximately 30-40 fold higher.

The number of transgene copies was calculated for each sample based on the assumption that there are approximately 7 pg of DNA per cell (Palmiter et al., 1983) and that the size of the pCMV-mfra2 plasmid is 4.8 kb. Thus, it was calculated that 50 fg contains approximately 9,500 copies of the transgene and that 100 ng of genomic DNA contains approximately $1.4 \times 10^4$ cells (ie. $2.8 \times 10^4$ copies of the genome, as there are two copies of the genome per cell). For each transgenic line the intensity of the amplification product produced compared to the intensity of the 50 fg of plasmid band was multiplied by 9,500 (the number of copies of the transgene in 50 fg). This figure was then divided by $2.8 \times 10^4$ (the number of copies of the genome in 100 ng of genomic DNA) to obtain an estimate of the number of copies of the transgene per cell from that transgenic line. Therefore, the number of copies of the transgene for each transgenic line was calculated as 1-2 copies, 25 copies and 10-12 copies for lines 1, 2 and 3 respectively. There was no significant difference in the number of copies of the transgene in any of the five line 1 N1 mice. However, it is important to note that during this analysis the levels of transgene amplification obtained for each sample did vary slightly between different PCR experiments (data not shown).
100 ng of genomic tail DNA from founder 6526 (line 2), founder 6536 (line 3) and the first five transgenic N$_1$ offspring of line 1 (line 1A, 1B, 1C, 1D and 1E) was analysed by PCR, using primers CMV5' and F2Ex3c. Two identical reactions were set up for each template; one reaction was amplified for 25 cycles and the other was amplified for 30 cycles of the mouse detection program (see method 3.2.2). Reactions were also performed using 50 fg, 500 fg or 5 pg of the plasmid pCMV-mfra2, for quantitative comparison. The arrow indicates the 1.2 kb size of the fra-2 transgene fragment generated. M = DNA size markers ($\lambda$/HindIII plus φX174/HaeIII).
4.3.5 Northern blotting

A range of tissues were removed from transgenic offspring from sub-lines 1A to 1E, line 2 and line 3 and one control animal. RNA was extracted from the tissue samples and analysed by Northern blotting. The results are shown in figures 4.5 to 4.11. The fra-2 RNA from the transgene was 1.2 kb and was easily distinguishable from the endogenous fra-2 mRNA (approximately 6 kb).

For the five N1 sub-lines from transgenic line 1, the results showed the presence of 3 distinctive patterns of transgene expression, although there was a large degree of overlap in these patterns. Firstly, sub-lines A, B and C all expressed the transgene in bone, kidney, heart, large intestine, muscle, skin, small intestine, stomach and eye (figures 4.5 to 4.7). The level of expression in some of the tissues (for example, heart and large intestine) did vary slightly between animals. These similar expression results suggest that these 3 sub-lines were probably not distinct lines. Therefore, in subsequent analysis only one animal from either sub-line A, B or C was used in the analysis to represent all three sub-lines collectively.

Sub-line D also showed a similar pattern of expression, except there was no transgene expression detected in the skin and significant levels of expression in the spleen. Expression in the spleen was only seen in this sub-line (see figure 4.8).

Sub-line E had the most distinctive pattern of expression, with the transgene detected in bone, eye, muscle, pancreas, skin, small intestine, stomach, and weakly in the heart (see figure 4.9). Despite either degradation of RNA or low amounts of RNA loaded in the lanes for bone, muscle and pancreas (figure 4.9a), expression of the transgene was detected on the Northern blot suggesting there were significant levels of transgene expression in these tissues. This was the only sub-line to show expression in the pancreas.
Transgenic mice: Results

**Figure 4.5: Northern blot analysis of transgenic mouse line 1A tissues**

a) 5 µg of total RNA from each tissue was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.

b) The gels were blotted to nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with approximately 1 x10^6 cpm/ml of a ^32^P-labelled mouse fra-2 cDNA nick translated probe (method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) fra-2 RNA and the 1.2 kb transgene (T) fra-2 RNA. The positions of the 28s and 18s RNAs are indicated. RNA from the stomach of a littermate control sample was included for comparison.

S(c) = control stomach, B = Bone, Br = Brain, E(l) = Left Eye, E(r) = Right Eye, K = Kidney, H = Heart, LI = Large Intestine, L = Liver, Lg = Lung, M = Muscle, P = Pancreas, Sk = Skin, SI = Small Intestine, Sp = Spleen, S = Stomach, T = Thymus, Te = Testes, O = Ovary.
Transgenic mice: Results

(a) 

(b)
**Figure 4.6:** Northern blot analysis comparing *fra-2* RNA expression of transgenic mouse line 1B tissues and littermate control tissues (next 2 pages)

a) 5 µg of total RNA from each tissue was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.

b) The gels were blotted to nitrocellulose overnight in 20 X SSC (see section 3.2.3). The filters were hybridised with approximately 1 x10⁶ cpm/ml of a ³²P-labelled mouse *fra-2* cDNA nick translated probe (see method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) *fra-2* RNA and the 1.2 kb transgene (T) *fra-2* RNA. The positions of the 28s and 18s RNAs are indicated.

(for letter codes, refer to figure 4.5, except E = Eye, C = control and T= transgenic)
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![Image of gel with labeled bands (28s and 18s)]

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![Image of gel with labeled bands (28s and 18s)]
Figure 4.7: Northern blot analysis of transgenic mouse line 1C tissues

a) 5 µg of total RNA from each tissue was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.
b) The gels were blotted to nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with approximately 1 x 10^6 cpm/ml of a ^32P-labelled mouse fra-2 cDNA nick translated probe (see method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) fra-2 RNA and the 1.2 kb transgene (T) fra-2 RNA. The positions of the 28s and 18s RNAs are indicated. RNA from the stomach of a littermate control sample was included for comparison. (for letter codes, refer to figure 4.5, except E = Eye)
Transgenic mice: Results

(a)

(b)

S(c) B Br K H L Lg M P Sk Sl Sp S Th O E

28s 18s

28s 18s

E T
Transgenic mice: Results

Figure 4.8: Northern blot analysis of transgenic mouse line 1D tissues

a) 5 µg of total RNA from each tissue was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.
b) The gels were blotted to nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with approximately 1 x10^6 cpm/ml of a ^32P-labelled mouse fra-2 cDNA nick translated probe (see method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) fra-2 RNA and the 1.2 kb transgene (T) fra-2 RNA. The positions of the 28s and 18s RNAs are indicated. (for letter codes, refer to figure 4.5, except E = Eye)
Figure 4.9: Northern blot analysis of transgenic mouse line 1E tissues

a) 5 μg of total RNA from each tissue was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.

b) The gels were blotted to nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with approximately 1 x10^6 cpm/ml of a$^{32}$P-labelled mouse fra-2 cDNA nick translated probe (see method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) fra-2 RNA and the 1.2 kb transgene (T) fra-2 RNA. The positions of the 28s and 18s RNAs are indicated. (for letter codes, refer to figure 4.5, except E = Eye)
Transgenic mice: Results

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28s
18s

E
T
Figure 4.10: Northern blot analysis of transgenic mouse line 2 tissues

a) 5 μg of total RNA from each tissue was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.

b) The gels were blotted to nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with approximately 1 x 10^6 cpm/ml of a ^32P- labelled mouse fra-2 cDNA nick translated probe (see method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) fra-2 RNA and the 1.2 kb transgene (T) fra-2 RNA. The positions of the 28s and 18s RNAs are indicated. RNA from the stomach of a littermate control sample was included for comparison. (for letter codes, refer to figure 4.5, except E = Eye)
Transgenic mice: Results

(a) S(c) B Br H K Li L Lg M P Sk Sl Sp S Th O Te E

(b) S(c) B Br H K Li L Lg M P Sk Sl Sp S Th O Te E

28s 18s

E T
Figure 4.11: Northern blot analysis of transgenic mouse line 3 tissues

a) 5 μg of total RNA from each tissue was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.

b) The gels were blotted to nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with approximately 1 x10⁶ cpm/ml of a ³²P-labelled mouse fra-2 cDNA nick translated probe (see method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) fra-2 RNA and the 1.2 kb transgene (T) fra-2 RNA. The positions of the 28s and 18s RNAs are indicated. (for letter codes, refer to figure 4.5, except E = Eye)
All the sub-lines showed expression of the transgene in the eye and the level of expression seemed to correlate with the severity of the eye abnormality. Each of the sub-lines had the same site of transgene integration (refer to figure 4.3) and the same number of copies of the transgene (see figure 4.4), so it is unclear as to the reason for the distinct patterns of expression. However, this phenomenon of different patterns of transgene expression within the one line of animals with the CMV promoter has been reported previously (Furth et al., 1991).

The RNA expression patterns for transgenic lines 2 and 3 are shown in figures 4.10 and 4.11 respectively. No transgene mRNA was detected by Northern blotting for transgenic line 2, despite the high copy number of the transgene (see figure 4.4). Therefore, this line was not analysed further. Line 3 showed high transgene expression in bone, muscle and skin and low levels in heart and eye. There was no obvious phenotype present that correlated with the presence of the transgene, so this line was also not investigated any further. All subsequent analysis was only done on animals from transgenic line 1 (founder 6492).

### 4.3.6 Fra-2 may regulate its own promoter

Closer examination of the Northern blotting analysis, particularly figure 4.6 where control and transgenic RNA samples for each tissue were run side-by-side, identified circumstances where the level of endogenous fra-2 mRNA was reduced in the presence of transgenic fra-2 mRNA. This suggested that expression from the endogenous gene was being down-regulated. The analysis was repeated using transgenic and littermate control stomach RNA samples (figure 4.12). It was observed that the moderately high level of endogenous fra-2 mRNA in stomach was reproducibly reduced in the transgenic animals. It was hypothesised that the transgenic Fra-2 protein was down-regulating the endogenous fra-2 promoter, as there were moderately high levels of the transgene expressed in the stomach. The transgene RNA expression levels were unaffected because the construct made use
Figure 4.12: Negative regulation of the endogenous fra-2 expression in transgenic tissue

(a) 5 μg of total RNA from transgenic and control stomach were electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to check the quality of the RNA.

(b) The gel was blotted to nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with approximately 1 x10^6 cpm/ml of a ^32P-labeled mouse fra-2 cDNA nick translated probe (see method 3.2.11) overnight at 42°C and then washed in 1 X SSC/0.1% SDS followed by exposure to film for 24 hours. The arrows indicate the approximately 6 kb endogenous (E) fra-2 RNA and the 1.2 kb transgene (T) fra-2 RNA. The positions of the 28s and 18s RNAs are indicated. Tg = Transgenic, C = Control.
Transgenic mice: Results

of the CMV promoter and not the \textit{fra-2} promoter. This apparent auto-regulation of \textit{fra-2} expression was only seen in the stomach and not in other tissues where both the transgene and endogenous \textit{fra-2} gene were shown to be co-expressed by Northern blotting.

4.3.7 Western blotting

Tissues were removed from transgenic and littermate control mice from transgenic line 1. Total protein extracts were prepared and analysed by Western blotting (see method 4.2.9). Initial experiments were performed with the commercial antibody, Fra-2 L15 (Santa Cruz Biotechnologies) but no clear results were obtained (data not shown). Therefore, the $\alpha$F2-FL antibody was used (see method 4.2.7).

The results of one of the Western blots using transgenic line 1B total protein is shown in figure 4.13. Equivalent amounts of total protein were used for each pair of matched samples, so that total protein levels could be compared between the control and transgenic animal samples. As the transgene was not tagged in any way (to avoid any possible interference in the function of the transgenic Fra-2 protein), it was not possible to distinguish between protein produced from the transgene and protein produced from the endogenous gene. Therefore, the aim of the Western blotting experiment was to look for evidence of an overall increase in Fra-2 protein levels in the transgenic tissues compared to the control tissues. More subtle changes, such as a shift in the phosphorylation state, might also be detected by this analysis. All of the tissues analysed here, except for brain, showed significant RNA expression of the transgene (see figure 4.6).

Therefore, it was somewhat surprising that the differences between the Fra-2 protein in the transgenic and control samples, both quantitatively and
Transgenic mice: Results

**Figure 4.13:** Western blot analysis of Fra-2 expression in mouse tissues

10 µg of total protein extract (except for the eye samples, where only 5 µg of total protein extract was used) from transgenic and littermate control mice tissues were electrophoresed under denaturing conditions on 9% SDS polyacrylamide gels. The gels were electroblotted overnight onto nitrocellulose filters (see method 4.2.12) at 25 mAmps. Filters were incubated with 1:500 diluted αF2-FL polyclonal antibody followed by 1:10,000 diluted donkey anti-rabbit IgG antibody conjugated to HRP (Amersham) and then with 1:1500 diluted streptavidin conjugated HRP antibody (Amersham). Bound antibody was detected by ECL Western blotting (see method 4.2.9). The arrow identifies the 46 kDa Fra-2 species and the # and * identify bands that are missing in the heart transgenic sample compared to the heart control sample. C = Control, Tg = Transgenic and M = ECL protein molecular weight markers (Amersham).
**Transgenic mice: Results**

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Muscle

Eye

Stom

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Fra-2
transgenic mice: Results

qualitatively, were quite small. The results show alterations in the expression of Fra-2 protein only in bone, heart, kidney and stomach transgenic samples compared to their respective control samples. The bone and stomach samples show an increase in the total amount of Fra-2 protein in the transgenic samples. However, this increase in Fra-2 protein did not seem to adequately reflect the large amount of transgenic RNA produced in these tissues (compare figures 4.6 and 4.13). At this point it is important to note that the apparent increase in the level of Fra-2 protein in the transgenic brain sample compared to the control brain sample is actually caused by an artefact of the film being exposed to the filter. A shadowing effect has reduced the band intensity of the control brain sample. This effect has also hidden some of the upper bands in the transgenic bone sample.

The transgenic heart and kidney samples showed a shift in the Fra-2 protein bands in the transgenic samples compared to the control samples, such that there was an increase in the amount of the higher molecular weight species. This shift probably indicates an increase in the hyper-phosphorylated form of Fra-2 protein in these transgenic tissue samples. Interestingly, the transgenic heart sample also show the loss of two protein bands that are present in the control heart sample (see * and #). These may represent effects on target genes of Fra-2. The amount of protein used for the eye samples were low and therefore the results of the Western analysis were unclear for this tissue.

4.3.8 Immunofluorescence

Immunofluorescence was performed on tissues from transgenic and littermate control mice to confirm transgenic Fra-2 protein expression and determine its cellular localisation. The tissues analysed included bone, brain, eye, heart, kidney, muscle, large and small intestines, spleen, pancreas, skin and stomach. The optimal conditions for immunofluorescence were determined by attempts with a range of fixatives, namely Bouin’s solution, 10 % neutral buffered formalin,
Michel’s solution and Carnoy’s solution. Two different primary antibodies were used, Fra-2 L15 (Santa Cruz Biotechnologies) and αF2-FL (see method 4.2.7). The optimal results, which were finally used in the analysis, were obtained with fixing in Carnoy’s solution and then detection with the αF2-FL primary antibody. Transgenic animals from sub-lines B, D and E from line 1 were examined and the results obtained for each sub-line were the same, hence only the results obtained with sub-line 1B are shown.

The results obtained for bone, brain, heart, kidney, small and large intestine and spleen showed no clear difference in the expression pattern of Fra-2 protein between control and transgenic tissues (data not shown). In contrast, eyes, muscle, pancreas, skin and stomach all showed clear differences.

The cornea of the transgenic eye showed moderately strong Fra-2 immunofluorescence in the disorganised stroma and in the corneal epithelial layer (see figure 4.14). In comparison, the control cornea showed very little Fra-2 staining suggesting that the result seen in the transgenic cornea was due to the presence of the transgene. This pattern of putative transgene Fra-2 protein expression correlated with the area involved in the eye abnormality phenotype (see below).

The skeletal muscle from transgenic animals showed considerable Fra-2 expression in the muscle fibres as compared to the control skeletal muscle which showed no detectable expression of Fra-2 protein (see figure 4.15).

An interesting result was obtained with the pancreas samples. Fra-2 protein was detected in both control and transgenic pancreatic tissue samples (see figure 4.16). However, the transgenic tissue sample showed much higher levels of Fra-2 protein expression than the control tissue sample. All transgenic sub-lines from
Figure 4.14: Immunofluorescence analysis of Fra-2 expression in the eye

Eyes were fixed in Carnoy's solution for 6 hours and then stored in 100% ethanol until sectioned. Sections were incubated with αF2-FL antibody (diluted 1:40 in PBS/1% BSA), followed by FITC-conjugated anti-rabbit antiserum (diluted 1:50). Scale bars = 25 μm.

a) Control eye cornea. The well-organised corneal stromal layer (S) and corneal epithelial layer (E) show very low level Fra-2 staining.

Transgenic eye cornea. Fra-2 immunofluorescence was visible throughout the disorganised corneal stromal layer (S) and some staining was observed in the corneal epithelial layer (E). Arrows indicate the areas of immunofluorescence.
Transgenic mice: Results

(a) Control Cornea

(b) Transgenic Cornea

S E

178
Figure 4.15: Immunofluorescence analysis of Fra-2 expression in skeletal muscle

Muscle tissue was fixed and stained as described in figure 4.14. Scale bars = 25 μm.

a) Control skeletal muscle with no sign of Fra-2 staining in the muscle fibres.

b) Transgenic skeletal muscle showing increased levels of Fra-2 immunofluorescence staining in the muscle fibres.
Pancreas tissue was fixed and stained as described in figure 4.14. Scale bars: (a) & (b) 100 µm; and (c) & (d) 25 µm.

a) Low magnification image of a control pancreas with very little Fra-2 staining detectable.

b) Low magnification image of a transgenic pancreas with extensive Fra-2 immunofluorescence staining detected.

c) High magnification image of control pancreas showing some Fra-2 immunofluorescence staining present in the cells.

d) High magnification image of transgenic pancreas showing stronger and more extensive staining of Fra-2 as compared to the control pancreas.

Figure 4.16 Immunofluorescence analysis of Fra-2 expression in pancreas
Transgenic mice: Results

line 1 showed an increase in the Fra-2 immunofluorescence compared to littermate controls. This was in contrast to the results obtained by Northern blotting, which showed pancreatic *fra-2* expression only in sub-line 1E. The Northern blotting analysis was done much earlier than the immunofluorescence analysis. Therefore, earlier generations (N_2) of the mice were used for RNA expression analysis, whereas later generations (at least N_4) were used in the immunofluorescence analysis. Following the observation that all sub-lines had increased Fra-2 protein in the pancreas as determined by immunofluorescence, the Northern analysis was repeated using tissue from later generations of transgenic mice from sub-lines 1B and 1D. As expected, these sub-lines now showed pancreatic *fra-2* transgene expression (data not shown). This suggested some changes in transgene expression pattern had occurred with successive generations, so that all sub-lines appeared to show the same pattern of *fra-2* expression. Interestingly, the Northern blot analysis of tissues from the later generation also revealed that sub-line 1D animals had lost *fra-2* expression in the spleen.

An increase in Fra-2 protein was seen in transgenic skin as compared to control skin (see figure 4.17). Interestingly, the Fra-2 protein was seen in a similar region of the skin, the hair follicle, in both transgenic and controls tissues. A similar result was seen in stomach tissue, with an increase in Fra-2 levels in the transgenic animals in the same cell types as Fra-2 protein was detected in the control animals tissue samples (see figure 4.18).

4.3.9 Transgenic animals were smaller than littermate controls

Transgenic animals were noticeably smaller than their littermate controls. The weights of animals were recorded for several litters from the time of weaning over a period of 2-3 weeks. Animals representing a cross-section of all the
Transgenic mice: Results

Figure 4.17: Immunofluorescence analysis of Fra-2 expression in skin

Skin tissue was fixed and stained as described in figure 4.14. Scale bars: a) & b) 100 μm; c) & d) 25 μm.

a) & c) Control skin with Fra-2 immunofluorescence staining evident within the cells of the hair follicle.

b) & d) Transgenic skin with stronger Fra-2 immunofluorescence staining present within the hair follicle.

Arrows indicate the areas of immunofluorescence.
Figure 4.18: **Immunofluorescence analysis of Fra-2 expression in stomach**

Stomach tissue was fixed and stained as described in figure 4.14. Scale bars = 25 μm.

a) Control stomach showing some weak Fra-2 immunofluorescence staining present in the mucosal cells (arrow).

b) Transgenic stomach showing much stronger Fra-2 immunofluorescence staining in the same cell types as in control (arrow).
transgenic sub-lines were included in this analysis. Figure 4.19a and b shows an example of the results obtained from male and female mice. Transgenic and littermate control pairs are shown for each of the sub-lines. Taking all the results from the different sub-lines together, 53% of male and 100% of female N2 transgenic mice were 5-50% smaller than their littermate controls (p<0.001). For sub-line 1D, 100% of transgenic mice weighed, both male and female, were smaller than their littermate controls. To determine if weight differences were due to an overall decrease in size, individual organs were weighed from transgenic and littermate control mice and compared to the total body weight. A litter of mice from line 1D, including transgenic and littermate controls, was selected, weighed and then sacrificed. The individual organs were removed from the mice and weighed. The ratio of organ weight to the total body weight was compared for each animal (see figure 4.19c). There was no significant difference between the ratios of organ weight to total body weight for transgenic and control mice for any of the tissues analysed. This indicated that the transgenic mice were proportionally smaller than their littermate controls, suggesting a general reduction in growth.

4.3.10 Mouse behaviour experiment

It was observed that there was a reduction in the number of offspring produced from the transgenic mice. As discussed above, the transgenic mice also showed significant weight differences compared to their littermate controls. One possible explanation that might account for both observations, is that the transgenic mice could have an impaired sense of smell, reducing their ability to detect both pheromones and food. Therefore, a behaviour experiment was performed to determine if the reduced mating and smaller weights of the transgenic mice were caused by a decrease in the olfactory ability of the transgenic mice.
Figure 4.19: Weights of transgenic and control mice

(a) Male and (b) female mice were weighed on various days after weaning. Pairs of transgenic and littermate control animals, representative of all sub-lines, are shown. The transgenic animals are indicated with an asterisk, dashed lines and open symbols, whereas littermate controls are indicated by solid lines and filled symbols.

(c) Transgenic and littermate control mice were weighed and then sacrificed. The organs were removed and weighed separately. The ratio of organ weight to total body weight was determined to establish if transgenic animals were proportionally smaller than control animals. The transgenic animal (JM644) is indicated by an asterisk.

Sp = Spleen, K = kidney, St = Stomach, I = Intestine, Li = Liver, Lg = Lung, H = Heart, E = Eye, Br = Brain.
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(a) Weight (grams) vs. Age (days)

(b) Weight (grams) vs. Age (days)

(c) Ratio of organ weight/total body weight

Sp K St I Li Lg H E Br

JM6.4.4* JM6.4.5 JM6.4.6 JM6.4.7

(c) Ratio of organ weight/total body weight

(X 10^-3)
Four pairs of transgenic and littermate control animals were observed independently by two people for behavioural responses to different stimuli. The tests were performed in one room and the mice were kept in another room until tested. Each mouse was brought into the test room in a plastic bag and then quickly released into the test cage. Each mouse had its own plastic bag and test box so that odour from other mice did not interfere with the tests. The test solutions were soaked onto individual strips of 3 MM paper (2.5 x 8 cm). A fresh strip was used for each mouse. In some cases the strips were held directly under the mouse’s nose to obtain an immediate response. Behaviour was observed for 1 minute. The stimuli tested were water, saturated sucrose solution, sultanas, butanol and acetic acid. Generally, the mice sniffed the stimulus and then quickly seemed to become sensitised to the smell. Strong odours, like those from butanol and acetic acid, caused the mice to pull away if the test strip was held directly under the nose. There was no significant difference in the response to the stimulus by either transgenic or control animals. This suggested no difference in sense of smell in the transgenic mice compared to the control mice.

One noticeable difference was observed in the behaviour of the transgenic mice compared to the controls. The transgenic animals consistently appeared to be calmer than their littermate controls. For example, in response to the alarm from the timer used in the experiment the transgenic animals did not seem to be affected whereas the control animals became tense and hyperactive. The transgenic animals showed a similar response to the control animals if a vibration coincided with the noise. These results might suggest that the transgenic animals have a hearing defect or some other sensory deficiency.
4.3.11 Testes analysis

As mentioned above, older male transgenic mice, approximately 6 months of age, appeared to show a decrease in their fertility levels, as fewer off-spring were sired by these animals. To investigate this, both testes were removed from older transgenic mice; one testis was analysed for histology and the other testis was analysed for DNA content.

The histology of transgenic and littermate control testis is shown in figure 4.20. The control testis (a and c) shows fairly packed seminiferous tubules containing cells ranging from the outer spermatogonia to the mature sperm in the centre. Interstitial cells surround the tubules. In contrast, the seminiferous tubules from transgenic testis (figure 4.20b and d) contained large spaces and were less densely packed. The tubules appeared to contain all the different cell types but were lacking in total cell numbers. The numbers of interstitial cells surrounding the tubules was also greatly reduced. Young transgenic mice (approximately 6-8 weeks) did not show this difference in testicular tissue (data not shown) suggesting that there was a breakdown or reduction in the production of spermatids in older transgenic mice.

The different cell types within the seminiferous tubule (spermatogonia, spermatocytes, spermatids and sperm) represent various stages of meiotic division that give rise to haploid cells (1N) from diploid progenitors (2N), via a tetraploid intermediate (4N). Thus, one mechanism by which the relative numbers of spermatogenic cell types can be monitored is by DNA content. This can readily be analysed by propidium iodide staining followed by FACS. A propidium iodide staining experiment is shown in figure 4.21. The relative number of cells in each stage of the cell cycle appears to be very similar between the control and transgenic testes cell populations. The different phases of the cell cycle are indicated on the figure for the control sample only (red line). The population of
Transgenic mice: Results

Figure 4.20: Histology of transgenic and control testes

Testes were removed from 6-month-old transgenic and littermate control mice and fixed in 10% neutral buffered formalin for at least 24 hours. Tissues were sectioned and stained with hematoxylin & eosin. Scales: (a) & (b) 100X, (c) & (d) 250X magnification.

(a) & (c) The control testes shows the presence of seminiferous tubules with the interstitial cells between them. The seminiferous tubule is packed full of cells from the outer spermatogonia to the mature sperm mainly in the centre.

(b) & (d) The transgenic testes shows the seminiferous tubules contain a large empty space in the centre and thus are lacking in total cell numbers compared to the control testes. All cell types appear to be represented in the tubules but in lower numbers.

S = seminiferous tubules and I = interstitial cells.
Figure 4.21: DNA content analyses of testis cells from transgenic and control mice

Testicular cells from transgenic and littermate control mice were stained with propidium iodide (PI). Cells were fixed in cold 95% ethanol at 4°C for 48 hours, followed by washing in PBS. Cells were stained with 40 mg/ml PI solution. Cells were sorted according to their relative levels of PI by FACS analysis at 585 nm ± 42. The result shows a shift to the right in the transgenic sample (green line) compared to the control testes sample (red line). This indicates an increase in PI staining. The different phases of the cell cycle are indicated for the control testes sample. G = G₀/G₁ resting phase, S = DNA synthesis phase, M = G₂M mitotic division phase and D = doublets, triplets etc.
cells before the G0/G1 phase are from apoptotic cells and cellular debris. The transgenic sample (green line) cell cycle phases are shifted slightly to the right compared to the control. This indicates that the transgenic testis cells had increased propidium iodide staining compared to the control testis cells. There are two possibilities for this increase in staining; firstly, more DNA may be present either from more cells or larger cells, or secondly, the DNA may be in a less dense state, allowing the DNA to take up more propidium iodide. The results from the histology analysis do not suggest more cells or larger cells in the transgenic testis. Therefore, it seems likely that the DNA is less densely packed in the transgenic testis cells.

4.3.11 Evaluation of the eye phenotype in transgenic animals

The presence of the transgene could be predicted by examination of the eyes of each animal for each sub-line of transgenic line 1 in the later generations (greater than N2). PCR on tail DNAs was done to confirm the correlation of transgene presence and eye abnormality. The degree of eye abnormality did vary between animals and even between eyes of the same animal. This also appeared to correlate with the level of transgene expression, with the more severe phenotypes showing higher levels of transgene expression (refer to figure 4.5).

The eyes of several adult animals were examined, with the assistance of Dr. Arnie Leon, an ophthalmology expert. The affected eyes showed both diffuse and focal corneal opacities, which prevented examination of the ocular interior. In a few transgenic animals, the eyes were significantly smaller than in littermate controls, indicating microphthalmia. Figure 4.22 shows a control and a transgenic adult eye clearly revealing the abnormal physical characteristics of the transgenic eye. The corneal opacity is evident and a mucous discharge from the eye is also present.
Figure 4.22: Eyes from adult control and transgenic mice

a) Control adult mouse eye showing clear open eye.

b) Transgenic adult mouse eye showing small squinting eye with visible corneal opacity and mucous ocular discharge collecting on the exterior of the eye.
Adult eyes were removed from transgenic and littermate control mice for histological analysis (figure 4.23). The abnormality was determined to be anterior segment dysgenesis, which is characterised by the lack of an anterior chamber. There is also an absence of the corneal endothelium and Descemet's membranes and extensive adherence of the iridal tissue to the posterior surface of the cornea (compare figure 4.23c and d). Numerous small blood vessels were present in the corneal stroma and this vascularisation was accompanied by an increase in cellular elements. Calcium deposits were seen in the mid-corneal stroma and confirmed by Von Kossa staining (data not shown). There was also a focal corneal ectasia - an out-pouching of thinned cornea lined internally by pigmented iridal tissue. Further serial methods revealed corneal epithelial hyperplasia and hyperkeratosis overlying both a stromal calcium deposit and a region of intra-corneal haemorrhage. Towards the limbus the corneal stroma and pigmented iridal tissue were fused and thrown into dysplastic folds and rosettes. Generally the other regions of the eye, including the lens, retina, choroid, sclera, vitreous and optic nerve, were normal in the affected eyes. However, there were some animals that showed very severe eye abnormalities that affected the entire eye, such as microphthalmia and microphakia (figure 4.23e and f).

In the mouse, the eyes begin to develop from embryonic day 8.5 (E8.5) and continue until late in embryogenesis. To establish when the eye abnormality was occurring, embryonic and neonatal eyes were examined histologically at 11.5, 13.5, 14.5, 15.5, 16.5, 17.5 and 20.5 days post coitum. Newborn and 1-day old mice were also analysed. A male from sub-line 1A was mated with C57Bl6 females and the embryos were removed on the appropriate day. The embryonic heads were cut in half for histological analysis and another region of the embryo was taken for preparation of crude DNA that would be used for determination of transgenic status.
Figure 4.23. **Histological analysis of adult mouse eyes**

A) Control adult eye (4x magnification).

B) Transgenic adult eye showing abnormal anterior segment structure with extensive adherence of iris tissue to the posterior surface of the cornea, obliterating the anterior chamber. A corneal ectasia is also present in this section (arrowed). The lens and retina of this transgenic animal appear normal (4x magnification).

C) Higher magnification view of boxed inset in (A), showing corneal epithelium, relatively acellular corneal stroma containing a few keratocytes, corneal endothelium with underlying Descemet's membrane (arrowed), anterior chamber, iris and lens (40x magnification).

D) Higher magnification view of boxed inset in (B) illustrating increased cellular elements and vascularisation within the corneal stroma, absence of corneal endothelium, and adhesion of pigmented iris tissue to the posterior cornea (arrowed) (40x magnification).

Other examples of severely affected transgenic eyes.

E) In a neonatal mouse the ocular structure is severely disrupted. A microphakic lens with adjacent melanin pigment is completely surrounded by a massive inflammatory cell infiltrate (10x magnification).

F) In an adult mouse the eye is microphthalmic and the interior of the globe is composed almost entirely of highly dysplastic retinal tissue which is thrown into numerous folds and rosettes. The anterior uveal structures comprise an abnormal mass of melanin pigment adjacent to the cornea (4x magnification).

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The first gross difference between the control and transgenic embryos was seen in the cursory examination of the embryonic heads from E17.5 and older. The transgenic eye (figure 4.24b) was open, as opposed to the control eye (figure 4.24a, see arrow) which was closed. Histological analysis showed that this was due to a failure of the eyelid to fuse in transgenic animals and occurred earlier than E17.5 (figure 4.25). There was no apparent difference in the embryonic eyes up to and including E14.5 (compare figure 4.25a and b), but at E15.5 the transgenic eyes showed the first signs of a difference between littermate control and transgenic eyes. Firstly, there was a failure of the eyelids to fuse (compare figure 4.25c and d) and secondly, there were abnormalities in the developing cornea. The control eye (figure 4.25e) showed a well-organised corneal stroma and a clear endothelial cell layer (arrow). In contrast, the corneal stroma of the transgenic eyes (figure 4.25f) was disorganised and the endothelial cells were largely undefined.

At E17.5 the eyelid was still not fused in the transgenic mice. Indeed, eyelid fusion never occurred in transgenic animals. However, in both the control mouse eye (figure 4.25g) and the transgenic eye (figure 4.25h) keratinization (arrows) of the outer layer of the eyelid or eyelid bud was evident. This suggests that later signals, which normally follow the signal for eyelid fusion, have remained unaffected in the transgenic eye. At higher magnification (figure 4.25i and j) the disorganised stroma of the transgenic eye and the absence of endothelial and Descemet’s membranes (arrows) are clearly seen. The adherence of the iris tissue to the cornea and the lack of an anterior chamber are also evident.

In one-day-old transgenic mice, eyelid fusion was absent and the corneal stroma palpebral fissure contained an inflammatory infiltrate comprising mainly polymorphonuclear cells (figure 4.26). A keratitis was evident with polymorphonuclear cells infiltrating the posterior corneal stroma in some eyes (figure 4.26D large arrows) and the entire cornea in others. There was often an
Figure 4.24: **Mouse heads from embryonic day 17.5 control and transgenic mice**

a) Control embryonic head at day E17.5. The arrow indicates the closed eye of the embryo. The yellow appearance is due to the Bouin’s fixative.

b) Transgenic embryonic head at day E17.5 with the eye clearly open (arrow).
Figure 4.25: Histological analysis of embryonic mouse eyes

A) E14.5 control eye.
B) E14.5 transgenic eye showing no apparent difference from the control at this stage of development.
C) E15.5 control eye showing fusion of eyelid buds.
D) E15.5 transgenic eye showing absence of fusion of eyelid buds.
E) Higher magnification of boxed inset in panel (C) showing the iridocorneal angle of the E15.5 control eye. The corneal stroma is already well organised with regularly arranged, slim cellular elements. The corneal endothelial layer (arrowed) is also clearly differentiated from the stroma.
F) Higher magnification of boxed inset in panel (D) showing the iridocorneal angle of the E15.5 transgenic eye demonstrating poor differentiation of the corneal stromal and endothelial cell layers. Cells within the stroma appear rounded and unorganised, and the endothelial cells (arrowed) cannot be clearly defined.
G) E17.5 control eye showing keratinisation (arrowed) of the outer layer of the fused eyelids.
H) E17.5 transgenic eye. Eyelid fusion has failed in the transgenic eye. The lens and posterior segment of the eye appear similar to those of the control eye. Note the normal keratinisation (arrowed) of the outer layer of the unfused eyelids.
I) Higher magnification of boxed inset in panel (G) showing the E17.5 control eye iridocorneal angle. The arrow indicates the clearly defined endothelial cell layer.
J) Higher magnification of boxed inset in panel (H) showing the E17.5 transgenic eye iridocorneal angle, having poorly differentiated corneal stroma and endothelial cell (arrowed) layers compared with the control eye.

A, B, C, D, G and H = 10x magnification and E, F, I and J = 40x magnification.

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**Figure 4.26: Histological analysis of neonatal mouse eyes**

A) One-day-old control eye showing normal ocular structure. Note the fused eyelids covering the eye (10x magnification).

B) One-day-old transgenic eye showing failure of eyelid fusion, purulent exudate in the palpebral fissure, an inflammatory cell infiltrate in the posterior layers of the cornea, and haemorrhage and inflammatory cells (small arrows) between the lens and posterior surface of the cornea (10x magnification).

C) Higher magnification of boxed inset in panel (A) showing the iridocorneal angle of the control 1-day old eye (40x magnification).

D) Higher magnification of boxed inset in panel (B) showing the iridocorneal angle of the 1-day-old transgenic eye; the corneal inflammatory infiltrate is predominantly composed of polymorphonuclear cells (large arrows). The exudate within the palpebral fissure consists of a mixture of polymorphonuclear cells, keratin and other cellular debris (asterisk) (40x magnification).

accompanying hyperkeratosis of the corneal epithelium. The anterior chamber contained polymorphonuclear cells in addition to frank haemorrhage and a fibrinous exudate (figure 4.26B, small arrows). Some inflammatory cells were also occasionally observed within the palpebral conjunctiva.
4.4 Discussion

4.4.1 Three different transgenic lines were generated

The micro-injections produced three separate transgenic founders. The transgene expression analysis on these three lines revealed three entirely different patterns. Line 1 showed relatively high widespread transgene expression, line 2 showed no transgene expression and line 3 showed limited transgene expression. As might be expected, the site of integration and transgene copy number for each of these three lines also varied. Line 1 was shown to have one site of transgene integration and a relatively low transgene copy number per cell (1-2) (figures 4.3 & 4.4). Lines 2 and 3 both had at least 2 sites of transgene integration and very high copy numbers approximately 25 and 12 respectively (figures 4.3 and 4.4). However, the results from the Southern blot showing the site of integration and the PCR copy number estimation for line 2 were contradictory (compare figures 4.3 and 4.4). The Southern blot suggested a lower transgene copy number per cell for line 2 than for line 1, whereas the PCR analysis suggested a higher copy number per cell for line 2 than for line 1. The PCR was repeated many times with DNA prepared from different animals and the copy number determined by this method often varied in later generations (N2 and later). The DNA used for the Southern blot was prepared from an N2 animal. The Southern blot result is likely to be more reliable than the PCR analysis. The PCR analysis variability was probably due to a reduction in the number of copies of the transgene by homologous recombination events in later generations.

The expression of a transgene has been shown to be influenced by the number of copies of the transgene integrated within the genome. Transgenes that are integrated as high copy number arrays generally show a reduction in gene expression. This transcriptional silencing is often associated with heterochromatin formation and hypermethylation which repress transgene expression (Garrick et al., 1998). This phenomenon of reduction in transgene expression probably
accounted for the lack of phenotype observed in the transgenic line 2. A number of other factors can also influence the expression of the transgene, including the site of integration, the cell lineage, cis-acting control elements within the transgene and strain-specific differences in chromatin proteins (Martin and Whitelaw, 1996).

Transgenic line 3 showed a limited pattern of transgene expression, including significant expression in bone, muscle and skin and lower levels in kidney and eye. Despite this, no phenotype was observed in these animals in any tissue. There was also no phenotype observed in bone, muscle, skin or kidney for transgenic line 1 animals, which also showed expression of the transgene in these tissues. This suggests that in these tissues over-expression of fra-2 has no effect. However, transgenic line 3 mice did not develop the abnormal eye phenotype that was observed in the transgenic line 1 animals. This may indicate that in transgenic line 3 the fra-2 transgene was expressed in a different cell type within the eye than was the case in transgenic line 1.

4.4.2 Different patterns of transgene expression within line 1

The first five transgenic off-spring from line 1 showed 3 distinct patterns of transgene expression by Northern blot analysis and this resulted in each of these animals (and their offspring) being treated as a separate sub-line. The distinct patterns of expression were unexpected, given that all of these sub-lines appeared to have the same site of integration and the same number of copies of the transgene per cell. This phenomenon may have been due to the use of the CMV promoter, since a similar result has been reported previously with this promoter (Furth et al., 1991). Interestingly, in later generations all of the sub-lines gave the same pattern of transgene expression. This may be explained if the initial differential patterns were due to strain variations affecting the expression of the transgene. The founder mice were from a C57Bl6/SJL1 background.
(approximately 75:25 ratio) and then all future matings were done with C57Bl6 mice. Therefore, over successive generations, the mice would have all become very close to 100% C57Bl6. There are strain-specific modifiers of DNA methylation and expression (Allen et al., 1990). These have been shown to influence the pattern of transgene expression in mice from both mixed strain and inbred strain backgrounds (Chaillet, 1994). The C57Bl6 mouse strain has been shown to be highly methylated and this can cause a reduction in the expression of the transgene (Chaillet et al., 1995). In the pCMV-mfra2 transgenic mice, the earlier generations may have been influenced by different DNA modifiers than in the later generations, which would have all been affected by the C57Bl6 strain of modifiers.

4.4.3 Transgenic mice over-expressing Fra-2 lacked tumours

As discussed above, the expression of fra-2 was readily detectable in two of the three transgenic lines (1 and 3) and the gene was shown to be expressed (at both RNA and protein level) in a wide range of tissues in line 1. The absence of tumour development in these mice, up to 2 years later, suggests that fra-2 alone is not oncogenic. This does not exclude Fra-2 from having a role in oncogenesis, but suggests that over-expression of Fra-2 is not sufficient for tumour development.

Despite the known oncogenic potential of some of the AP-1 family members, namely c-jun (Maki et al., 1987) and c-fos (reviewed in Cohen and Curran, 1989), all AP-1 family members for which transgenic mice have been reported, (ie. c-jun (Grigoriadis et al., 1993), c-fos (Grigoriadis et al., 1993; Rüther et al., 1987; Rüther et al., 1989), fosB (Grigoriadis et al., 1993) and fosB2 (Carrozza et al., 1997)) with the exception of c-fos, have also shown a lack of tumour development. In this regard, fra-2 appears to be a typical member of the AP-1 transcription family. Therefore, c-fos appears to be the exception in this family of genes, in that over-expression of c-fos alone can cause tumours. Interestingly,
transgenic mice over-expressing both c-fos and c-jun showed an enhanced rate of tumour development compared to mice only over-expressing c-fos (Van Dam et al., 1993). This suggests some cooperative role for these two genes in the development of the tumours.

Over-expression of fra-2 can weakly transform CEF (Foletta et al., 1994; Nishina et al., 1990) and fra-2 mRNA levels are affected in a number of cell lines transformed by other oncogenes (Kralova et al., 1998; Mehta et al., 1997; Murakami et al., 1997; Suzuki et al., 1994). fra-2 is also known to be over-expressed in a number of human tumour cell lines (Matsui et al., 1990). These results suggest Fra-2 may play some role in cellular transformation. However, it is either the case that other cellular changes are required for transformation to occur, or the Fra-2 protein might have to be modified in some way to realise its oncogenic potential. This is the case with Jun. In the viral form of Jun, v-Jun, there is a deletion of the phosphorylation controlled transactivation δ domain (Smeal et al., 1991), which greatly enhances the transforming ability of this viral gene.

4.4.4 Few phenotypes were observed in the transgenic mice over-expressing fra-2

Mice from transgenic line 1 did show high levels of transgene expression in many tissues, but only 3 phenotypes were observed in the mice: a change in the testes of older male mice, reduced body weight and an eye abnormality. The majority of tissues appeared to be unaffected in these mice, despite high levels of transgene expression. This may be attributed to the high levels of endogenous fra-2 expressed in a number of murine tissues, both during embryogenesis (Carrasco and Bravo, 1995) and in the adult mouse (Foletta et al., 1994), such that any further increases in expression do not have any effect on normal cellular function. The activity of Fra-2 may also be strictly controlled, for example by
phosphorylation. Fra-2 is a known phosphoprotein and phosphorylation has been reported to enhance its activity as a transactivator (Murakami et al., 1997). Hence, even in circumstances where the Fra-2 protein levels are high, only a small proportion of the protein may be functionally active.

However, while this last point may be true, the results obtained with these transgenic mice suggest that other mechanisms were responsible for the lack of phenotype in most tissues. The expression of the transgenic fra-2 RNA was widespread as detected by Northern blotting, but only some of these tissues (eye, heart, kidney, muscle, pancreas, skin and stomach) showed any significant differences in protein expression levels between transgenic and littermate control mice. Although the immunofluorescence staining might not have identified all of the Fra-2 protein that was present (due to high auto-fluorescent backgrounds in some tissues and since fixatives and antibody combinations required for optimum results can vary between tissues (Foletta, 1995)), the Western blotting results are likely to be a reliable indicator of protein levels. By Western blotting analysis, no transgenic tissue contained particularly high levels of Fra-2 protein. This may indicate that there is some level of translational control on the fra-2 mRNA, such that high mRNA levels don’t necessarily result in high protein levels. This translational control is not specific for the transgenic RNA, as closer examination of endogenous mRNA and protein expression level analysis (work performed by Victoria Foletta (Foletta, 1995)) does show a similar effect. The high mRNA levels observed in the ovary and stomach do not correspond to high protein levels in these tissues; the level of Fra-2 protein expression in ovary and stomach is similar to that observed in the other Fra-2 expressing tissues, for example, in bone, heart and thymus.

There are regulatory processes that can affect the rate of protein translation. For example, some mRNA species are very unstable, as seen with the c-fos gene (Veyrune et al., 1995). The selection of the mRNA by the ribosomal complex and/or identification of the translational start codon can also impact on protein
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Translation. Initiation factors involved in these processes may be limiting or inactive in some tissues, as seen in the control of GCN4 translation (Hinnebusch, 1994). There have also been reports of expressed transgene RNA fragments inhibiting the expression of the endogenous genes (Cameron and Jennings, 1991). For example, in transgenic plants containing copies of a functional transgene involved in flower colouration, expression of the transgene actually reduced the colour in the plants rather than enhancing it (Van der Krol et al., 1990).

Interestingly, there was evidence that the presence of the transgene was leading to reduced expression of the endogenous fra-2 gene in some circumstances. Since Fra-2 is a transcription factor, the effect can easily be explained by the postulate that Fra-2 may regulate expression from its own promoter. In support of this, the murine fra-2 gene does contain an AP-1 site within its promoter region (Foletta et al., 1994) and this site has been shown to be bound by a variety of AP-1 complexes (Foletta, 1995). Transient transfection studies in mouse and chicken cells have shown that the c-Jun/Fra-2 AP-1 complex can suppress fra-2 promoter activity (Foletta, 1995; Sonobe et al., 1995).

From the Northern blotting results, only the stomach appeared to show this down-regulation of the endogenous fra-2 mRNA. There are two possible explanations for why this phenomenon was not seen in other tissues. Firstly, down-regulation of the endogenous gene by the transgenic Fra-2 could only occur if the endogenous gene and the transgene were being expressed in the same cells within the tissue. As expected from the Northern blotting result, the immunofluorescence staining of the stomach showed co-expression of the endogenous fra-2 gene and the transgene in the same mucosal cells. However, the immunofluorescence results for the skin also showed a common localisation of transgenic and endogenous Fra-2 in the hair follicle, whereas the Northern blot did not show down-regulation of the endogenous fra-2 mRNA in skin.
Thus, the second reason for lack of this down-regulation phenomenon in some circumstances might relate to the functional activity of Fra-2 in those situations. Fra-2 binds to DNA as part of a heterodimeric complex. Therefore, auto-regulation may depend on the presence of a particular protein partner in the cell. Thus, the level of the protein partner, and not the level of Fra-2, may be the limiting factor in the functional activity of AP-1 complexes containing Fra-2. The requirement of a protein partner by Fra-2 may also explain why some tissues lacked any phenotype, despite the expression of the fra-2 transgene in those tissues.

Therefore, the lack of phenotype in most tissue might reflect one of the following circumstances.

(i) the transgene was not expressed at all;
(ii) transgenic RNA was transcribed but was not translated into protein;
(iii) transgenic protein was translated but not modified correctly to produce functional protein; or
(iv) functional Fra-2 protein was made from the transgene, but absence of protein partners limited the levels of functional complexes containing Fra-2.

Presumably, only in those three circumstances discussed below did the cellular conditions permit some manifestation of the presence of the transgene.

Another important consideration in any random insertion transgenic mouse experiment is the impact of the site of integration of the transgene in relation to the phenotypic outcome observed in the resulting animals. With only one transgenic line showing any phenotype, the site of the transgene integration or positional effects on the transgene cannot be excluded as the cause of the eye abnormality in this study. However, the correlation between the level of transgene expression and the degree of severity of the eye abnormality suggests that it was, in fact, the expression of the transgene that caused the development of the eye abnormality.
4.4.5 Testes

In mice, spermatogenic cycles begin every 12 days and the whole process takes 34.5 days. The process is not simultaneous in all tubules. Not all cells complete spermatogenesis; degeneration occurs at the first and second meiotic divisions ((Bronson et al., 1966) and references within). The testes of transgenic mice over-expressing Fra-2 developed and functioned normally until approximately 6 months of age. Mice up to 6 months old successfully produced viable offspring, indicating an active spermatogenic cycle. This was confirmed by histology and FACS analysis of younger male transgenic mice (data not shown).

However, the results from the histology and DNA content experiments (figures 4.20 and 4.21) with the testes from older (at least 6 months) male transgenic mice suggested a degeneration of cells in the transgenic testes. Interestingly, cells from the transgenic testes showed increased propidium iodide staining. As discussed earlier, this might indicate either an increase in cell size or less dense packing of the DNA. As the histology of the testes showed that there was no significant difference in the cell size between transgenic and control testes, then it seems likely that the DNA of the transgenic testes cells was less densely packaged and therefore able to incorporate more propidium iodide stain. The FACS analysis also showed no significant difference in the relative number of apoptotic or necrotic cells between the control and transgenic testes cell populations. However, the histological analysis revealed that the transgenic testis contained less cells and large, acellular regions in the centre of the seminiferous tubules. Therefore, the degeneration may have occurred by a reduction in the spermatogenic cycle over time, so that the total number of cells in the transgenic testes was not maintained, even though the relative number of spermatogenic cell types was unchanged.

Interestingly, a similar effect on the testes was observed in transgenic mice expressing anti-sense fra-2 (Foletta, 1995). It is unlikely that both the anti-sense transgene and the sense transgene used in the present study were coincidently
integrated into sites where they affected the activity of a gene involved in spermatogenesis, and so other explanations must be found.

One possibility is that the presence and expression of any transgene causes a disruption in spermatogenesis as the transgene animals age. As there have been a large number of transgenic animal studies reported where no such phenomenon was observed, this is unlikely to be the explanation. However, it cannot be completely ruled out that there is some degree of non-specificity in the phenomenon of disrupted testis function observed in this study and the previous study using anti-sense fra-2.

A second possible explanation is that the over-expression of sense fra-2 from the transgene resulted in a net reduction in overall Fra-2 protein levels in the testes by the same phenomenon as described earlier, whereby gene expression in a transgenic plant was inhibited by sense RNA transcribed from a transgene (Cameron and Jennings, 1991).

A third explanation for the similar phenotype in transgenic mice expressing sense or anti-sense fra-2 may be that the relative level of Fra-2 compared to other Fos family members may be more important than the absolute level of Fra-2 protein. The Fos family of proteins can only bind DNA when they are in a heterodimeric complex with a protein partner (e.g. a Jun family member). Thus, the relative levels of all the Fos family members will determine which heterodimeric combinations of proteins occur. Therefore, any increase or decrease in the level of one of these Fos proteins would disrupt this balance, leading to changes in the availability of protein partners for the other Fos family members. This would lead to changes in the total array of AP-1 complexes, and since the different dimeric complexes have different affinities for individual binding sites and different functional activities, this could result in significant changes to the overall pattern of gene expression.
A hypothetical scheme for how this could explain the phenomenon whereby both increases and decreases in the level of Fra-2 protein have the same net effect on spermatogenesis is shown in Figure 4.27. In this hypothetical system, only four AP-1 family members are considered: Fos, Fra-2, Jun and JunD. The expression of a gene, x, whose protein product X is involved in promoting spermatogenesis, is positively regulated by Fos-Jun complexes and negatively regulated by Fos-JunD complexes. In this system, the role of Fra-2 is to bind to JunD and limit the formation of Fos-JunD complexes, which repress expression of gene x. If the level of Fra-2 increases dramatically, then not only would Fra-2 form complexes with JunD, it would also compete effectively for binding to Jun. Therefore reducing the number of Fos-Jun complexes and resulting in reduced transcription of gene x. On the other hand, if the level of Fra-2 decreased significantly, then the number of Fos-JunD complexes would increase, resulting in down-regulation of gene x expression. Although this scheme is very much a simplification of the interplay between competing transcription factors, it does illustrate how it might be possible for both an increase and a decrease in the amount of Fra-2 protein to produce the same net phenotype.

The results in the testes are interesting given that fra-2 transgene expression was barely detected in the testes by Northern blotting. The Northern blot on line 1B tissue RNA samples did show a very low level of fra-2 transgene expression, thus the level of fra-2 transgene expression may have been at the limits of detection by Northern blotting. The CMV promoter has been shown to be active in the testes but produces low levels of transcript as compared to its activity in other tissues (Furth et al., 1991). The immunofluorescence results showed no significant difference in the pattern or amount of Fra-2 protein between the control and transgenic testes. However, there was a high auto-fluorescent background in these samples and as discussed above, the immunofluorescence conditions may not have been optimal for this tissue, particularly if the level of Fra-2 protein was low.
A hypothetical scheme for how increases and decreases in Fra-2 levels can cause the same net effect on spermatogenesis. In this hypothetical system, only four AP-1 family members are considered: Fos, Fra-2, Jun and JunD. The expression of a gene, x, whose protein product X is involved in promoting spermatogenesis, is positively regulated by Fos-Jun complexes and negatively regulated by Fos-JunD complexes. In this system, the role of Fra-2 is to bind to JunD and limit the formation of Fos-JunD complexes, which repress expression of gene x. Furthermore, in the proposed system, Fra-2-Jun and Fra-2-JunD complexes do not have a high affinity for the AP-1 site in the promoter of gene x. If excess Fra-2 is present, then not only would Fra-2 form complexes with JunD, it would also compete effectively for binding to Jun, thereby reducing the number of Fos-Jun complexes and resulting in reduced transcription of gene x. On the other hand, if there was insufficient Fra-2, then the number of Fos-JunD complexes would increase, resulting in down-regulation of gene x expression. Blue circles = Fra-2, red circles = Fos, green circles = c-Jun and orange circles = JunD.
Excess Fra-2

Insufficient Fra-2

Gene x

AP-1

Spermatogenesis

Protein X
The expression of endogenous $fra-2$ mRNA in the testes has been detected by RT-PCR (Cohen et al., 1993; Foletta et al., 1994). Fra-2 is known to be expressed during the onset and shutdown of spermatogenesis in spermatocytes and spermatids of the seasonal breeder, the European red fox (Cohen et al., 1993). Fra-2 protein has also been shown to be expressed in the Leydig cells of mouse testes (Foletta, 1995) and its expression is induced in response to human chorionic gonadotrophin (hCG) in the murine Leydig cell line, MA-10 (Suzuki et al., 1998). In unstimulated MA-10 Leydig cells three $fra-2$ transcripts were detected: 6 kb, 2.3 kb and 1.75 kb. After hCG stimulation, the 6 kb and 2.3 kb transcripts increased and peaked within 1 hour. Leydig cells are stimulated by hCG to secrete testosterone and high testosterone levels in the testes are required for normal spermatogenesis to occur (Suzuki et al., 1998). Therefore, Fra-2 could potentially be involved in the regulation of testosterone gene expression in Leydig cells. It is possible that at low levels of Fra-2 protein, testosterone gene expression is promoted, while at higher levels of Fra-2 protein, testosterone gene expression is inhibited. As discussed earlier, disruption of spermatogenesis in transgenic mice did not occur until approximately 6 months of age. The reason for the latency in phenotype development may be attributed to a gradual build up in transgenic Fra-2. The RNA from many testes-specific genes, e.g. PGK-2, has been shown to be stored as nonpolysomal RNA as a form of translational control (Eddy et al., 1991). If transgenic $fra-2$ RNA was also stored in this manner, then with time the levels may be significant enough to have an effect in the testes.

There have been reports of other transgenic and knockout mice that have shown male infertility and degeneration in the seminiferous tubules of the testes. These include the transgenic mice over-expressing follistatin (Guo et al., 1998) and mice that have lost a functional expression of the estrogen receptor (ER) (Eddy et al., 1996). The follistatin gene has been shown to be TPA inducible and thought to signal through the cAMP-PKA pathway (Miyanaga and Shimasaki, 1993). All members of the AP-1 transcription factor family are induced by TPA and cAMP has been shown to induce expression of Fos proteins, including Fra-2, in the Leydig cell line, MA-10 (Li et al., 1997). AP-1 complexes can also directly interact with the ER and inhibit its activity (Tzukerman et al., 1991). Therefore if Fra-2 is involved in these regulatory activities, over-expression of Fra-2 may cause a similar testicular disruption through a similar
signalling pathway. Excess Fra-2 may interact with ER and inhibits its function and/or induce expression of either follistatin or other genes in that signalling pathway.

There are a number of other genes potentially regulated by Fra-2 which, if their expression were to be deregulated, might result in downstream effects on the spermatogenic cycle. For example, putative AP-1 binding sites have recently been identified in the promoters of two mouse genes: testis-specific protein kinase 1 (tesk1) (Toshima et al., 1998) and cysteine-rich secretory protein-1 (CRISP-1) (Schwidetzky et al., 1997). Tesk1 is a protein serine-threonine kinase that is expressed predominantly in testicular germ cells. The CRISP-1 protein is expressed in the testicular epididymis and is involved in final stages of spermatogenesis. Further investigation into the expression patterns of these genes in the transgenic mice over-expressing Fra-2 would be required to establish a role for Fra-2 in the regulation of expression of these genes.

4.4.6 Weight differences

The transgenic mice over-expressing Fra-2 were consistently smaller than their littermate controls. This was seen as an overall reduction in weight, rather than any decrease in the amount of body fat or size of any particular tissue. A behavioural experiment did not reveal any difference between transgenic and control mice in their ability to smell and food was invariably found in the gastrointestinal tract of all sacrificed animals. Therefore, it was unlikely that weight differences were due to any effects on feeding in the transgenic mice. The weight reduction could potentially be linked to the expression of the fra-2 transgene in the pancreas. The pancreas is involved in the digestive pathway and secretes many hormones from the islet of Langerhans, including insulin, glucagon, somatostatin and pancreatic polypeptide (Ganong, 1987). The pancreas plays an important role in the regulation of growth. In diabetes mellitus, insulin levels are reduced and the patients often show a reduction in weight. The expression of insulin can be inhibited by somatostatin and recently the mouse somatostatin receptor type 5 gene has been shown to contain AP-1 binding sites within its promoter (Baumeister et al., 1998).
There are two potential mechanisms for the reduction in weight observed in the transgenic mice:

(i) Fra-2 could directly negatively regulate the expression of target genes, such as insulin, which promote growth; or

(ii) An excess of Fra-2 could compete with other AP-1 members, possibly c-Fos or Fra-1, for protein partners, thereby reducing the number of functional AP-1 complexes that are available to regulate those genes involved in growth promotion.

Another possible explanation for the smaller size of the transgenic animals is that Fra-2 may play an anti-proliferative role in some circumstances. Over-expression of Fra-2 might therefore have a net slowing effect on growth, although clearly, cell proliferation is still occurring in the transgenic animals. This would also provide an alternative explanation for the lack of tumour formation, and may also explain the lack of eyelid fusion in the transgenic animals (see below). An anti-proliferative function of Fra-2 could be mediated by one or more of the following mechanisms:

(i) up-regulating the expression of genes that encode proteins with anti-proliferative functions;

(ii) down-regulating the expression of genes that encode proteins that promote proliferation;

(iii) competing for complex formation and/or DNA binding sites with other transcription factors and thereby interfering with the function of those complexes.

4.4.7 Eye abnormality

Adult eyes from transgenic mice showed ocular abnormalities suggestive of anterior segment dysgenesis. This was characterised by corneal abnormalities, including an absence of corneal endothelial and Descemet’s membranes, disruption of the corneal stroma and adherence of the iridal tissue to the cornea. These defects collectively result in an absence of an anterior chamber in the eye. This phenotype closely
Transgenic mice: Discussion

resembles that seen in two congenital conditions of the human eye, namely Peters’ anomaly and Rieger’s anomaly (Sheilds et al., 1985).

Examination of embryonic eyes revealed that the first gross differences between transgenic and littermate control mice occurred at E15.5. At this stage the first signs of corneal disruption were visible and there was a lack of proliferation in the eyelid buds, and corresponding absence of eyelid fusion. At later stages of development, cellular infiltration of inflammatory cells into the cornea and anterior chamber was evident. This neonatal keratitis was probably the cause of the adult anterior segment dysgenesis phenotype and may be a consequence of the lack of eyelid fusion. The premature exposure of the eyes may cause irritation, resulting in corneal injury and therefore inflammation. The eyelids develop as folds of the surface ectoderm, which proliferate to form upper and lower lids that fuse and then separate again approximately 10 days after birth (Tripathi et al., 1991). In the transgenic eyes, the initial signals that produce the eyelid buds, as well as the later signals such as those that result in keratinisation of the outer eyelid are still occurring. Therefore, as discussed above, the failure of eyelid fusion appears to be a lack of cellular proliferation signals that promote the growth of the eyelid buds into eyelids. Specific factors have been identified in chicken embryonic eyes that have proliferative and anti-proliferative functions. They are found in the anterior chamber and the vitreous humor of the eye, respectively (Hyatt and Beebe, 1993). These factors, or similar factors, may play a role in controlling proliferation of mouse eyelids and Fra-2 could potentially be involved in regulating the expression of such factors.

A similar mechanism could also explain some of the other defects in the transgenic eyes that resulted in anterior segment dysgenesis. The corneal endothelium is formed through the flattening of two or three layers of cells into a monolayer. These cells secrete intermittent basal lamina which form the basis of the Descemet’s membrane (Tripathi et al., 1991). A lack of proliferation signals or the presence of anti-proliferative signals, caused by over-expression of Fra-2, might disrupt the formation of the initial endothelial cell layers. This would then inhibit all subsequent events leading to the development of the corneal endothelium and Descemet’s membrane.
Further investigation into the role Fra-2 may play in cellular proliferation, particularly of endothelial cells, would be informative on this point.

The mouse eye develops from several regions of the embryonic forebrain and development begins at E8.5 (reviewed in Freund et al., 1996; Tripathi et al., 1991). The lens, eyelids and corneal epithelium develop from the surface ectoderm and the corneal endothelium and stroma develop from the neural crest tissues of the developing embryo (Tripathi et al., 1991). Eye development involves the mutual interactions of developing regions of the eye to obtain the precise and coordinated process of eye development (Saha et al., 1992). Thus, inappropriate development of one region of the eye can have effects on the development of other ocular structures. For example, the lack of a lens in transgenic mice expressing diptheria toxin under the control of a αA-crystallin promoter causes concomitant reduction in eye size, classically microphthalmia (Harrington et al., 1991). Therefore some of the major ocular disruptions seen in some transgenic mice, like microphthalmia or microphakia (refer to figures 4.23e and f), may have been a consequence of this alteration in the coordinated signalling between the developing regions of the eye, rather than a direct effect of transgene expression. However, direct transgene involvement cannot be ruled out based on the analysis performed here.

A vast number of transcription factor genes have be shown to be involved in eye development (reviewed in Freund et al., 1996). Whether Fra-2 is just another factor to be added to this list of transcription factors or whether it is linked to any of these factors is yet to be determined. The questions of how over-expression of fra-2 leads to eye abnormalities and possible downstream target genes of Fra-2 need further investigation. Attempts to answer these questions are pursued in the following chapter.
Chapter 5

Target genes of Fra-2
5.1 Introduction

In the previous chapter transgenic mice over-expressing fra-2 were described. The major phenotype in these mice was an eye abnormality. The next step was to investigate possible mechanisms for the development of the eye abnormality and identify any potential target genes of Fra-2.

A search of the literature revealed a number of knockout and transgenic mice which had developed eye abnormalities (reviewed in Götz, 1995). The three main eye abnormalities were microphthalmia, cataracts and anterior chamber malformations.

TGFα knockout mice have been reported to show an abnormality in the anterior chamber of the eye, including open eyes at birth and corneal inflammation (Luetteke et al., 1993; Mann et al., 1993). It was also reported that there was a reduction in the weight of homozygous pups compared to their heterozygote littermates in TGFα knockout mice (Luetteke et al., 1993).

Anterior chamber malformations have also been reported with transgenic over-expression of two genes, T-cell receptor δ (Tamura et al., 1995) and TGFα (Decsi et al., 1994; Reneker et al., 1995). The T-cell receptor δ transgenic mice expressed the T-cell receptor δ gene under the control of its own promoter. The mice showed anterior segment dysgenesis, corneal opacities and adherence of the iris tissue to the cornea (Tamura et al., 1995). Transgenic mice over-expressing TGFα under the control of the lens specific αA crystallin promoter also showed an eye abnormality. There were corneal disruptions, absence of endothelial and Descemet's membranes, cellular infiltration of the anterior chamber and delayed eyelid closure (Decsi et al., 1994; Reneker et al., 1995); this collection of defects are similar to those observed in the fra-2 transgenic mice. However, TGFα
transgenic mice also showed lens and retinal abnormalities that were not seen in the fra-2 transgenic mice.

Interestingly, daily injections of TGFα in normal newborn mice have been shown to cause precocious eye opening, premature tooth eruption and reduced growth rates (Smith et al., 1985; Tam, 1985). These effects all correlated well with the dose of TGFα administered. The similarity between the eye defects resulting from altered levels of TGFα and those observed in the fra-2 transgenic mice suggested a potential link between Fra-2 and TGFα.

There are a number of transcription factor genes which are known to be involved in mammalian eye development (reviewed in Freund et al., 1996). Two of these factors, Pax6 and Mitf, when mutated, have been shown to produce similar phenotypes to those seen in the transgenic mice over-expressing Fra-2. Briefly, Pax6 mutations produce a variety of phenotypes that usually involve the anterior chamber of the eye. Pax6 is also known to be expressed in the developing eye including the cornea (Walther and Gruss, 1991). Mitf is a bHLH-Zip protein that is associated with microphthalmia (small eyes) and has also been shown to cause inner ear defects, osteopetrosis and failure of tooth eruption (Hodgkinson et al., 1993). The latter two of these phenotypes are similar to those observed in c-Fos knockout mice. These highlight potential candidate genes that may interact with Fra-2 and lead to the development of the eye abnormality.

There are two main strategies that can be employed to investigate the target genes of Fra-2. Firstly, directly looking at potential target genes of Fra-2 determining if Fra-2 affects the expression of these genes. Initially experiments would include analysis of the expression patterns of these genes in the fra-2 transgenic mice. Secondly, a more general approach of searching for a range of genes whose expression may be affected by an increase in Fra-2 levels. This involves the use of
experimental protocols such as PCR-Select differential screening (CLONTECH) to identify differentially expressed genes.

The PCR-Select differential screening protocol (CLONTECH) is based on suppression subtractive hybridisation (Diatchenko et al., 1996). The PCR-select differential screening protocol involves the subtraction of identical sequences from two cDNA populations and then enrichment of the differentially expressed cDNAs. It uses a suppression PCR strategy to exclude (or greatly reduce) amplification of those products which are identical in both populations (refer to figure 5.1). The procedure is explained briefly below.

The first step is the production of two double-stranded (ds) cDNA populations. These cDNA populations are digested with Rsa I, a four base cutting restriction enzyme that produces blunt ends. The digested ds cDNAs are divided into 3 groups, driver cDNA, which requires no further modifications, and tester-1 and tester-2 cDNA populations, which require ligation of separate adaptors, adaptor 1 or adaptor 2R respectively. Two hybridisation steps are then performed. In the first hybridisation an excess of driver cDNA from cDNA population 1 is mixed with a sample of each tester cDNA from cDNA population 2 in separate tubes. Identical sequences in the two cDNA populations should anneal together in each of the reactions. The second hybridisation step involves the mixing together of the first two hybridisation reactions with extra freshly-denatured driver cDNA (population 1). This encourages any identical sequences between the two cDNA populations, that have not previously annealed, to anneal. It also allows those sequences that are only in the tester population to form ds cDNA molecules having one strand from each tester sample, ie. with different adaptors on each strand of the cDNA. These ds tester molecules are then amplified by PCR using primers specific to the different adaptors. A second PCR amplification with nested primers specific to the different adaptors further enriches these differentially expressed cDNAs. The procedure is also performed in the opposite
Figure 5.1: Suppression subtractive hybridisation method

Schematic diagram of the suppression subtractive hybridisation method reproduced from Diatchenko et al., 1996 (Diatchenko et al., 1996). The solid lines represent Rsa I digested tester and driver cDNA. The solid and clear boxes together represent adaptor 1 and individually correspond to the PCR primer 1 and nested PCR primer 1 sequences respectively. The shaded box and clear box containing a dot together represent adaptor 2R and individually correspond to the PCR primer 2R and nested PCR primer 2R sequences respectively.
direction, with population 2 driver and population 1 tester cDNAs to obtain the differentially expressed sequences from both populations. This protocol has been used successfully to clone a novel member of the tumour necrosis factor (TNF) cytokine family, called TRANCE (Wong et al., 1997).
5.2 Methods

Solutions used in this chapter are listed in alphabetical order in appendix A. The oligonucleotide primers used in this chapter are described in appendix B.

5.2.1 In vitro transcription

One microgram of previously linearised plasmid DNA was *in vitro* transcribed using 150 units/60 μl reaction of T7 RNA polymerase (New England BioLabs). The reactions used the T7 promoter carried within the plasmid vector. The reaction contained 10 mM DTT, 0.1 mg BSA, 120 units of RNasin (Promega), 0.133 mM m7GppG (Pharmacia) and 0.667 mM of each dNTP in 1 X transcription buffer. The reaction was incubated at 40°C for 1 hour and then 1 unit of RQ1 DNase I (Promega) was added followed by a second incubation step at 37°C for 15 minutes. The reaction was purified by phenol extraction (see method 3.2.1) and then the newly transcribed RNA was ethanol precipitated with 2.5 M ammonium acetate. The RNA pellet was dissolved in water and the concentration of the RNA was determined by optical density at 260 nm as described in method 2.2.5. A small aliquot of each RNA sample was analysed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel to check the quality of the RNA.

5.2.2 In vitro translation

Radioactively labelled and unlabelled proteins were translated from 0.5 μg of *in vitro* transcribed RNA. Proteins were translated in reticulocyte lysates (Promega) with 22.2 μM amino acid mixture either minus or plus methionine depending on whether the proteins were to be labelled or not, respectively. The labelled proteins were labelled with 25 μCi of 35S methionine. The reactions were incubated at 30°C for 30 minutes and then 1 μl of each labelled protein, in 1 X dissociation buffer, was analysed by SDS-PAGE on a 13% polyacrylamide gel in 1 X Laemmli.
buffer. Following electrophoresis the gel was fixed in 25% isopropanol/10% acetic acid for 30 minutes and then incubated in Amplify (Amersham) for 20 minutes. The gels were then dried using a BioRad gel dryer (model 383) at 80°C for 20 minutes and then exposed to film.

5.2.3 In vitro association experiment

One microlitre of each in vitro translated protein to be tested was added together with 4 μl of reticulocyte lysate (Promega). Proteins were generally assayed by mixing one labelled protein with either none, one or two unlabelled proteins. The proteins were allowed to associate at 37°C for 45 minutes and then 100 μl of RIPA buffer was added to each reaction. The samples were vortexed and then centrifuged for 5 minutes at 14,000 rpm (Eppendorf, 5415C). The supernatant was collected and mixed with 1.5 μl of the appropriate antibody and incubated at 4°C for 2 hours. Following this, 25 μl of Staphylococcus aureus suspension in RIPA buffer was added and the reactions were incubated for a further 15 minutes at 4°C. The Staphylococcus aureus suspension had been previously prepared from PANSORBIN cells (Calbiochem) according to the manufacturer’s instructions with extensive washing in RIPA buffer. The reaction was centrifuged for 30 seconds at 14,000 rpm (Eppendorf, 5415C) and the supernatant was removed. The pellets were washed in 3 x 500 μl of RIPA buffer and then resuspended in 25 μl of 1 X dissociation buffer. The samples were frozen in a dry ice-ethanol bath and kept at -70°C overnight. The following day, the samples were thawed and the pellets were resuspended. The samples were boiled for 3 minutes and then centrifuged for 2 minutes. A 15 μl aliquot was removed from each sample supernatant and electrophoresed under denaturing conditions on an 11% SDS polyacrylamide gel in 1 X Laemmli buffer. The gels were fixed in 25% isopropanol/10% acetic acid for 30 minutes and then incubated in Amplify
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(Amersham) for 20 minutes. The gels were then dried on a BioRad gel dryer (Model 383) at 80°C for 20 minutes and exposed to film.

5.2.4 mRNA preparation

mRNA was prepared from tissue or cell extracts using the QuickPrep micro mRNA purification protocol (Pharmacia) according to the manufacturer’s instructions. Briefly, tissues were homogenised using a small plastic hand-held homogeniser in 400 µl of extraction buffer. Cells were not homogenised, but were pelleted and then resuspended by vortexing in 400 µl of extraction buffer. 800 µl of elution buffer was added and the samples were vortexed thoroughly to mix. The samples were centrifuged at 14,000 rpm (Eppendorf, 5415C) for 1 minute and 1 ml of each sample supernatant was added to 1 ml of Oligo(dT) cellulose. The Oligo(dT) cellulose had previously been pelleted by centrifugation for 1 minutes at 14,000 rpm (Eppendorf, 5415C). The samples were mixed by inversion for 3 minutes and then centrifuged at 14,000 rpm (Eppendorf, 5415C) for 10 seconds. The supernatant was removed and the sample pellets were washed in 5 x 1 ml of High-salt buffer by repeated rounds of resuspension by inversion followed by centrifuging for 10 seconds at 14,000 rpm (Eppendorf, 5415C). Following this, the sample pellets were washed twice in 1 ml of Low-salt buffer as described above. The sample pellets were then resuspended in 300 µl of Low-salt buffer and transferred to a Microspin column (Pharmacia) in an Eppendorf tube and centrifuged for 5 seconds at 14,000 rpm (Eppendorf, 5415C). The effluent was discarded and the column was washed in 3 x 500 µl of Low-salt buffer by centrifugation for 5 seconds at 14,000 rpm (Eppendorf, 5415C). The column was placed in a fresh Eppendorf tube and the mRNA was eluted in 2 x 200 µl of pre-warmed (to 65°C) Elution buffer by centrifugation at 14,000 rpm (Eppendorf, 5415C) for 5 seconds. The eluted mRNA samples were centrifuged for 1 minutes
at 14,000 rpm (Eppendorf, 5415C) to remove any fine particle contaminants and the supernatant was placed in a fresh Eppendorf tube.

The mRNA samples were precipitated by adding 10 µl of Glycogen solution, 40 µl of 2.5 M potassium acetate and 1 ml of ice-cold 95% ethanol and incubating at -20°C for 1-2 hours. The mRNA samples were pelleted by centrifugation at 14,000 rpm (Eppendorf, 5415C) for 5 minutes at 4°C. The pellet was air dried and then resuspended in 10 µl of distilled water. The mRNA concentration was determined by optical density at 260 nm as described in method 2.2.5. The quality of a 1 µl aliquot of each mRNA sample was checked on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel.

5.2.5 SMART cDNA synthesis (CLONTECH)

SMART double-stranded (ds) cDNA was prepared from 1 µg aliquot of mRNA using the SMART PCR cDNA synthesis protocol (CLONTECH) as per the manufacturer’s instructions. Briefly, to prepare the first cDNA strand, the mRNA sample, 2 µM of the SMART cDNA synthesis primer (CLONTECH) and 2 µM of the SMART II oligonucleotide (CLONTECH) were mixed together in a total volume of 5 µl. The reaction was incubated at 70°C for 2 minutes to allow the primers to anneal. Following this incubation step, 200 units of Superscript II reverse transcriptase (GibcoBRL), 2 mM DTT and 1 mM of each dNTP all in 1 X SMART first strand buffer were added. The reaction was incubated at 42°C for 1 hour and then 450 µl of TE buffer was added to each sample. A final incubation step of 7 minutes at 72°C was performed and then these first strand cDNA samples were stored at -20°C until required.

To prepare double-stranded (ds) SMART cDNA, 1 µl aliquots of each first strand cDNA sample were diluted with 9 µl of distilled water. Triplicate reactions were
prepared for each mRNA sample. Each reaction contained 1X Advantage KlenTaq Polymerase mix (CLONTECH), 0.2 mM of each dNTP and 0.2 μM of the SMART PCR primer in 1X KlenTaq PCR buffer. The reactions were overlaid with 2 drops of mineral oil (Sigma) and placed in a pre-heated (to 95°C) thermal cycler (MJ Research Inc, PTC-100) and incubated for 1 minute at 95°C prior to beginning the SMART PCR cycles. The tubes were subjected to a previously determined optimal number (see below) of SMART PCR cycles: 95°C for 15 seconds; 65°C for 30 seconds; and 68°C for 6 minutes. The reactions were terminated by the addition of 2 μl of 0.5 M EDTA and a 5 μl aliquot of each ds cDNA sample was analysed on a 1.2% agarose/1X TAE/1 μg/ml ethidium bromide gel.

The optimum number of SMART PCR cycles was determined by subjecting one tube for each sample to 15 cycles of SMART PCR. A 15 μl aliquot was removed from the reaction to a clean Eppendorf tube and the remaining reaction was returned to the thermal cycler for a further 3 cycles. The removal of a 15 μl aliquot was repeated to collect samples after 15, 18, 21 and 24 SMART PCR cycles. A 5 μl aliquot from each of these samples was then analysed on a 1.2% agarose/1X TAE/1 μg/ml ethidium bromide gel. The optimum number of cycles was determined for each sample by comparing the amount of PCR amplification product produced from each cycle sample. The optimal number of cycles was one cycle less than that needed to reach the plateau of amplification product.

5.2.6 SMART cDNA purification

Two ds cDNA sample reactions were mixed together and then purified by phenol extraction (see method 3.2.1). The samples were concentrated to a volume of 40-70 μl by the addition of 700 μl of n-butanol and vortexed thoroughly to mix. The samples were centrifuged for 1 minute at 14,000 rpm (Eppendorf, 5415C) and the
upper n-butanol organic phase was discarded. The sample was carefully applied to a CHROMA SPIN-1000 column (CLONTECH), which had previously been equilibrated with 1 X TNE buffer. 25 μl of 1 X TNE buffer was applied to the column and allowed to completely drain through the column, before another 150 μl of 1 X TNE buffer was applied and again allowed to completely drain through the column. The column was transferred to a clean Eppendorf tube and the purified ds cDNA sample was eluted from the column with 320 μl of 1 X TNE buffer.

5.2.7 Double-stranded cDNA synthesis

The PCR-Select cDNA subtraction protocol (CLONTECH) was employed to prepare ds cDNA samples. The protocol was followed as per the manufacturer’s instructions. Briefly, first strand cDNA was prepared from a 2 μg aliquot of each mRNA sample. The mRNA was mixed with 2 μM of the cDNA synthesis primer to a total volume of 5 μl. The reaction was incubated at 70°C for 2 minutes and then cooled on ice for 2 minutes. Following this, 1 mM of each dNTP and 20 units of AMV reverse transcriptase (CLONTECH) in 1 X First-strand buffer were added. The reactions were incubated at 42°C for 1.5 hours and then terminated by placing on ice. The reactions were immediately used in the second strand cDNA synthesis procedure.

The second cDNA strand was synthesised using the first strand cDNA reactions, 1 X Second-strand enzyme cocktail (CLONTECH) and 0.2 mM of each dNTP in 1 X Second-strand buffer. The reaction was incubated at 16°C for 2 hours and then 6 units of T4 DNA polymerase (CLONTECH) was added and the tubes were incubated for a further 30 minutes at 16°C. The reaction was terminated by adding 4 μl of 20 X EDTA/glycogen mixture. The ds cDNA was purified by phenol extractions (see method 3.2.1) and then ethanol precipitated with 40 μl of 4 M
ammonium acetate and 300 μl of 95% ethanol. The sample was centrifuged at 14,000 rpm (Eppendorf, 5415C) for 20 minutes. The pellet was washed in 500 μl of 80% ethanol and was then allowed to air dry. The dry ds cDNA sample was resuspended in 50 μl of distilled water.

5.2.8 Preparation of driver and tester cDNA samples

The PCR-Select cDNA subtraction protocol (CLONTECH) was employed to prepare driver and tester cDNA samples to be used in subtractive hybridisations with two mRNA populations. The protocol was followed as per the manufacturer’s instructions. The ds cDNA samples were digested with 15 units of Rsa I restriction enzyme (CLONTECH) (see method 3.2.8). Following digestion, 2.5 μl of 20 x EDTA/glycogen mix was added to terminate the reaction. The samples were purified by phenol extractions (see method 3.2.1) followed by ethanol precipitation with ammonium acetate as described above. The DNA pellet was washed in 80% ethanol, air-dried and then resuspended in 5.5 μl of distilled water. An aliquot of these Rsa I digested ds cDNA samples for each mRNA population was removed to serve as the driver cDNA samples.

1 μl of each Rsa I digested ds cDNA sample was diluted with 5 μl of distilled water. For each of these diluted samples, three adaptor ligation reactions were prepared. Two of the reactions contain 2 μl of the diluted Rsa I digested ds cDNA sample, 2 μM of Adaptor and 400 units of T4 DNA ligase (containing 300 μM ATP) in 1 X ligation buffer to a total volume of 10 μl. The reactions contained either Adaptor 1 or Adaptor 2R and were called tester-1 and tester-2 samples respectively. The third reaction, called the unsubtracted tester control, was prepared by removing 2 μl aliquots from the tester-1 and -2 samples and mixing them together in a separate tube. All the reactions were incubated overnight at 16°C and then 1 μl of EDTA/glycogen mixture was added to terminate the
reactions. The samples were incubated at 72°C for 5 minutes to denature the ligase.

5.2.9 Ligation and Subtraction efficiency analysis

Ligation efficiency and subtraction efficiency analyses were performed using a PCR based strategy. For the ligation efficiency analysis, PCR was performed with two sets of primers to detect a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The PCR was designed to detect G3PDH both with and without the adaptors ligated to it. A 1 μl aliquot of each 1:200 diluted (in water) adaptor ligated cDNA sample was used in the reactions. For each of the tester-1 and 2 samples two reactions were prepared. The first reaction contained G3PDH 3' primer and G3PDH 5' primer and would detect G3PDH without adaptor. The second reaction contained G3PDH 3' primer and PCR primer 1, which would detect G3PDH with adaptor ligated. All primers were at 0.4 μM final concentration. The reactions also contained 0.2 mM of each dNTP and 1 X Advantage cDNA polymerase mix in 1 X Advantage PCR reaction buffer in a total volume of 25 μl. The reactions were overlaid with 50 μl of mineral oil (Sigma) and incubated at 75°C for 5 minutes to extend the adaptors. Following this, the reactions immediately commenced 20 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 2.5 minutes. A 9 μl aliquot of each reaction was analysed on a 2 % agarose/1 X TAE/1 μg/ml ethidium bromide gel in 1 X loading buffer.

For the subtraction efficiency analysis PCR was performed using the G3PDH 3' and G3PDH 5' primers. PCR amplifications were performed on forward subtracted, reverse subtracted, and both unsubtracted cDNA populations. The PCR was as described for the ligation efficiency test except 5 μl aliquots were removed after 18, 23, 28 and 33 cycles of PCR. The PCR cycle was 94°C for 30
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seconds, 60°C for 30 seconds and 68°C for 2 minutes. The 5 µl aliquots were analysed on a 2% agarose/1 X TAE/1 µg/ml ethidium bromide gel.

5.2.10 PCR-Select cDNA hybridisation subtractions and PCR

The PCR-Select cDNA subtraction protocol (CLONTECH) was employed to perform subtractive hybridisation and PCR amplification on two mRNA populations. The protocol was followed as per the manufacturer’s instructions. Two initial hybridisation reactions were prepared for each subtraction. A 1.5 µl aliquot of Rsal digested driver cDNA was mixed with either 1.5 µl of tester sample 1 or 1.5 µl of tester sample 2 in pre-warmed (to room temperature) 1 X Hybridisation buffer. The reactions were overlaid with one drop of mineral oil (Sigma) and then incubated at 98°C for 1.5 minutes, followed by 68°C for 8 hours. A second hybridisation step was prepared by diluting another 1 µl aliquot of driver cDNA (1:4) in 1 X Hybridisation buffer and then a 1 µl aliquot of this diluted driver cDNA was denatured at 98°C for 1.5 minutes. This freshly denatured driver cDNA was simultaneously mixed with the first two hybridisation samples, such that the two initial hybridisation samples were mixed only in the presence of freshly denatured driver. The sample was incubated overnight at 68°C. The following day, 200 µl of dilution buffer was added to the hybridisation reaction and the reaction was mixed by pipetting. The reaction was incubated at 68°C for 7 minutes.

A 1 µl aliquot of each diluted final hybridisation reaction and 1 µl of unsubtracted tester control (diluted 1:1000 in distilled water) were used separately in primary PCR amplification reactions. The PCR reactions also contained 0.2 mM of each dNTP, 0.4 µM of PCR primer 1 and 1 X Advantage cDNA polymerase mix in 1 X Advantage PCR reaction buffer in a total volume of 25 µl. The reactions were overlaid with 50 µl of mineral oil (Sigma) and incubated at 75°C for 5 minutes to
extend the adaptors, followed immediately by 27 cycles of 94°C for 30 seconds, 66°C for 30 seconds and 72°C for 1.5 minutes.

A 1 µl aliquot of each 1:10 diluted (in distilled water) primary PCR reaction was used in a secondary PCR reaction. The reaction was as described above except using Nested PCR primer 1 and Nested PCR primer 2R (CLONTECH). Twelve cycles of 94°C for 30 seconds, 68°C for 30 seconds and 72°C for 1.5 minutes were performed. A final incubation step of 5 minutes at 72°C was performed. A 9 µl aliquot of each primary and secondary PCR was analysed on a 2% agarose/1 X TAE/1 µg/ml ethidium bromide gel in 1 X loading buffer.

5.2.11 Purification of PCR products

PCR products were purified using the Advantage PCR-Pure protocol (CLONTECH) as per the manufacturer's instructions. Briefly, approximately 3 volumes of SALT solution were mixed with the PCR reaction and then 5 µl of BIND solution was added (for up to 1 µg of DNA with an extra 1 µl of BIND for every additional 1 µg of DNA). The samples were incubated for 5 minutes at room temperature to allow the BIND to bind the DNA and then centrifuged for 1 minute at 14,000 rpm (Eppendorf, 5415C). The supernatant was discarded. 1 ml of WASH solution was added to the pellet and the sample was mixed by pipetting and then centrifuged at 14,000 rpm (Eppendorf, 5415C) for 1 minute. All traces of the supernatant were carefully removed and the pellet was air-dried. The pellet was resuspended by pipetting in 15 µl of distilled water and then incubated for 5 minutes at room temperature. The DNA was eluted by centrifuging at 14,000 rpm (Eppendorf, 5415C) for 1 minute.
Random primer labelling was used to prepare cDNA population probes labelled with [α\(^{32}\)P]dCTP according to the PCR-Select differential screening protocol (CLONTECH). Briefly, 50-100 ng of cDNA was mixed with distilled water to a final volume of 9 μl and then denatured at 95°C for 8 minutes. The samples were cooled on ice and then labelled using 2-5 units of Klenow enzyme (-exo) (CLONTECH). Each reaction also contained 3 μl of reaction buffer (-dCTP), 2 μl of random primer mix and 50 μCi of [α\(^{32}\)P] dCTP. The reactions were incubated at 37°C for 30 minutes and terminated by the addition of 5 μl of Stop solution. The probes were purified using a NICK column (Pharmacia) and 2 x 1 μl aliquots were removed to determine the specific activity of the probe.

The BigDye terminator cycle sequencing protocol (Applied Biosystems) was used to sequence plasmid DNA samples. The reactions contained 300-600 ng of plasmid DNA, 5 pmole of T7 or SP6 oligonucleotide primers, 8 μl of Terminator ready reaction mix and distilled water up to a final volume of 20 μl. The samples were mixed well and then transferred to capillary PCR tubes and placed in a thermal cycler (Corbett Research FTS-1 thermal sequencer). The sequencing cycle began with 5 minutes at 95°C followed by 30 cycles of 95°C for 30 seconds, 55°C for 10 seconds and 60°C for 4 minutes. The samples were then purified by precipitation with 2 μl of 3M sodium acetate and 50 μl of 95% ethanol on ice for 10 minutes. The tubes were centrifuged at 14,000 rpm (Eppendorf, 5415C) at 4°C for 20 minutes. The pellet was washed with 500 μl of 70% ethanol and centrifuged for 5 minutes at 14,000 rpm (Eppendorf, 5415C). The pellet was dried in a Savant SpeedVac Concentrator for 10 minutes. The sequencing reactions
were then taken to the Biomolecular Resource Facility (JCSMR) for electrophoresis and sequence analysis.

5.2.14 Cell culture and transfections

Swiss 3T3 fibroblasts were cultured in DMEM plus 10% FCS at 37°C/5% CO₂ until confluent and then the media was removed and the cells were washed in 2 x 5 ml of PBS. The cells were trypsinised in pre-warmed (to 37°C) 0.025% trypsin solution for 1 minute and then the excess trypsin was removed. The cells were washed off the flask in 5 ml of DMEM plus 10% FCS and the cells were counted. For cell maintenance purposes the trypsinised cells were split 1:20 in DMEM plus 10% FCS on 250 ml (75 cm²) flasks (Becton Dickinson). For transfection experiments, the cells were plated onto 100 mm dishes at 4 x 10⁵ cells/plate in DMEM plus 10% FCS and incubated overnight at 37°C/5% CO₂. The following day the media was removed from the plates and replaced with 4 ml of fresh DMEM plus 10% FCS. For each plate to be transfected, plasmid DNA was mixed with DOSPER (Boehringer Mannheim) at a ratio of 1:2.5 (w/v). The volume was made up to 100 μl with HBS buffer, pH 7.4. The DNA-DOSPER mixture was incubated for 15 minutes at room temperature and then added dropwise to the cells. The plates were incubated for 6 hours at 37°C/5% CO₂ and then another 4 ml of DMEM plus 10% FCS was added. The plates were incubated overnight at 37°C/5% CO₂. The following day the media was removed and the cells were washed in 3 x 5 ml of PBS and then cultured in 10 ml of DMEM plus 10% FCS overnight at 37°C/5% CO₂. The transfected cells were harvested by removing the media and then washing in 3 x 5 ml PBS. The cells were trypsinised as described above and the cells were washed off in 5 ml of DMEM plus 10% FCS or 5 ml of PBS.
5.3 Results

5.3.1 RT-PCR analysis of other genes involved in eye development

A review of the literature has identified a number of instances in which an alteration in the expression or function of a particular gene has resulted in a similar eye abnormality to that observed in the fra-2 over-expressing transgenic mice. As outlined in the Introduction section of this chapter, in three of these cases – namely, TGFα, Pax6 and Mitf – it was plausible to consider some connections with Fra-2. Therefore, as a starting point, an experiment was performed to determine if the expression of any of these genes was affected by the over-expression of Fra-2 in the transgenic eye.

The expression levels of Mitf, Pax6 and TGFα were compared in control and transgenic eyes by RT-PCR analysis. RNA was prepared from control eyes and left and right transgenic eyes separately as described in method 4.2.4. cDNA was prepared (see method 2.2.6) and used in a PCR with specific primers for each of the genes; Mitfpr1, Mitfpr2, Pax6pr1, Pax6pr2, mTGFpr3 and mTGFpr4. The results of the PCR (figure 5.2) show no detectable expression of Mitf in any sample. Pax6 appeared to be down-regulated in the transgenic eyes and there was no significant difference in the level of TGFα in control and transgenic eye RNA samples. The expression of fra-2 was readily detectable and increased in transgenic eyes, as shown earlier by Northern blotting (figures 4.5-4.9).

5.3.2 TGFα stimulation

It appeared that TGFα expression was unaffected in the transgenic mice over-expressing fra-2, suggesting that the TGFα gene is not a downstream target of Fra-2. Since TGFα is a growth factor that exerts its effect through receptor-
Figure 5.2: RT-PCR for Mitf, Pax6 and TGFα expression levels in eyes

RT-PCR with Mitf, Pax6 and TGFα specific primers: Mitfpr1, Mitfpr2, Pax6pr1, Pax6pr2, mTGFpr3 and mTGFpr4 (product sizes 1007 bp, 922 bp and 423 bp respectively) was performed on control and transgenic eye RNA. The RNA was reverse transcribed using AMV reverse transcriptase (see method 2.2.6). The PCR amplifications were performed using AB Taq polymerase for 30 cycles of the mouse detection PCR program (see method 3.2.2). The amplification products were run on a 0.8% agarose/TAE/1 μg/ml ethidium bromide gel. The arrows indicate the Pax6 and TGFα PCR amplification products.

C = control eye, L = left transgenic eye, R = right transgenic eye, W = water and M = DNA size markers (λ/HindIII plus φX174/HaeIII).
mediated signalling, another possibility for a relationship between TGF\(\alpha\) and Fra-2 is that Fra-2 is an effector molecule in the TGF\(\alpha\) signalling pathway. Therefore, investigations into whether TGF\(\alpha\) is upstream of \(fra-2\) in the signalling pathway were undertaken. Swiss 3T3 fibroblasts were employed for this analysis. Serum starved cells were stimulated with 25ng/ml of TGF\(\alpha\) and then RNA (method 4.2.4) and protein (method 4.2.8) were prepared from the cells at 0, 1, 2, 4 and 8 hours post stimulation. The expression of \(fra-2\) was assessed by Northern (figure 5.3a) and Western blotting (figure 5.3b). The results show an induction in \(fra-2\) mRNA expression within 1-hour and a corresponding induction and phosphorylation of Fra-2 protein within 1-2 hours. This suggested that Fra-2 could be downstream of TGF\(\alpha\) in the signalling pathway.

To establish if TGF\(\alpha\) stimulation could also lead to expression of Mitf and/or Pax6, RNA was prepared from the TGF\(\alpha\) stimulated cells and used in an RT-PCR reaction. Mitf and Pax6 specific oligonucleotide primers: Mitfpr1, Mitfpr2, Pax6pr1 and Pax6pr2, were used in the PCR. The result (see figure 5.4) shows an induction in Mitf expression with TGF\(\alpha\) stimulation within 1 hour. The level of Mitf was reduced within 4 hours, but was still detectable and remained present for at least 8 hours post-stimulation. There was no detectable expression of Pax6 at any time.

### 5.3.3 Mitf

Mitf is a member of the bHLH-Zip protein family. These proteins have been shown to dimerise with other proteins through their leucine zipper motif. As discussed earlier in chapter 1, three of the bHLH-Zip proteins have been shown to interact with members of the AP-1 transcription family: FIP, MyoD and USF. They have been shown to interact with c-Fos (Blanar and Rutter, 1992), c-Jun
Figure 5.3: Time course of fra-2 induction in Swiss 3T3 fibroblasts following TGFα stimulation

RNA or protein was extracted from unstimulated cells (0) and from cells stimulated with 25 ng/ml TGFα for 1, 2, 4, or 8 hours. The RNA and protein samples were prepared as described in methods 4.2.4 and 4.2.8 respectively.

a) 2 μg of total RNA from each time sample was electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filter was hybridised with 0.6 x 10^6 cpm/ml of a 32P labelled mouse PCR-generated fra-2 specific probe (see method 3.2.10) overnight at 42°C and washed in 1 X SSC/0.1% SDS followed by exposure to film for 6 days. The arrow identifies the fra-2 mRNA.

b) 10 μg of total protein extract from each time sample were electrophoresed under denaturing conditions on 11% SDS polyacrylamide gels. The gels were electroblotted overnight onto nitrocellulose filters (see method 4.2.12) at 25 mAmmps. The filters were incubated with 1:500 diluted αF2-FL polyclonal antibody followed by 1:10,000 diluted donkey anti-rabbit IgG antibody conjugated to HRP (Amersham) and then with 1:1500 diluted streptavidin conjugated HRP antibody (Amersham). Bound antibody was detected by ECL Western blotting (see method 4.2.9). The square bracket indicates the various phosphorylated forms of Fra-2.

M = ECL protein molecular weight markers (Amersham).
Swiss 3T3 fibroblasts were stimulated with 25 ng/ml of TGFα for 0, 1, 2, 4 or 8 hours and RNA was extracted from the cells. The RNA was reverse transcribed using AMV reverse transcriptase (see method 2.2.6). PCR was performed with Mitf and Pax6 specific primers: Mitfpr1, Mitfpr2, Pax6pr1 and Pax6pr2 (product sizes 1007 bp and 922 bp respectively). AB Taq polymerase and 30 cycles of the mouse detection PCR program (see method 3.2.2) were used for the PCR amplification. The PCR amplification products were analysed on a 0.8% agarose/1 X TAE/1 µg/ml ethidium bromide gel. The arrow indicates the Mitf PCR products. Water (W) was used as a negative control. M = DNA size markers (λ/HindIII plus φX174/HaeIII).

Figure 5.4: RT-PCR for Mitf and Pax6 expression levels after TGFα stimulation
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(Bengal et al., 1992) and Fra-1 (Pognonec et al., 1997) respectively. Since, both Mitf and fra-2 expression are induced by TGF\(\alpha\) in a similar time-frame, and given that alterations affecting the normal expression of both gene produces similar eye defects, it was of interest to determine whether Mitf and Fra-2 could also interact in the same way as other AP-1 and bHLH-Zip proteins.

The pBS-ORF1 plasmid containing the coding region of the murine Mitf gene was a kind gift from Dr. Heinz Armheiter. The pBS-ORF1 and pGEMZfra-2 plasmids were used to prepare in vitro transcribed RNA (method 5.2.1). This RNA was then used to prepare labelled and unlabelled proteins (see method 5.2.2). Proteins were also prepared for c-Fos, c-Jun and JunB. An aliquot of each of the prepared labelled proteins is shown in figure 5.5a. These labelled and unlabelled proteins were used in various combinations in an in vitro association experiment (see method 5.2.3) to determine if Mitf interacted with Fra-2 or any of the other AP-1 transcription factor family members that were analysed (c-Jun, JunB and c-Fos).

The results of the in vitro association experiment are shown in figure 5.5b. The in vitro association experiment, as expected, showed the interactions of c-Jun with c-Fos (lanes 7 and 8), c-Jun with Fra-2 (lane 9) and JunB with Fra-2 (lane 10). However, there was no significant interaction between Mitf and Fra-2 (lane 12) or Mitf and c-Fos (lane 13) or Mitf and c-Jun (lane 14). There was also no interaction between Mitf and an AP-1 complex (i.e. if the AP-1 members were present as dimers) (lanes 15-17).

The in vitro association result also shows the presence of faint bands in the reactions. Firstly, the faint Mitf band present in lanes 4-6 is probably due to a low level of non-specific immunoprecipitation of the Mitf protein by the antibodies used in those reactions. Therefore the faint Mitf band present in lanes 12-17 is
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**Figure 5.5:** In vitro association experiment to check if Mitf and Fra-2 can interact with each other

Mitf and fra-2 RNAs were transcribed in vitro (see method 5.2.1) from linearised plasmids pBS-ORF1 and pGEMZfra-2, respectively. Proteins were translated in vitro (see method 5.2.2) in reticulocyte lysates, with and without $^{35}$S-methionine, to produce labelled and unlabelled proteins. c-Fos, c-Jun and JunB were also in vitro translated with and without $^{35}$S-methionine.

a) A 1 µl aliquot of each $^{35}$S-methionine labelled protein was electrophoresed under denaturing conditions on a 13% SDS-PAGE gel.

b) An in vitro association experiment was performed. The proteins were mixed together (1 µl of each) and allowed to associate at 37°C for 45 minutes. 100 µl of RIPA buffer was added followed by 1.5 µl of the antibody indicated, to immunoprecipitate the protein complexes (see method 5.2.3). Proteins were then resolved on an 11% SDS-PAGE gel. In the reactions described below, protein 1 was the labelled protein and proteins 2 and 3 were unlabelled.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Protein 1</th>
<th>Protein 2</th>
<th>Protein 3</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c-Fos</td>
<td>-</td>
<td>-</td>
<td>α-Fos</td>
</tr>
<tr>
<td>2</td>
<td>c-Jun</td>
<td>-</td>
<td>-</td>
<td>α-Jun</td>
</tr>
<tr>
<td>3</td>
<td>Fra-2</td>
<td>-</td>
<td>-</td>
<td>α-Fra-2</td>
</tr>
<tr>
<td>4</td>
<td>Mitf</td>
<td>-</td>
<td>-</td>
<td>α-Fos</td>
</tr>
<tr>
<td>5</td>
<td>Mitf</td>
<td>-</td>
<td>-</td>
<td>α-Jun</td>
</tr>
<tr>
<td>6</td>
<td>Mitf</td>
<td>-</td>
<td>-</td>
<td>α-Fra-2</td>
</tr>
<tr>
<td>7</td>
<td>c-Fos</td>
<td>c-Jun</td>
<td>-</td>
<td>α-Jun</td>
</tr>
<tr>
<td>8</td>
<td>c-Jun</td>
<td>c-Fos</td>
<td>-</td>
<td>α-Fos</td>
</tr>
<tr>
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<td>Fra-2</td>
<td>-</td>
<td>α-Fra-2</td>
</tr>
<tr>
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<td>JunB</td>
<td>Fra-2</td>
<td>-</td>
<td>α-Fra-2</td>
</tr>
<tr>
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<td>c-Fos</td>
<td>Fra-2</td>
<td>-</td>
<td>α-Fra-2</td>
</tr>
<tr>
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<td>Mitf</td>
<td>Fra-2</td>
<td>-</td>
<td>α-Fra-2</td>
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<td>c-Fos</td>
<td>-</td>
<td>α-Fos</td>
</tr>
<tr>
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<td>c-Jun</td>
<td>-</td>
<td>α-Jun</td>
</tr>
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<td>c-Jun</td>
<td>α-Fra-2</td>
</tr>
<tr>
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<td>Fra-2</td>
<td>JunB</td>
<td>α-Fra-2</td>
</tr>
<tr>
<td>17</td>
<td>Mitf</td>
<td>c-Fos</td>
<td>c-Jun</td>
<td>α-Fos</td>
</tr>
</tbody>
</table>
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(a) Mitf Fra-2 JunB Jun Fos

(b) cFos cJun Fra-2

Mitt JunB

kD

200
130
79
42.6
32.7

10 11 12 13 14 15 16 17

kD

200
130
79
42.6
32.7
probably not significant. These lanes 12-17 also contain faint Fra-2, c-Fos, c-Jun and/or JunB bands, but each of these proteins were added to the reactions in their unlabelled version. Therefore, it is likely that a low level of translation of these proteins occurred during the association reactions.

5.3.4 SMART cDNA synthesis

To broaden the search for potential target genes of Fra-2, the PCR-Select differential screening protocol was employed. The pCMV-mfra2 transgenic and littermate control mice were used as the source of mRNA for the screen. In light of the fact that the first histological difference between the control and transgenic mice was observed at E15.5, it was decided to perform the differential analysis using E15.5 eye tissue. Therefore E15.5 eyes were removed from pCMV-mfra2 transgenic and littermate control mice and mRNA was prepared from the eyes as described in method 5.2.4. Considering the small quantities of mRNA obtained from E15.5 mouse eyes, the SMART PCR cDNA synthesis protocol (CLONTECH) was used to prepare the ds cDNA required for the screening protocol (see method 5.2.5).

The principles of the SMART protocol are outlined in figure 5.6. Briefly, the first strand of cDNA is synthesised using a modified oligo(dT) primer (SMART CDS primer) and when the reverse transcriptase reaches the 5' end of the mRNA template, it adds a few extra nucleotides, primarily deoxycytidines, to the 3' end of the newly synthesised cDNA strand. The SMART II oligonucleotide primer, which contains an oligo(G) sequence at its 3' end, anneals with the deoxycytidine sequence and allows the addition of a few extra nucleotides that will provide priming sequences for PCR. This produces a full-length single stranded cDNA containing the complete mRNA sequence and sequences complementary to the
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**Figure 5.6: Flow chart of SMART cDNA synthesis procedure**

The SMART II oligonucleotide, CDS primer, and PCR primer all contain a stretch of identical sequence. This figure was reproduced from CLONTECH SMART PCR cDNA synthesis protocol handbook (PT3041-1).
SMART oligonucleotide and CDS primer at the 3' and 5' ends respectively. The CDS primer and SMART oligonucleotide contain a stretch of identical sequence, which matches the SMART PCR primer. Therefore PCR amplification with the SMART PCR primer is used to produce ds cDNA.

5.3.5 PCR-Select differential subtraction

The SMART ds cDNA was used in the PCR-select hybridisation and differential screening protocol (see method 5.2.9) to search for differentially expressed cDNAs from transgenic and control E15.5 eye cDNA populations. This protocol involves the subtraction of identical sequences from two cDNA populations and enriches for the differentially expressed cDNAs. Tester and driver cDNA populations were prepared from both the transgenic and control E15.5 eye cDNA populations (see method 5.2.8). The PCR-select hybridisation and differential screening protocol is outlined in figure 5.7. This protocol was followed to produce forward and reverse subtracted cDNA populations. For the forward subtraction, transgenic E15.5 eye cDNA tester populations were used with the control E15.5 eye cDNA driver population. The reverse subtraction used the control E15.5 eye cDNA tester populations and the transgenic E15.5 eye cDNA driver population.

The protocol relies on sufficient ligation of the adaptors to the tester cDNA populations. Therefore, a ligation efficiency test was performed to confirm the success of these ligation reactions for both the control and transgenic tester populations (see method 5.2.9). Figure 5.8a shows the results from the ligation efficiency analysis. The result shows the presence of a band of G3PDH + adaptor in each of the tester populations (C1, C3, T1 and T3). The presence of these bands indicates that a significant proportion of the G3PDH cDNA had adaptors ligated to them. Therefore the subtraction procedure was continued.
Figure 5.7: Flow chart of the experimental set-up for PCR-select cDNA subtraction and differential screening

This flow chart was modified from that in CLONTECH’s PCR-select differential screening protocol handbook (PT3138-1). To prepare forward subtracted and non-subtracted probes, follow this procedure as described above. For reverse subtracted and non-subtracted probes, use driver cDNA in place of the tester cDNA and tester cDNA in place of the driver cDNA.
**Figure 5.8: Ligation and subtraction efficiency analysis of control and transgenic E15.5 eye cDNA subtraction experiment**

PCR amplification reactions were performed using 1 X Advantage cDNA polymerase mix (see method 5.2.9). The PCR products were analysed on 2% agarose/1 X TAE/1 μg/ml ethidium bromide gels in 1 X loading buffer. M = DNA size markers (λ/HindIII plus φX174/HaeIII).

a) Ligation efficiency analysis. Ligation reactions (see method 5.2.8) were performed between tester cDNA populations and adaptors (either 1 or 2R) for both control (C) and transgenic (T) E15.5 eye cDNA tester populations. PCR amplification was performed with two sets of primers to detect glyceraldehyde-3-phosphate dehydrogenase (G3PDH) with and without adaptors ligated. Set I (G3PDH3' and G3PDH5') recognise G3PDH cDNA without adaptors (0.5 kb) and set II (G3PDH3' and PCR primer 1) recognises the G3PDH cDNA with adaptors ligated (1.2 kb). Arrows indicate the bands produced from each pair. The PCR was performed on both tester 1 and 2 cDNA populations. The numbers 1 to 4 represent the different reaction combinations: 1 & 3 = tester with adaptor ligated (adaptor 1 or 2R respectively) as the template and the set I primer combination. 2 & 4 = tester with adaptor ligated (adaptor 1 or 2R respectively) as the template and the set II primer combination.

b) Subtraction efficiency analysis. PCR-select subtraction reactions (see method 5.2.10) were performed with control and transgenic E15.5 eye cDNA populations. The efficiency of the subtraction reactions was determined by removing 5 μl aliquots after 18, 23, 28 and 33 cycles of PCR amplification. The PCR amplifications were performed with unsubtracted control eye cDNA (C), forward subtracted (F), reverse subtracted (R) and transgenic eye unsubtracted cDNA (T) using the primers G3PDH3' and G3PDH5'.
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(a)

G3PDH + Adaptor

G3PDH

(b)

G3PDH
Hybridisation and PCR amplification steps were performed to produce forward and reverse subtracted cDNA populations as described in method 5.2.10. The forward subtracted cDNA population was used to identify cDNAs that were up-regulated in the transgenic eye samples. The reverse cDNA population was used to identify cDNAs that were down-regulated in the transgenic E15.5 eyes.

To confirm the subtraction procedure had been successful a subtraction efficiency test was performed (see method 5.2.9). Figure 5.8b shows the results from the subtraction efficiency analysis. The results show a reduction in the level of G3PDH in both the reverse and forward subtraction samples compared to the unsubtracted samples; this is most evident after 28 cycles of PCR. This suggested that the subtractions were successful.

The forward and reverse subtracted cDNA populations were ligated into the pGEM-T vector (Promega) as described previously in chapter 3 method 3.2.4, except the reactions were left to ligate at 4°C for 48 hours. A 2 μl aliquot of each reaction was transformed into 50 μl of JM109 high efficiency competent cells (Promega) as described in method 3.2.4, using SOC medium.

5.3.6 Screening for differentially expressed cDNAs

Bacterial colonies were picked, at random, from the ligation plates. The colonies were grown in 100 μl of LB, containing 50 μg/ml of ampicillin, in a 96-well plate at 37°C for a minimum of 2 hours. Five LB agar plates containing 50 μg/ml of ampicillin were prepared for each set of 96 clones. One was used as a master plate and the other 4 had Hybond-C nitrocellulose filters overlaid onto them. A small aliquot of each bacterial culture was transferred to each filter using a sterile pipette tip. The plates were incubated overnight at 37°C. The following day, the
filters were removed from the plates and denatured, neutralised and dried as described in method 3.2.13.

Two secondary PCRs (method 5.2.10) for each of the forward and reverse subtraction samples were purified (method 5.2.11) and then the adaptors were removed by restriction digests with Rsa I, Sma I and Eag I restriction enzymes (see method 3.2.8). The reactions were purified using the Advantage PCR-Pure protocol as described in method 5.2.11. The purified PCR samples were then used to prepare subtracted probes. Forward and reverse subtraction probes were prepared as described in method 5.2.12. Unsubtracted probes were also prepared for both transgenic and control eye cDNA populations using the driver cDNA populations.

Each set of 4 filters was hybridised separately with one of the 4 probe populations; the two unsubtracted (from fra-2 transgenic and littermate control eyes), forward and reverse probes generated with the PCR-select differential screening protocols. The hybridisations were performed as described in method 3.2.12, except 200 μl of denatured blocking solution and 200 μl of 20 X SSC were added to 40 ml of 1 X pre-hybridisation solution. 100 μl of denatured blocking solution and 100 μl of 20 X SSC were added to 20 ml of 1 X hybridisation solution. Figure 5.9 is an example of the results obtained, showing the forward differential screen hybridisation filters. Sample number 61 (see arrow 1) shows an example of a gene whose activity was down-regulated in the transgenic E15.5 eye sample. In contrast, sample number 96 (see arrow 2) shows an example of a gene whose activity has been up-regulated in the transgenic E15.5 eye sample. A total of 192 colonies were screened for the forward and reverse subtraction samples, 96 for each.

From the hybridisation screening, 34 clones were identified as being differentially expressed. These clones were picked into 4 ml of LB containing 50 μg/ml of
Figure 5.9: Differential screening colony hybridisation filters for control and transgenic E15.5 eye subtraction

Nitrocellulose filters were overlaid onto four 1.5% LB agar plates containing 50 μg/ml ampicillin and 96 bacterial colonies were randomly picked onto these filters and grown overnight at 37°C. Four identical filters were prepared for each set of 96 to enable screening with 4 probes; forward and reverse subtracted probes and transgenic and control E15.5 eye cDNA population unsubtracted probes. All the probes were denatured and added at 1 x 10^6 cpm/ml to the hybridisation solution and the filters were hybridised overnight at 42°C (see method 3.2.12). The following day the filters were washed briefly in 2 X SSC/0.5% SDS at room temperature and then at 68°C for 30 minutes. They were then washed twice in 0.2 X SSC/0.1% SDS at 68°C for 30 minutes. The filters were exposed to film overnight.

a) Filter hybridised with forward subtracted probe.
b) Filter hybridised with reverse subtracted probe.
c) Filter hybridised with unsubtracted transgenic E15.5 eye cDNA population probe.
d) Filter hybridised with unsubtracted control E15.5 eye cDNA population probe.

The arrows identify examples of potential differentially expressed cDNA's: #61 and #96.
Target genes of Fra-2: Results
ampicillin. The cultures were grown overnight with shaking at 37°C. Plasmid DNA was prepared from each culture as described in method 3.2.6(iii). The clones were sequenced (see method 5.2.13) and then nucleic acid homology searches, using the NCBI BLAST program, were performed to identify the putative differentially expressed genes. It is important to note that the sequences obtained were often incomplete and contained unidentified nucleotides. However, the sequence information that was obtained was generally complete enough to enable identification of the genes. A total of 12 independent putative differentially expressed mRNAs were identified, namely: β-crystallin, γ-A-crystallin, γ-B-crystallin, γ-C-crystallin, γ-F-crystallin, α-tubulin isotype m-α-1, cyclophilin, cytochrome c oxidase subunit I, human nuclear pore complex associated protein translocated promoter region (Tpr), rat β-globin, LLRep3 and elongation factor Tu (EF-Tu). In many cases the differentially expressed cDNAs were identified in more than one sequenced clone. The sequences obtained showed 81-100% homology with the mRNA sequences identified in the homology searches (see table 5.1).

5.3.7 Virtual Northerns

To confirm the differential expression of the 12 subtracted cDNAs, Virtual Northerns were performed. Virtual Northern blots are generated using SMART cDNA instead of total or poly A+ RNA and can provide similar information obtained from a standard Northern blot. Virtual Northern blots are useful when there is a limited amount of the sample material as was the case for E15.5 eye RNA. Virtual Northern blots were prepared using SMART ds cDNA samples for both control and transgenic E15.5 eye samples. The SMART ds cDNA samples were electrophoresed on 1.2% agarose/1XTAE/1 μg/ml ethidium bromide gels. Southern blots were performed using PCR probes prepared from the cloned cDNAs (see method 5.2.10). The results are shown in figure 5.10.
Table 5.1: Summary of genes identified in PCR-select differential screen with transgenic and littermate control E15.5 eye mRNA

<table>
<thead>
<tr>
<th>Figure</th>
<th>Subtracted cDNA</th>
<th># clones</th>
<th>Level of expression in transgenic E15.5 eyes compared to control</th>
<th>Fold difference in expression between transgenic and control eye</th>
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Target genes of Fra-2: Results

Figure 5.10: Virtual Northern for cDNAs identified from the PCR select differential screen performed on E15.5 eye samples

Colonies identified as putative differentially expressed cDNAs from the PCR select differential screen performed on control and transgenic E15.5 eye cDNA populations were picked and plasmid DNA was prepared (see method 3.2.6(iii)). Sequence information was obtained (see section 5.2.12) and used to identify the cDNA insert. These cloned inserts were used as templates with nested PCR primer 1 and nested PCR primer 2R for the generation of 32P labelled PCR probes (see section 3.2.10). The probes were used in hybridisations with Virtual Northern blots on control and transgenic E15 eye RNA samples. SMART ds cDNA reactions (see method 5.2.5) for transgenic and control E15.5 eye samples were run on a 1.2% agarose/1 X TAE/1 μg/ml ethidium bromide gel and then blotted (see method 3.2.9) overnight onto nitrocellulose filters in 20 X SSC. The filters were hybridised with 32P-dCTP labelled PCR generated probes overnight at 42°C. The following day filters were washed in 1 X SSC/0.1% SDS at 55°C and then the filters were exposed to film for the times indicated. The filters were re-used by stripping them in 0.1 X SSC/0.1% SDS at high temperatures. Filters were used up to 4 times each.

a) An example of the cDNA gel used for the Virtual Northern analysis.

b) Virtual Northern analysis of mouse β-Crystallin cDNA (predicted size = 669 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film overnight.

c) Virtual Northern analysis of mouse γ-A-Crystallin cDNA (predicted size = 794 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the bands obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film for 2 hours

d) Virtual Northern analysis of mouse γ-B-Crystallin cDNA (predicted size = 525 bp) in control and transgenic E15.5 eye cDNA populations. The arrow

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indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film overnight.

e) Virtual Northern analysis of mouse $\gamma$-Crystallin cDNA (predicted size = 527 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film overnight.

f) Virtual Northern analysis of mouse $\gamma$-F-Crystallin cDNA (predicted size = 594 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film overnight.

g) Virtual Northern analysis of mouse $\alpha$-tubulin isotype m-$\alpha$-1 cDNA (predicted size = 1593 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film for 5 days.

h) Virtual Northern analysis of mouse cyclophilin cDNA (predicted size = 494 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film for 1.5 hours.

i) Virtual Northern analysis of mouse cytochrome c oxidase subunit I cDNA (predicted size = 1099 bp) in control and transgenic E15.5 eye cDNA populations. The arrows indicate the bands obtained. Predicted to be down regulated from the PCR select subtractive screen. Filter exposed to film overnight.

j) Virtual Northern analysis of human nuclear pore complex associated protein Tpr cDNA, (predicted size = 7049 bp) in control and transgenic E15.5 eye cDNA populations. The arrows indicate the bands obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film for 2 hours.

k) Virtual Northern analysis of mouse elongation factor Tu cDNA (predicted size = 1722 bp) in control and transgenic E15.5 eye cDNA populations. The
arrow indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film for 2 hours.

l) Virtual Northern analysis of mouse LLRep3 cDNA (predicted size = 1728 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the band obtained. Predicted to be down-regulated from the PCR select subtractive screen. The filter was exposed to film for 1.5 hours.

m) Virtual Northern analysis of mouse β-globin cDNA (predicted size = 441 bp) in control and transgenic E15.5 eye cDNA populations. The arrow indicates the band obtained. Predicted to be up-regulated from the PCR select subtractive screen. The filter was exposed to film for 2 hours.
Target genes of Fra-2: Results

(a) kb 23.1 4.4 2.3 1.3 0.6

(b) C T

(c) C T

(d) C T

(e) C T

(f) C T

(g) C T

CT ~ • ~ • ~
Target genes of Fra-2: Results
All the cDNAs except β-globin (figure 5.10m) showed a slight reduction in the cDNA expression level in the transgenic eye as compared to the control eye. The differences in band intensity between the control and transgenic samples were estimated using a LKB Bromma UltraScan XL enhanced laser densitometer. Table 5.1 shows a summary of the results obtained. The level of expression was generally 1.5 to 2 fold higher in the control eye samples. Four cDNAs, γ-A-crystallin, α-tubulin, cyclophilin and elongation factor Tu (figure 5.10c, g, h, k), showed relatively high levels of expression in both the control and transgenic eye population samples. However, there was a reduction in the level of expression of these RNAs in the transgenic eye population. The Tpr protein (figure 5.10j) cDNA Virtual Northern blot result showed the presence of two products (approximately 400 and 700 bp), which were much smaller than expected (7049 bp). This is probably due to the SMART cDNA synthesis producing only partial cDNA molecules due to the very large size of the Tpr mRNA. Large mRNA species can have significant secondary structure that may prevent a good read-through by the RT enzyme. Interestingly, except for Tpr, all the Virtual Northern blots gave the predicted size band, plus additional bands, some of which might be minor, incomplete cDNAs generated by the SMART protocol.

5.3.8 Transfection of Swiss 3T3 fibroblasts with pCMV-mfra2

The differential screening of the transgenic and control E15.5 eye samples identified a few genes that may have been affected by the over-expression of Fra-2. However, the very small size of the E15.5 eyes and the restricted areas of the eye showing abnormalities made it difficult to prepare a distinct population of those cells over-expressing Fra-2. It was therefore decided to pursue the differential screening approach in another system.

Swiss 3T3 fibroblasts were transiently transfected with pCMV-mfra2, the same plasmid that was used to make the transgenic mice (see methods 5.2.15).
Transfected and non-transfected Swiss 3T3 fibroblasts were cultured and then harvested for mRNA preparation (see method 5.2.4). Transiently transfected cells were used rather than stably transfected cells as transiently transfected cells do not incorporate the transfected plasmid into their genome. Stably transfected cells would have required significant characterisation of several clones to ensure the results obtained were not artifactual, (ie. due to positional effects). Therefore, although in transiently transfected cell populations only 5-10% of the cells may contain the transfected plasmid, this did create a system where the only difference between the two cell populations was the level of Fra-2 being expressed. Figure 5.11a shows a Northern blot of RNA from the Fra-2 transfected and non-transfected Swiss 3T3 fibroblast cells. The result indicates that the pCMV-mfra2 plasmid was highly expressed in the transfected cell population.

A Western blot was performed using protein extracts from pCMV-mfra2 transfected Swiss 3T3 fibroblasts to determine the levels of Fra-2 protein expressed as the amount of transfected pCMV-mfra2 plasmid was increased. Figure 5.11b shows the results from this Western blot. Since the transgene protein cannot be distinguished from the endogenous protein, the result measures the total amount of Fra-2 protein expressed in the cells. The blot shows that the level of Fra-2 protein expression increased as the amount of transfected pCMV-mfra2 plasmid was increased. This result suggests that the transfected cells did have higher levels of Fra-2 protein, as expected. However, the increases in the level of protein expression were relatively low compared to the high level of transgene RNA expression observed in the Northern blot (compare figure 5.11a and b).
Target genes of Fra-2: Results

Figure 5.11: Swiss 3T3 fibroblasts transfected with pCMV-mfra2

Swiss 3T3 fibroblasts were transfected with 10 μg of pCMV-mfra2 plasmid combined with 25 μl of DOSPER transfection reagent (Boehringer Mannheim) and HBS pH 7.4 up to 100 μl. Following a 48 hour incubation at 37°C/5% CO2, mRNA was extracted from the cells (see method 5.2.4). Non-transfected Swiss 3T3 fibroblasts were used as a control.

a) 2 μg of mRNA from pCMV-fra2 transfected (FT) and non-transfected cells (NT) were electrophoresed on a 0.8% agarose/1 X MOPS/2.2 M formaldehyde gel. The gel was stained in ethidium bromide, viewed under UV light and photographed to determine the quality of the RNA.

b) The gel was blotted overnight onto nitrocellulose in 20 X SSC (see method 3.2.9). The filter was hybridised with approximately 1 x 10^6 cpm/ml of a PCR generated ^32P-labeled fra-2 cDNA probe (see method 3.2.10) at 42°C overnight and then washed in 1 X SSC/0.1% SDS at 55°C. The filter was exposed to film for 5 days. The arrows indicate the endogenous (Endo) and transgene (Tg) fra-2 RNA products.

c) Swiss 3T3 fibroblasts were transfected with 0, 2.5, 5, 10 or 15 μg of pCMV-mfra2 plasmid (see method 5.2.14). Following a 48 hour incubation at 37°C/5% CO2, protein was prepared from the cells (see method 4.2.8). 10 μg of total protein extract from each sample was electrophoresed under denaturing conditions on a 9% SDS polyacrylamide gel. The gel was electroblotted overnight onto a nitrocellulose filter (see method 4.2.12) at 25 mAmps. The filter was incubated with 1:500 diluted αF2-FL polyclonal antibody followed by 1:10,000 diluted donkey anti-rabbit IgG antibody conjugated to HRP (Amersham) and then with 1:1500 diluted streptavidin conjugated HRP antibody (Amersham). Bound antibody was detected by ECL Western blotting (see method 4.2.9). The arrow identifies the 46 kDa Fra-2 species and M = ECL protein molecular weight markers (Amersham).
Target genes of Fra-2: Results

(a) and (b) show gel electrophoresis results with bands labeled as 28S and 18S.

(c) shows a western blot analysis with bands labeled Fra-2 at different kDa sizes: 97.4, 68, 46, 31, and 20.1.
5.3.9 Differential screening of transfected versus non-transfected mRNA populations

Double-stranded cDNA was prepared from the fra-2 transfected and non-transfected cell populations (see method 5.2.7). These ds cDNA populations were used in a PCR-select hybridisation screen to search for genes whose expression may be affected by an increase in Fra-2 expression. Tester and driver cDNA populations were prepared for the fra-2 transfected and non-transfected cell ds cDNA populations (see method 5.2.8). Then PCR-select hybridisation and screening protocols (refer to figure 5.7) were followed as described previously for the E15.5 eye samples. Forward and reverse subtracted cDNA populations were prepared from the tester and driver populations (method 5.2.10). The forward subtraction used the fra-2 transfected cell tester cDNA population and the non-transfected cell driver cDNA population. The reverse subtraction used the non-transfected cell tester cDNA population and the fra-2 transfected cell driver cDNA population.

As described previously for the PCR select differential subtraction with the transgenic and control E15.5 eyes a ligation efficiency test was performed (result 5.3.5). The results (data not shown) showed that approximately 25% of the G3PDH cDNA samples had adaptors ligated to them. This level of ligation was significant enough to continue the hybridisation screening procedure. The subtraction procedure (see method 5.2.10) was completed and a subtraction efficiency test (data not shown) was performed as described earlier for the transgenic and control E15.5 eye subtraction (see result 5.3.5). The subtraction efficiency test showed that both the forward and reverse subtractions were successful in decreasing the RNA levels of genes that were expressed in both cell populations.

To confirm the enrichment of differentially expressed cDNAs by the subtraction protocol a second subtraction efficiency test was performed. This subtraction
efficiency test was designed to detect an increase in the level of *fra-2* cDNA in the forward subtraction population compared to the reverse subtraction population. A secondary PCR for each of the forward and reverse subtraction procedures was electrophoresed on a 1.5% agarose/1 X TAE/1 μg/ml ethidium bromide gel. The gel was blotted onto Hybond-C nitrocellulose (method 3.2.9) and probed with a PCR generated *fra-2* probe (see methods 3.2.10 and 3.2.12). The results (figure 5.12) show the selective increase in *fra-2* cDNA level in the forward reaction and the lack of detectable *fra-2* cDNA in the reverse subtraction. This indicated that enrichment for the differentially expressed *fra-2* cDNA was successfully achieved in the forward subtraction cDNA population.

The forward and reverse subtracted cDNA populations were ligated into the pGEM-T vector and transformed into JM109 high efficiency competent cells as described above (results 5.3.6). The cloned forward and reverse subtracted cDNA populations were screened for differentially expressed cDNAs using unsubtracted non-transfected and *fra-2* transfected cDNA populations and the forward and reverse subtraction probes. The colony hybridisation was performed with 2 x 10^6 cpm/ml of each probe as described above in results 5.3.6.

A total of 576 colonies were screened: 384 for the forward subtraction and 192 for the reverse subtraction. From the hybridisation screening 62 clones were identified as being differentially expressed. These clones were picked into 4 ml of LB containing 50 μg/ml of ampicillin. The cultures were grown overnight with shaking at 37°C. Plasmid DNA was prepared from the bacterial cultures as described in chapter 3 method 3.2.6(iii). The clones were sequenced (see method 5.2.12) and then nucleic acid homology searches using the NCBI BLAST program were performed to identify the putative differentially expressed genes. A total of 22 different mRNAs were identified: MHC Class I heavy chain precursor,
**Figure 5.12:** Southern analysis of *fra-2* cDNA expression levels in forward and reverse subtracted cDNA's populations

a) A PCR-select differential screen (see method 5.2.10) was performed on cDNA prepared from non-transfected and pCMV-fra2 transfected cells. The secondary PCR products obtained from the forward and reverse PCR-select subtractions were electrophoresed on a 1.5% agarose/TAE/1 µg/ml ethidium bromide gel. The gel was viewed under UV light and photographed.

b) The gel was blotted overnight onto nitrocellulose in 20 X SSC (see method 3.2.9). The filter was hybridised (see method 3.2.12) overnight at 42°C with 0.4 x 10^6 cpm/ml of a *fra-2* PCR generated probe (see method 3.2.10) and subsequently washed in 1 X SSC/0.1% SDS at 55°C. The filter was exposed to film for 3 days. The arrow indicates the *fra-2* cDNA.

F = forward subtracted reaction, R = reverse subtracted reaction and M = DNA size markers (λ/HindIII plus φX174/HaeIII).
migratory inhibitory protein (MIF), cyclin D1, A10 or phosphoglycerate dehydrogenase, ST2/T1, Ras GTPase activating protein (GAP) binding protein (G3BP), Stearoyl CoA desaturase 2, mouse complement component C3, pyruvate kinase, prothymosin α, RANTES, Tax responsive element binding protein 107, proliferin, ribosomal protein L18, acidic ribosomal phosphoprotein PO, rat tricarboxylate carrier, human Arp2/3, human BH-protocadherin (PCDH7), secretory leukocyte protease inhibitor, SPARC, rat neurodegeneration associated protein-1, and frizzled-1. The sequenced clone sequences showed 76-99% homology with the mRNA sequences identified in the homology searches.

5.3.10 Confirmation of differential expression

To confirm the differential expression of the identified cDNAs, Northern blots were performed. The Northern blots were prepared using non-transfected and fra-2 transfected Swiss 3T3 fibroblast RNA samples (Northerns). The cDNAs that showed a differential expression pattern in the Northern blots were then checked for a differential expression pattern in the transgenic and control eyes by Virtual Northern analysis. The gels used for the Northern and the Virtual Northern analysis are shown in figure 5.13.

Table 5.2 shows a summary of the results obtained for the Northern analysis. The differences in band intensity produced between the non-transfected and fra-2 transfected samples were estimated using a LKB Bromma UltraScan XL enhanced laser densitometer.

Four sequenced clones showed 99% homology to the mouse MHC class I heavy chain precursor H2-Kb mRNA. Their expression was up-regulated in the fra-2 transfected cells. Northern blot analysis to confirm the differential expression (figure 5.14a) showed that the MHC class I heavy chain precursor mRNA levels
Table 5.2: Summary of genes identified in PCR-select differential screen with fra-2 transfected and non-transfected Swiss 3T3 fibroblast mRNA (this page and next)

nd = not determined

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<th>Figure</th>
<th>Subtracted cDNA</th>
<th># clones</th>
<th>Level of expression in Fra-2 transfected fibroblasts</th>
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<td>1</td>
<td>decrease</td>
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Northern blots were performed to check if cDNAs identified by the PCR-select differential screening protocol were truly differentially expressed between the pCMV-mfra2 transfected and non-transfected Swiss 3T3 fibroblasts. Colonies identified as putative differentially expressed cDNAs were picked and plasmid DNA was prepared (see method 3.2.6(iii)). Sequence information was obtained (see section 5.2.12) and used to identify the cDNA insert. These cloned inserts were used as templates with nested PCR primer 1 and nested PCR primer 2R for the generation of $^{32}$P labelled PCR probes (see section 3.2.10). The probes were used in hybridisations with Northern blots on pCMV-mfra2 transfected and non-transfected Swiss 3T3 fibroblast mRNA. To establish if the cDNAs were differentially expressed in transgenic and control E15.5 eyes, Virtual Northern were also performed.

a) An example of the RNA gel used for the Northern blots. 5 µg aliquots of non-transfected (NT) and pCMV-mfra2 transfected (FT) Swiss 3T3 fibroblast RNA was run on a 0.8% agarose/1 X MOPS/2.2M formaldehyde gel. The RNA gel was stained in ethidium bromide, viewed under UV light and photographed.

b) An example of the cDNA gel used for the Virtual Northern blots. PCR reactions from the SMART cDNA synthesis procedure for control E15.5 eye (C) RNA and transgenic E15.5 eye (T) RNA were run on a 1.2% agarose/TAE/1 µg/ml ethidium bromide gel. The gel was viewed under UV light and photographed.
Target genes of Fra-2: Results

(a) (b)

Figure 3.4b illustrates the gel images of PCR products of mRNA isolated from different cell lines. The bands at 23.1, 4.4, 2.3, 1.35, and 0.6 kb correspond to specific genes identified as targets of the Fra-2 protein. The gel shows the presence of these bands in both the control (C) and treated (T) samples, indicating the activation of these genes under the influence of Fra-2.
**Figure 5.14:** Northern/Virtual Northern analysis of MHC Class I heavy chain precursor mRNA expression in *fra-2* transfected cells and *fra-2* transgenic eyes

Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 2 x 10^6 cpm/ml of a ^32^P labelled mouse major histocompatibility complex (MHC) class I heavy chain precursor cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 1567 bp is predicted using this probe.

a) Northern analysis of the MHC Class I heavy chain precursor mRNA (indicated by arrow) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film overnight.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The product size obtained for the MHC Class I heavy chain precursor cDNA (indicated by arrow) is smaller than predicted. The filter was exposed to film overnight.
were higher in the fra-2 transfected cells. The Virtual Northern result (figure 5.14b) was inconclusive. The detected product band did show an increase in the transgenic eye compared to the control eye. However, the band obtained in the Virtual Northern result (approx. 300 bp) was smaller than the expected cDNA product of 1567 bp.

A single subtracted clone showed 99% homology to murine migratory inhibitory protein (MIF) and was identified as being up-regulated in the fra-2 transfected cells. The Northern blot (figure 5.15a) showed an increase in the level of the MIF mRNA expression in the fra-2 transfected cells compared to the non-transfected cells. However, the Virtual Northern analysis (figure 5.15b) showed no significant difference between the levels of MIF cDNA in the transgenic and control eyes.

One sequenced clone showed 79% homology to the murine cyclin D1 mRNA. However, there were a number of unidentified nucleotides in the sequence obtained for this clone, so there may actually be up to 93% homology to the murine cyclin D1 mRNA. This cyclin D1-related cDNA clone was identified as being up-regulated in the fra-2 transfected cells. The Northern blot (figure 5.16a) showed no detectable expression of the cyclin D1-related mRNA in the non-transfected cells and a low level of expression in the fra-2 transfected cells. The Virtual Northern analysis (figure 5.16b) results also showed a slight increase in the cyclin D1-related mRNA expression in the transgenic eyes compared to the control eyes.

Two sequenced clones showed 93% homology to murine A10 cDNA or 91% homology to rat D-3-phosphoglycerate dehydrogenase. The A10 cDNA clone was identified as being up-regulated in the fra-2 transfected cells. The Northern blot (figure 5.17a) showed an increase in the level of A10 mRNA expression in the fra-2 transfected cells compared to the non-transfected cells. In contrast, the
Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 1.6 x $10^6$ cpm/ml of a $^{32}$P labelled mouse MIF cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 345 bp is predicted using this probe.

a) Northern analysis of MIF mRNA (indicated by arrow) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The arrow indicates the MIF cDNA. The filter was exposed to film for 2 hours.
Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 0.3 x 10^6 cpm/ml of a 32P labelled mouse cyclin D1 cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 3737 bp is predicted using this probe.

a) Northern analysis of cyclin D1 mRNA (indicated by arrow) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The arrow indicates the cyclin D1 cDNA. The filter was exposed to film for 4 days.
Figure 5.17: Northern/Virtual Northern analysis of A10 mRNA expression in fra-2 transfected cells and fra-2 transgenic eyes

Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 0.4 x 10^6 cpm/ml of a ^32P labelled mouse A10 (phosphoglycerate dehydrogenase) cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 1589 bp is predicted using this probe.

a) Northern analysis of the A10 mRNA (indicated by arrow) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The arrow indicates the A10 cDNA. The filter was exposed to film for 3 days.
Virtual Northern analysis (figure 5.17b) showed a decrease in the level of A10 mRNA in the transgenic eyes compared to the control eyes.

A single subtracted clone showed 76% homology to murine ST2/T1 mRNA. However, there were a number of unidentified nucleotides in the sequence obtained for this clone, so there may be up to 95% homology to the murine ST2/T1 mRNA. The ST2/T1 cDNA clone was identified as being up-regulated in the fra-2 transfected cells. As expected, the Northern blot (figure 5.18a) showed an increase in the level of ST2/T1 mRNA expression in the fra-2 transfected cells compared to the non-transfected cells. However, the Virtual Northern analysis (figure 5.18b) showed a decrease in the level of ST2/T1 mRNA in the transgenic eyes compared to the control eyes. Three product bands were detected in the Virtual Northern blot: the upper product band (see arrow) is of the predicted 2.67 kb size. All the product bands showed a decrease in the level of expression in the transgenic eye.

One subtracted clone showed 98% homology to murine Stearoyl CoA desaturase 2 mRNA and was identified as being up-regulated in the fra-2 transfected cells. As predicted, the Northern blot (figure 5.19a) showed a slight increase in the level of Stearoyl CoA desaturase 2 mRNA (see arrow 1) expression in the fra-2 transfected cells compared to the non-transfected cells. However, there are also an extra two bands at approximately 2 kb and 2.2 kb (arrow 2), which also showed an increased level of expression in the fra-2 transfected cells compared to the non-transfected cells. These extra bands produced a stronger signal than the upper band. The Virtual Northern analysis (figure 5.19b) showed a slight decrease in the level of Stearoyl CoA desaturase 2 mRNA expression in the transgenic eyes compared to the control eyes. The Virtual Northern detected a smear from approximately 0.3 kb to 1.4 kb with distinct product bands at approximately 0.3 kb and 0.4 kb. These are much smaller than the predicted 5 kb Stearoyl CoA desaturase 2 mRNA.
Figure 5.18: Northern/Virtual Northern analysis of ST2 mRNA expression in fra-2 transfected cells and fra-2 transgenic eyes

Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 0.3 x 10^6 cpm/ml of a ^32P labelled mouse ST2 cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 2665 bp is predicted using this probe.

a) Northern analysis of the ST2 mRNA (indicated by arrow) in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 4 days.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The arrow indicates the ST2 cDNA. The filter was exposed to film overnight.
Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 1.4 x 10^6 cpm/ml of a ^32^P labelled mouse Stearoyl CoA desaturase cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 5032 bp is predicted using this probe.

a) Northern analysis of the Stearoyl CoA desaturase mRNA (indicated by arrow 1) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. Additional smaller products were also detected (indicated by arrow 2). The filter was exposed to film for 3 days.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The product size is smaller (indicated by arrows) than predicted for the Stearoyl CoA desaturase cDNA. The filter was exposed to film for 5 days.
Figure 5.20 shows the results for cDNAs which were identified as being up-regulated in the fra-2 transfected cells by the PCR select subtractive screen but showed no significant difference, or very little difference, in the mRNA expression levels between transfected and non-transfected cells as determined by Northern blotting. RANTES and pyruvate kinase were each identified by homology to two sequenced clones. Four mRNAs were each identified by homology to a single clone: prothymosin α, Tax responsive binding protein 107, murine proliferin and L18 ribosomal protein. The Northern blots for Tax responsive binding protein 107, prothymosin α and RANTES (figures 5.20a to c) showed a slight increase in the mRNA expression of the gene in the fra-2 transfected cells compared to the non-transfected cells. The prothymosin α Northern blot detected two product bands: the lower band (see arrow) was of the predicted 1.2 kb size. Pyruvate kinase, proliferin and L18 ribosomal protein Northern blots (figures 5.20d to f) showed no significant difference in the expression of the mRNAs between the fra-2 transfected and non-transfected cells. In all of these samples, except for pyruvate kinase, the level of mRNA was very low in both cell populations.

Two subtracted clones showed 96% homology to murine G3BP mRNA and were identified as being down-regulated in the fra-2 transfected cells. As predicted, the G3BP Northern blot (figure 5.21a) showed a decrease in the level of G3BP mRNA expression in the fra-2 transfected cells compared to the non-transfected cells. The Virtual Northern analysis (figure 5.21b) for this mRNA was inconclusive. There is a smear on the gel with two cDNA bands at 0.7 kb and 1 kb and both bands were present at a decreased level in the transgenic eye samples. However, both products were considerably smaller than the expected 2.7 kb species.

One subtracted clone showed 95% homology to murine complement component C3 mRNA and was identified as being down-regulated in the fra-2 transfected
Target genes of Fra-2: Results

**Figure 5.20:** Northern blot analysis on subtracted clones identified as being over-expressed in pCMV-mfra2 transfected cells

Gels similar to those shown in figure 5.14a were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 0.5-1.2 x 10^6 cpm/ml of a ^32^P labelled mouse cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film.

a) Northern analysis murine tax responsive element binding protein 107 mRNA (indicated by arrow, predicted size = 950 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

b) Northern analysis of murine prothymosin α mRNA (indicated by arrow, predicted size = 1191 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 5 days.

c) Northern analysis of murine RANTES mRNA (indicated by arrow, predicted size = 548 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film overnight.

d) Northern analysis of murine pyruvate kinase mRNA (indicated by arrow, predicted size = 2134 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 5 days.

e) Northern analysis of murine proliferin mRNA (indicated by arrow, predicted size = 790 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 5 days.

f) Northern analysis of murine L18 ribosomal protein mRNA (indicated by arrow, predicted size = 632 bp) expression in pCMV-mfra2 transfected (FT) as
compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 5 days.
Target genes of Fra-2: Results

(a) [Image]

(b) [Image]

(c) [Image]

(d) [Image]

(e) [Image]

(f) [Image]
Figure 5.21: Northern/Virtual Northern analysis of G3BP mRNA expression in fra-2 transfected cells and fra-2 transgenic eyes

Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 4 x 10^6 cpm/ml of a 32P labelled mouse G3BP cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probes were prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 2686 bp is predicted using this probe.

a) Northern analysis of the G3BP mRNA (indicated by arrow) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The product (indicated by arrow) is smaller than predicted for the G3BP cDNA. The filter was exposed to film for 3 days.
cells. The Northern blot (figure 5.22a) showed a low level of complement component C3 mRNA expression in the non-transfected cells and no detectable expression in the fra-2 transfected cells. The Virtual Northern analysis (figure 5.22b) was inconclusive. It showed a smear from approximately 0.7 kb to 6.5 kb with a distinct product band at approximately 2.3 kb, which is smaller than the predicted murine complement component C3 mRNA size of 6.4 kb. However, the result did show a slight decrease in the level of mRNA expression in the transgenic eyes compared to the control eyes.

Figure 5.23 shows the results for cDNAs that were identified as being down-regulated in the fra-2 transfected cells by the PCR select subtractive screen but showed no significant difference, or very little difference, in the mRNA expression levels between transfected and non-transfected cells, as determined by Northern blotting. Three sequenced clones showed homology to rat Arp2/3 mRNA, two clones showed homology to murine SPARC and one clone showed homology to each of the following mRNAs: rat tricarboxylate carrier, murine acidic phosphoprotein PO, murine BH-protocadherin (PCDH7), murine secretory leukocyte protease inhibitor, rat neurodegeneration associated protein-1 and mouse frizzled-1. The Northern blot for tricarboxylate carrier cDNA clone (figures 5.23a) showed a slight increase in the mRNA expression of the gene in the fra-2 transfected cells compared to the non-transfected cells. All the other mRNAs showed no significant difference in the mRNA expression levels between the cell populations (figure 5.23b to h). The SPARC cDNA clone Northern blot (figure 5.23f) showed multiple product bands but they generally showed no significant difference in expression levels between the two mRNA samples. The neurodegeneration associated protein-1 and frizzled-1 (figure 5.23g and h) both showed very low levels of mRNA expression in both cell populations.
Figure 5.22: Northern/Virtual Northern analysis of Complement component C3 mRNA expression in fra-2 transfected cells and fra-2 transgenic eyes

Gels similar to those shown in figure 5.14 were blotted onto nitrocellulose overnight in 20 X SSC (see method 3.2.9). The filters were hybridised with 1.1 x 10^6 cpm/ml of a ^32^P labelled mouse complement component C3 cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probe was prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1 X SSC/0.1% SDS at 55°C and then exposed to film. A band of 6435 bp is predicted using this probe.

a) Northern analysis of the complement component C3 mRNA (indicated by arrow) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 9 days.

b) Virtual Northern analysis on control (C) and transgenic (T) E15.5 eye cDNA populations. The product (indicated by arrow) is smaller than predicted for the complement component C3 cDNA. The filter was exposed to film overnight.
Target genes of Fra-2: Results

**Figure 5.23:** Northern blot analysis on subtracted clones identified as being down-regulated in pCMV-mfra2 transfected cells (next 2 pages)

Gels similar to those shown in figure 5.14a were blotted onto nitrocellulose overnight in 20X SSC (see method 3.2.9). The filters were hybridised with 0.4-2.5 x 10^6 cpm/ml of a ^32P labelled mouse cDNA PCR probe overnight at 42°C (see method 3.2.12). The PCR probes were prepared using nested PCR primer 1 and nested PCR primer 2R as described in method 3.2.10. The following day the filters were washed in 1X SSC/0.1% SDS at 55°C and then exposed to film.

a) Northern analysis of murine acidic phosphoprotein PO mRNA (indicated by arrow, predicted size = 1094 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

b) Northern analysis of tricarboxylate carrier mRNA (indicated by arrow, predicted size = 2986 bp for rat mRNA) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

c) Northern analysis of murine Arp2/3 mRNA expression (indicated by arrow, predicted size = 1428 bp for human mRNA) in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 5 days.

d) Northern analysis of murine BH-protocadherin (PCDH7) mRNA (indicated by arrow, predicted size = 4454 bp for human mRNA) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

e) Northern analysis of murine secretory leukocyte protease inhibitor mRNA (indicated by arrow, predicted size = 671 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.
Target genes of Fra-2: Results

f) Northern analysis of murine SPARC mRNA (indicated by arrow, predicted size = 2079 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 5 days.

g) Northern analysis of neurodegeneration associated protein-1 mRNA (indicated by arrow, predicted size = 4758 bp for rat mRNA) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 3 days.

Northern analysis of murine frizzled-1 mRNA (indicated by arrow, predicted size = 1881 bp) expression in pCMV-mfra2 transfected (FT) as compared to non-transfected (NT) Swiss 3T3 fibroblasts. The filter was exposed to film for 5 days.
Target genes of Fra-2: Results

(c) [Image of gel with bands]

(b) [Image of gel with bands]

(a) [Image of gel with bands]
Target genes of Fra-2: Results

(h) 

(g) 

(f) 

(e)
5.4 Discussion

5.4.1 Links between TGFα and Fra-2

As described earlier, a literature search revealed that transgenic and knockout TGFα mice develop a similar eye phenotype to that observed in the fra-2 transgenic mice. Thus, experiments were performed to investigate potential links between these genes. There was no significant difference in the amounts of TGFα mRNA detected in the fra-2 transgenic eyes compared to the littermate control eyes (see figure 5.2). However, expression of fra-2 RNA and protein was shown to be up-regulated by TGFα treatment of Swiss 3T3 fibroblasts in vitro (see figure 5.3), indicating that Fra-2 may be a downstream effector of TGFα signalling. Thus, the results that were observed in the fra-2 transgenic mice might explain, at least in part, some of the results observed in the TGFα knockout and transgenic mice.

TGFα is a member of the epidermal growth factor family of proteins. Other members of this gene family have been shown to induce the expression of AP-1 family members. For example, TGFβ has been shown to induce the expression of c-fos and junB and EGF is known to induce the expression of many of the AP-1 family members, including c-jun, junB, c-fos and fosB (reviewed in Herdegen and Leah, 1998). The fra-2 promoter contains an SRE (Foletta et al., 1994) and TGFα probably exerts its effects on fra-2 through this promoter element in the same way EGF enhances c-fos expression through the SRE promoter element in the c-fos promoter.

TGFα signals through the EGF receptor (EGFR) (reviewed in Derynck, 1988). Numerous studies have shown that the EGFR is expressed in corneal epithelium and throughout the eyelid in humans, rabbits and mice (Berkowitz et al., 1996; Li and Tseng, 1995; Reneker et al., 1995; Wong et al., 1996). TGFα has also been
found in the cornea (references within Reneker et al., 1995). The EGFR signals via a tyrosine kinase activity and this activity is lost in wa-2 mice (Luetteke et al., 1994). The wa-2 mice have a point mutation in the kinase activity of the EGFR protein but do retain the ability to bind TGFα. Crosses between TGFα transgenic mice and wa-2 mice lack the signalling migratory response observed in the TGFα transgenic mice but still show anterior segment dysgenesis (Reneker et al., 1995). This suggests that there are at least two signalling pathways downstream of TGFα – EGFR interaction: one that involves the tyrosine kinase activity and another that does not. Fra-2 may be involved in this second signalling pathway, since the fra-2 transgenic mice also show the anterior segment dysgenesis phenotype.

TGFα clearly has an effect on the corneal epithelium: in TGFα knockout mice the corneal epithelium is thinner (Mann et al., 1993) and in TGFα transgenic mice there is hyperplasia of the corneal epithelium (Decsi et al., 1994; Reneker et al., 1995). TGFα has also been shown to have effects on other tissue epithelium and has been suggested to play a major role as an epithelial cell mitogen, particularly for secretory epithelium (Sandgren et al., 1990). Transgenic mice over-expressing TGFα show primary effects in epithelia of many tissues, including liver, intestines, pancreas, skin, coagulation glands and mammary epithelium. The TGFα appears to selectively promote growth of the epithelium. However, only in a few of these tissues is malignant transformation observed, for example, in the mammary epithelium and liver (Jhappen et al., 1990; Matsui et al., 1990; Sandgren et al., 1990; Vassar and Fuchs, 1991). Fra-2 is also known to be expressed in many developing epithelia, including stomach, skin (including the hair follicle), kidney, oral and intestinal epithelium (Carrasco and Bravo, 1995). Immunofluorescence staining in the transgenic mice reported here also detected Fra-2 expression in the pancreas of control and transgenic mice. This suggests that TGFα and Fra-2 may be important factors involved in the signalling pathways...
involved in epithelial cell growth. However, if this is the case, it raises a number of questions.

Firstly, why were there not more effects observed on epithelial cells in the fra-2 transgenic mice? Fra-2 and TGFα are both expressed in the pancreas and hair follicles and the fra-2 transgenic mice did show increased Fra-2 expression in these tissues. However, despite the potential link between Fra-2 and TGFα, the fra-2 transgenic mice did not show any gross phenotypic effects on either tissue up to 2 years of age, unlike the TGFα transgenic and knockout mice, which showed abnormalities in hair growth. The lack of effects on these other epithelia may be due to one or more of the following factors:

(i) Insufficient Fra-2 protein levels to have any observable effect in these cells.
(ii) Lack (or insufficient levels) of an appropriate protein partner for Fra-2 to bind to and form an active AP-1 complex with.
(iii) Limited levels of other important components of the signalling pathway which are also regulated by TGFα.

Secondly, why do TGFα knockout mice and TGFα transgenic mice show a similar phenotype of anterior segment dysgenesis? If Fra-2 is a downstream signalling effector of TGFα, then the principles that apply to Fra-2 will also apply to TGFα. A mechanism was described earlier in chapter 4 (see figure 4.27) to explain how either increases or decreases in Fra-2 levels could lead to a similar phenotype. The hypothesis suggested was that it is the balance between the relative levels of all AP-1 family members that is important, rather than the absolute levels of the individual proteins. Therefore, this phenomenon would also apply when TGFα expression levels are altered, if TGFα signals through Fra-2, since the consequent imbalance in Fra-2 levels would also lead to changes in the array of total AP-1 complexes and changes in gene expression. Thus, the same eye phenotype would develop whether TGFα levels were increased or decreased.
5.4.2 Mitf and other transcription factor genes

The earlier literature search also identified similar eye abnormalities to those observed in the *fra-2* transgenic mice resulting from disruptions to Mitf and Pax6 proteins. Therefore the links between these proteins and Fra-2 were also investigated.

There are four possible explanations for the observation that Mitf mutations produce similar eye phenotypes to those found in the *fra-2* transgenic mice:

(i) Fra-2 regulates Mitf expression or Mitf regulates Fra-2 expression.
(ii) Fra-2 and Mitf proteins interact directly.
(iii) Fra-2 and Mitf proteins interact indirectly.
(iv) The two results are unrelated.

These possibilities will be discussed below in relation to the results obtained from the experiments involving Mitf and Fra-2.

The RT-PCR analysis on mouse eyes did not detect any Mitf expression in either transgenic or control eyes (see figure 5.2). This result, together with the fact that both genes can normally be expressed in eye tissue, suggests that neither Mitf nor Fra-2 regulates the expression of the other. The absence of any detectable Mitf expression in control eyes, where endogenous *fra-2* expression can be detected, indicates that Mitf does not regulate *fra-2* expression in this tissue. Likewise, since Fra-2 normally functions in the eye, it can be assumed that protein partners and cellular conditions for Fra-2 function are present in this tissue. Therefore, the absence of any Mitf expression in the transgenic eyes suggests that increased Fra-2 expression is not sufficient to increase Mitf expression in the eye.

Interestingly, Mitf mRNA was detected in Swiss 3T3 fibroblast extracts following TGFα stimulation. The kinetics of the Mitf expression was similar to that of *fra-2* expression following TGFα stimulation. This result also supports the conclusion that the Mitf and Fra-2 do not regulate the expression of each other, since the
expression of the gene encoding the regulator would be expected to precede the expression of the target gene.

The *in vitro* association experiment did not show any direct interaction between Mitf and Fra-2 or any of the other AP-1 members analysed (c-Fos, c-Jun, JunB). Mitf also did not interact with a complete AP-1 complex.

However, this experiment did not rule out an indirect interaction between Mitf and Fra-2, through the association of each protein with a common intermediary protein. One good candidate for such a protein is the CBP/p300 transcription cofactor. Mitf has been shown to interact with CBP *in vitro* (Sato et al., 1997). The N-terminus DNA binding and dimerisation domains of Mitf have been shown to interact with the CPB2 domain of CBP/p300. The CPB2 domain of CPB/p300 is the domain that is known to interact with E1A, p/CAF and c-Fos. Mutations in Mitf that inhibit aspects of its function, for example mutations that affect its DNA binding ability, result in phenotypes such as failure of eyelids to fuse, osteopetrosis, small or absent eyes and failure of incisor eruption. Mutations in Mitf may indirectly affect AP-1 protein function through this interaction with CBP.

How can mutations in Mitf and over-expression of Fra-2 both lead to abnormal eyes and how might this relate to CBP/p300? Under normal circumstances, Mitf may effectively compete with Fra-2 for the binding of CBP/p300. When Mitf is mutated, it can no longer bind CBP/p300; this allows Fra-2 to bind CBP/p300 and this complex leads to abnormal eyes. In the case of the transgenic eyes that are over-expressing Fra-2, the Fra-2 concentration would be increased so that it can now effectively compete with Mitf for the binding of CBP/p300. In this circumstance, Fra-2-CBP/p300 complexes would be favoured and would once again lead to abnormal eyes. In this scenario, Mitf regulates Fra-2 function by restricting its access to a cofactor protein.
All transgenic mice showed the presence of an eye abnormality, however, not all transgenic mice had microphthalmia (small eyes). This may be explained by the fact that the effects of Mitf on Fra-2 would depend on the level of Fra-2 expression. Hence, an alteration on the size of the transgenic mouse eyes would only occur if significant Fra-2 expression occurred. In support of this, the severity of eye abnormality did correlate with the level of fra-2 expression in the fra-2 transgenic eyes.

Therefore, the analysis of Mitf that was undertaken suggests that Mitf and Fra-2 do not regulate each other’s expression and do not interact directly. To confirm the hypothesis that Mitf and Fra-2 interact indirectly, further experiments would need to be undertaken, including analysis to determine if Fra-2 does bind CBP/p300. The final possibility is that the results obtained with mutated Mitf and transgenic mice over-expressing fra-2 are unrelated. This possibility cannot be excluded on the basis of the results that were obtained in this analysis.

PAX6 is thought to play a fundamental role in the development of the eye and mutations in PAX6 can lead to aniridia and Peter’s anomaly in humans (reviewed in Prosser and Van Heyningen, 1998). In the developing mouse eye, Pax6 is generally expressed in all ocular structures, with Pax6 first detected at embryonic day 8.5 (Walther and Gruss, 1991). Regulation of Pax6 has been suggested to occur at the transcriptional level and a number of transcription factors have been identified that act as a transcriptional activator of Pax6, including c-Myb (Plaza et al., 1995).

Recently two Pax6 promoters have been identified, P0 and P1 (Xu et al., 1999). These promoters produce mRNA transcripts that are differentially expressed during eye development. The P0 transcripts are more abundant in the developing cornea than the P1 transcripts. Interestingly, the P0 transcripts are also expressed in the pancreas. Pax6 has been detected in all mature endocrine cells, the cells that
secrete the growth hormones glucagon, insulin, somatostatin and pancreatic polypeptide (Xu et al., 1999).

The level of Pax6 amplification product detected in the fra-2 transgenic eyes compared to the littermate control eyes was reduced, suggesting that over-expression of fra-2 may result in a decrease in the levels of Pax6 mRNA. In contrast, no Pax6 expression was detected in Swiss 3T3 fibroblasts either before or after TGFα stimulation in vitro. This may reflect a cell specific expression pattern for Pax6, such that Pax6 is expressed in the eye but not in fibroblasts. In support of this, Pax6 has been detected in the developing eye, brain, pituitary, olfactory bulbs and olfactory epithelium but not in adult kidney, heart, intestine, liver, lung, spleen, testis and uterus (Walther and Gruss, 1991).

Therefore Fra-2 / AP-1 could potentially negatively regulate the expression of P0 transcripts both in the cornea and pancreas. Thus, the reduction in Pax6 levels in the fra-2 transgenic mice compared to littermate control mice may have, at least in part, lead to the reduction in weight of the transgenic mice and some of the eye abnormalities observed. Alternatively Fra-2 may indirectly affect Pax6 expression by affecting another protein that positively regulates the expression of Pax6. Future experiments could include the analysis of the Pax6 promoter for the presence of an AP-1 site.

Interestingly, alterations in the expression of many transcription factors have been associated with eye abnormalities (reviewed in Cvekl and Piatigorsky, 1996; Freund et al., 1996). These transcription factors are from at least seven different protein families. Therefore, normal eye development may rely on a highly ordered cascade in the expression patterns of many transcription factor genes and cooperation between the proteins they encode. Hence, an alteration in any one of these transcription factors may disrupt the ordered development of the eye and lead to eye abnormalities. Future investigations into the functions of AP-1 (including Fra-2) in the eye are required to obtain a better understanding of the
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role played by this transcription factor family in eye development and the relationship of it to other transcription factors functioning in the eye.

Although the approach discussed above, of looking at genes known to be involved in eye development and/or abnormalities, did suggest a couple of Fra-2 target genes, a different approach to identify a broader array of Fra-2 target genes was also attempted. For this, the CLONTECH PCR-select differential screening protocol was employed and the results are discussed below.

5.4.3 Identification of genes regulated by Fra-2

(a) Important considerations when interpreting the results

The use of the SMART cDNA and PCR-select differential screening protocols did produce some interesting results. However, there are some important points that must be considered when interpreting the results obtained from these procedures.

Firstly, the quality of the starting RNA is very important in obtaining full-length double-stranded cDNA molecules. Poor quality degraded RNA will still be amplified and may affect the final results. This is important for two reasons. The first reason is that efficiency will be reduced if some of the cDNA molecules for a particular species are full-length while others are truncated. The second reason is that the Virtual Northern analysis, used to confirm the results of the differential screening, also relies on the SMART cDNA synthesis procedure. Thus, if only partial cDNAs are synthesised, this will produce bands and possibly smears on the Virtual Northern blots that are smaller than the predicted size of the mRNA being analysed. This will compromise the interpretation of the result. It is also important to use equal amounts of starting RNA, as this will affect the number of false positives obtained.
Secondly, PCR is the basis of both of these procedures and therefore PCR variability can also contribute to false positives.

Thirdly, the PCR-select differential screening protocol relies on the presence of Rsa I restriction sites in the cDNAs. Therefore, any cDNAs that lack these sites will not be analysed by this method. The size of the Rsa I fragments generated is also important, as smaller molecules are more efficiently hybridised, amplified and cloned. Cloning efficiency is likely to have been a problem in the screening experiments described here, as fra-2 was not detected as a differentially expressed gene in either of the PCR-select differential screens. The mouse fra-2 gene does contain Rsa I sites and the forward subtracted cDNA population from the fra-2 transfected versus non-transfected Swiss 3T3 fibroblast screen did show high levels of fra-2 cDNA compared to the reverse subtracted cDNA population (see figure 5.12).

Fourthly the number of differences between the two cDNA populations being tested is important. If there are few differentially expressed cDNAs and smaller differences in the levels of expression of the genes, then higher backgrounds result.

The final point to consider concerns the interpretation of the results against the original rationale for performing a differential analysis: namely, to identify genes whose expression is regulated by Fra-2. Since the only difference between the cell populations being compared (ie. transfected versus non-transfected fibroblasts and transgenic versus control E15.5 eyes) was the over-expression of Fra-2, it might be presumed that other alterations in gene expression were a result of this over-expression.

However, for any gene whose expression was altered in the transfected/transgenic cells compared to the control, there are actually four possible reasons:

(i) The expression of that gene was directly regulated by Fra-2.
(ii) The expression of that gene was directly or indirectly affected by the site of integration of the transgene (Note: This point does not apply to the results obtained with the transfected cells, since the transfected plasmid generally does not integrate into the genome during a transient transfection.)

(iii) The expression of that gene may have been affected as a result of a generalised cellular disruption caused by the expression of an introduced gene, which might be independent of the actual transgene.

(iv) The expression of that gene may have been a downstream effect of alterations in cellular processes (for example induction of differentiation, inhibition of cellular proliferation, etc). The induced change in cellular processes will be associated with a cascade of changes in gene expression patterns which are not directly regulated by the introduced gene, in this case Fra-2.

With all of the above caveats in mind, the differential screening analysis presented in this chapter can, at best, only identify possible candidate genes whose expression may be regulated by Fra-2. Further experimental evidence is required to confirm the relationship between Fra-2 and the genes identified. Time did not permit a more detailed investigation of any of these candidate genes. Therefore, the remainder of this discussion presents results of a literature search undertaken to establish further links between Fra-2 and the candidate genes, with a view to identifying those genes most worthy of further investigation.

(b) Potential downstream target genes of Fra-2 in the eye

The results obtained in the PCR-select differential screen with transgenic versus control E15.5 mouse eye cDNA populations identified the following genes: crystallin genes β, γ-A, γ-B, γ-C and γ-F, m-α-1 tubulin, cyclophilin, cytochrome c oxidase subunit I, Tpr, EF-Tu, β-globin and LLRep3, as potential targets of Fra-2.
The crystallin genes $\beta$, $\gamma$-A, $\gamma$-B, $\gamma$-C and $\gamma$-F, all showed a decrease in the expression levels of the crystallin genes in the transgenic eyes compared to the littermate control eyes. The down-regulation in crystallin gene expression may account for the cataracts observed in some of the transgenic eyes. Crystallin proteins are the major soluble proteins in the lens and can be divided into several classes. There are the $\alpha$- and $\beta$-crystallins that are expressed in all animals and other crystallin genes which are species specific, for example the $\gamma$-crystallins that are found only in mammals (Wistow and Piatigorsky, 1988). The crystallins are temporally and spatially expressed in the lens. The $\gamma$-crystallin genes are all simultaneously switched on in the embryonic lens but switched off individually after development. The $\gamma$-E and $\gamma$-F genes are switched off early in development and the $\gamma$-B gene is switched off later (Klok et al., 1998 and references within).

The regulation of the crystallin genes has been studied extensively but is still not completely understood. Some of the factors that have been shown to regulate the expression of the crystallin genes include the Pax6 protein, AP-1 proteins, SOX proteins, USF, CREB or CREM proteins and Maf proteins (Cvekl and Piatigorsky, 1996; Cvekl et al., 1994; Kamachi et al., 1995; Klok et al., 1998; McDermott et al., 1997; Nishiguchi et al., 1997).

The $\beta$-crystallin promoter contains an AP-1-CREB consensus sequence and is negatively regulated by the AP-1 complex composed of JunD and Fra2 in fibroblasts (McDermott et al., 1997). In the eye CREB family members positively regulate the same site. Also primary lens epithelial cells transfected with a retroviral c-Jun expression vector were shown to have decreased levels of $\beta$-crystallin (Rinaudo and Zelenka, 1992). Rinaudo et al. hypothesised that AP-1-CRE elements function as enhancer or repressor elements of the crystallin genes as the AP-1 and CREB protein ratios change during lens development (Rinaudo and Zelenka, 1992). Therefore in the case of the $\beta$-crystallin gene, the reduction in the expression is probably directly due to the increase in the levels of Fra-2, which negatively regulates the expression of the gene.
The γ-crystallin genes are regulated by γbox regulatory elements, which resemble Maf recognition elements (MARE) (Klok et al., 1998). As mentioned in chapter 1, Maf proteins can regulate transcription only as heterodimers with other Maf proteins or members of the AP-1 family (Kerppola and Curran, 1994), particularly Fos proteins (Kataoka et al., 1994; Kataoka et al., 1995). The γ-B-crystallin promoter contains two AP-1 sites and at least one of these is involved in the regulation of the gene (Klok et al., 1998). The SOX proteins also regulate the expression of the γ-crystallin genes, particularly Sox1 (Kamachi et al., 1995; Nishiguchi et al., 1997). Sox1 protein is essential for the activation of some γ-crystallin genes (A, B, D and F) and is required for maintaining the levels of all γ-crystallin genes during development (Nishiguchi et al., 1997). The SOX proteins are members of the HMG box protein family. This family of proteins has been shown to require cooperation with other factors that bind to neighbouring sites to enhance transcription (Giese and Grosschedl, 1993). AP-1 proteins have been shown to facilitate the binding of the HMG box protein SRY in vitro by disruption of the nucleosome (Ng et al., 1997).

The results from the search of the literature suggest four possible mechanisms as to how Fra-2 may down-regulate the expression of the γ-crystallin genes in the fra-2 transgenic mice. These mechanisms may act separately or cooperatively to produce the decrease in gene expression.

Firstly, AP-1 may directly bind a regulatory element in the crystallin gene promoter region. An AP-1 complex comprising the Fra-2 protein may act to repress the transcription of the genes, as suggested for the β-crystallin promoter.

Secondly, as mentioned above, Fra-2 may down-regulate the expression of the Pax6 gene, which is known to up-regulate the expression of many crystallin genes. Therefore the effects on the γ-crystallin genes would be a secondary effect of Fra-2 over-expression.
Thirdly, the MARE sites in the γ-crystallins γ-boxes may be bound by a Maf protein-Fra-2 protein complex in the fra-2 transgenic mice, which down-regulates the expression of the γ-crystallin genes.

Finally, in the case of the SOX proteins, if Fra-2 could interact with an adjacent site to the SOX protein-binding site, then the binding of Fra-2 to this site may interfere with the binding of the SOX protein to its own binding site. This would then inhibit transcriptional activation of the γ-crystallin gene by the SOX protein(s).

All the crystallin genes, particularly all γ-crystallins, are regulated by some common factors. Despite this, not all crystallin genes were identified as being down-regulated by the over-expression of Fra-2 in the E15.5 eye. This may be due to the screening procedure, the differential patterns of crystallin gene expression in the mouse embryonic eye or the cellular sites of Fra-2 transgene expression. The immunofluorescence analysis did show that Fra-2 was expressed in the lens of the transgenic eyes.

The expression of the m-α-1 tubulin gene appeared to be down-regulated in the fra-2 transgenic mouse eyes. The tubulin proteins are the principle subunits of microtubules and are considered to be house-keeping genes. The effect on tubulin gene expression may be an indirect effect of increased Fra-2 expression, as no AP-1 sites have been identified in the m-α-1 tubulin gene promoter to date.

Cytochrome c oxidase subunit I mRNA levels were also decreased in the fra-2 transgenic mice compared to their littermate controls. Cytochrome c oxidase subunit I gene is encoded by the mitochondrial DNA genome (Bibb et al., 1981). The cytochrome c oxidase protein is made up of six protein subunits, with subunits I, II and III being mitochondrial encoded genes and subunits IV, V and VI being nuclear encoded genes (Wiesner et al., 1992). The cytochrome c oxidase
protein is associated with mitochondrial proliferation. The AP-1 transcription factor has been suggested to play a role in regulating the expression of some of the nuclear encoded subunits of cytochrome c oxidase and therefore, in the initiation of mitochondrial proliferation (Xia et al., 1997).

Nuclear encoded genes are also thought to play a role in regulating the expression of cytochrome c oxidase subunit I (Pel et al., 1992). Interferon α/β has been shown to negatively regulate the expression of cytochrome c oxidase subunit I in vitro and this regulation is mediated by interferon-responsive nuclear genes (Shan et al., 1990). AP-1 proteins, in complexes with other transcription factors, have been shown to regulate the expression of interferon genes (reviewed in Foletta et al., 1998). A recent report using gossypol acetic acid treated rodent liver cells to investigate genes induced in response to oxidative stress revealed that c-fos levels were increased in a dose-dependent manner. A subtractive hybridisation experiment using these cells also identified a three-fold and five-fold induction of cytochrome c oxidase subunits I and II mRNA, respectively (Hutchinson et al., 1998). Therefore, the AP-1 transcription factor family may play a role in both positively and negatively regulating the expression of the cytochrome c oxidase subunit I and II genes. The results from work presented in this chapter suggests that Fra-2 negatively regulate the expression of cytochrome c oxidase subunit I gene. Future experiments would involve searching for AP-1 sites within the cytochrome c oxidase gene promoter.

Cyclophilin was also down-regulated in the fra-2 transgenic mice. Cyclophilin is an abundant cytosolic protein expressed in most tissues. It is a highly conserved protein that is thought to have a important role in T lymphocyte signal transduction (Walsh et al., 1992) and a role in many tissues in response to stimuli that alter cellular metabolism and induce differentiation or proliferation (Harding et al., 1986). No AP-1 sites have been identified in the cyclophilin gene promoter to date. Therefore, the effect on cyclophilin gene expression may be an indirect effect of increased Fra-2 expression, such that changes in cyclophilin gene
expression may result from changes in cellular processes, ie. induction of differentiation.

Tpr expression was also down-regulated in the fra-2 transgenic eyes compared to the control eyes. Tpr is a coiled-coil protein that is localised to the intranuclear filaments of the nuclear pore complex and may play a role in molecular trafficking (Byrd et al., 1994). Recently the Tpr protein was implicated in the process of mRNA export from the nucleus (Bangs et al., 1998). A search of the literature did not reveal any evidence to support or rule out a role for Fra-2/AP-1 in directly regulating the expression of the Tpr gene.

If Fra-2/AP-1 complexes do not directly regulate Tpr expression, an interesting possibility is that the effects on Tpr expression may have been an indirect effect of changes in the cellular processes occurring within the cells (eg. decreased proliferation, increased differentiation, etc.), which were induced by increased Fra-2 levels. Therefore, the changes in cellular processes in these mouse eyes may have led to a decrease in the requirements for mRNA export, which then produced a negative feedback, thereby reducing the level of Tpr expression.

The expression of LLRep3 mRNA was also down-regulated in the fra-2 transgenic mouse eyes compared to the littermate control eyes. LLRep3 is a highly conserved repetitive DNA sequence family, in mouse and humans. There are approximately 200 copies of the gene, which lead to the expression of a single abundant mRNA (Heller et al., 1988). The expression of this gene family is highest in rapidly dividing cells such as early mouse embryos and testis (Heller et al., 1988). The LLRep3 mRNA encodes for the ribosomal protein S2. This S2 protein is involved in the binding of aminoacyl-tRNAs to ribosomes and it also affects the fidelity of the translation of mRNA (Suzuki et al., 1991).

A search of the literature did not reveal any information suggesting that Fra-2 directly regulates the expression of the S2 protein. Alternatively, as discussed
above with Tpr expression, the decreased LLRep3 mRNA levels may be an indirect consequence of reduced proliferation of the eyelid buds. The reduced proliferation of the cells may have led to a decrease in the requirements for the S2 protein.

EF-Tu expression was also down-regulated in the fra-2 transgenic mouse eyes compared to littermate control mouse eyes. EF-Tu is an important factor in protein translation and is the carrier of aminoacyl tRNAs to the ribosome. EF-Tu is thought to be regulated at the level of transcription (Roth et al., 1987) but there is no evidence to suggest that Fra-2/AP-1 is involved in this regulation. However, a role for Fra-2 in negatively regulating EF-Tu cannot be excluded.

Interestingly, a reduction in EF-Tu expression is associated with cellular differentiation (Roth et al., 1987). There are also other important signs of cellular differentiation including: a decrease in total RNA content; a decrease in general RNA synthesis; an increase in the rate of globin gene transcription; and a decrease in the cellular protein content (Ganguly and Skoultchi, 1985; Parker and Housman, 1985).

In this context, it is interesting to note that the last four genes, namely tubulin, cyclophilin, Tpr and LLRep3, may have all been indirectly affected by Fra-2, resulting from a change in cellular processes occurring within the cell. Therefore, all these results taken together suggest a general theme involving the switching from proliferation to differentiation associated with an increase in the level of Fra-2 present in the cell.

The last of the genes whose expression was identified as being altered in the transgenic eyes was β-globin. β-globin expression was increased slightly in the fra-2 transgenic eyes (see figure 5.10m). This result also supports the idea of Fra-2 function being associated with induction of differentiation, such that the increase in Fra-2 expression in the eye may have induced cellular differentiation.
of some cells, which in turn led to the observed changes in the expression of many of these genes.

In terms of β-globin, another and not mutually exclusive possibility is that Fra-2 could be involved in the regulation of β-globin expression. In support of this, the β-globin locus control region has been shown to contain AP-1-like motifs and AP-1 is known to affect globin gene expression (Fleenor and Kaufman, 1993; Moi and Kan, 1990). c-Jun inhibits the activation of the β-globin gene (Moi and Kan, 1990) and another leucine zipper protein NF-E2-related factor 2 (Nrf 2) has been shown to enhance β-globin expression (Moi et al., 1994). Fra-2 may interact with Nrf 2 or directly bind the β-globin locus control region to enhance transcription. Alternatively, Fra-2 may sequester c-Jun, which then allows more Nrf 2 to bind the AP-1-like element and therefore increase the expression of the β-globin gene.

Figure 5.24 shows a summary of the potential target genes of Fra-2 identified by the PCR-select differential screen on fra-2 transgenic versus control E15.5 eyes and highlights how changes in the expression of these genes may have led to the phenotypes observed in the transgenic mice. Taking all of these potential target genes of Fra-2 together, the general result suggests a role for Fra-2 in the induction of differentiation. Fra-2 may switch the cellular signals from a proliferation signal to a differentiation signal. The resulting reduction in proliferation probably accounts for the lack of eyelid fusion and reduced weight of the transgenic animals.
Figure 5.24: Schematic representation showing a summary of potential target genes of Fra-2 identified in the eye

A schematic representation showing a summary of the potential target genes of Fra-2 identified by the PCR-select differential screen on transgenic and control E15.5 eye mRNA extracts and how changes in the expression levels of these genes may have led to the phenotypes observed in the transgenic mice. The red arrows represent negative effects of the above protein on the corresponding gene and the green arrows represent positive effects on the corresponding gene. The black arrows indicate the phenotypic consequences that alterations in the preceding signalling pathway can result in. The solid lines represent direct effects on the corresponding gene and the dashed lines represent indirect effects on the corresponding gene.
Many potential target genes of Fra-2 were identified from the PCR-select differential screen on Swiss 3T3 fibroblasts transfected with the pCMV-mfra2 construct versus non-transfected cells. However, the Northern blot analysis showed that a number of these genes were, in fact, not significantly differentially expressed. This included RANTES, pyruvate kinase, prothymosin α, Tax responsive protein 107, proliferin, L18 ribosomal protein, Arp2/3, SPARC, tricarboxylate carrier, PCDH7, ribosomal phosphoprotein PO, leukocyte protease inhibitor, neurodegeneration associated protein-1 and frizzled-1. The identification of these false positives highlights the shortcomings of differential screening procedures in general, and emphasises the importance of independent confirmation of the result.

There were 8 genes that did appear to be truly differentially expressed between the fra-2 transfected and non-transfected Swiss 3T3 cells, namely MHC class I heavy chain precursor (H-2D^b), MIF, cyclin D1, A10 (phosphoglycerate dehydrogenase), ST2, G3BP, SCD2 and complement component C3.

When the differential screen results were confirmed by Northern and Virtual Northern blotting, it was found that some of the genes had the same expression pattern in fra-2 transgenic eyes and fra-2 transfected fibroblasts, whereas other genes had different expression patterns in the two situations. For example, the MHC class I heavy chain gene was up-regulated in both circumstances, whereas the A10 gene was down-regulated in the transgenic eyes and up-regulated in the transfected fibroblasts. These results reflect the complexity of gene transcription. If the expression of a given gene only relied on the presence or absence of a single transcription factor complex, then it would be expected that the same expression pattern would be observed for all the genes identified in this analysis in both circumstances of over-expressed Fra-2. In reality, gene expression usually depends upon the interplay of more than one transcription factor complex on the
promoter. In different cell types, the array of transcription factor complexes that are present and functionally available varies. Therefore, the expression pattern for a particular target gene of Fra-2 will depend, not only on the presence or absence of Fra-2, but also on the presence or absence of other transcription factor complexes.

The mRNA encoding the MHC class I heavy chain precursor (H-2D\textsuperscript{b}) was up-regulated in the fra-2 transfected cells compared to the non-transfected cells. A similar up-regulation of the MHC class I heavy chain mRNA was observed in the fra-2 transgenic eye compared to the littermate control eyes, as shown by Virtual Northern analysis. The correct assembly of the MHC class I molecule involves association of the MHC class I heavy chain with an invariant \(\beta_2\)-microglobulin light chain and an 8-10 residue peptide. This complex is then transported out of the endoplasmic reticulum (ER) to the cell surface. If either the \(\beta_2\)-microglobulin light chain or the peptide is absent, then the complex is not expressed on the cell surface but is degraded (Salter and Cresswell, 1986; Sege et al., 1981). The MHC class I molecules play an important role in immune recognition and they function as target structures recognised by MHC-restricted antigen-specific cytotoxic T-lymphocytes (CTL) (Zinkernagel and Doherty, 1979).

The MHC class I molecules are expressed in nearly all nucleated somatic tissues, although the levels may vary (Singer and Maguire, 1990). The expression of MHC class I molecules is regulated mainly at the level of transcription; transcription is enhanced by various cytokines and inhibited by hormones (reviewed in van den Elsen et al., 1998). Interestingly, MHC class I molecules have been shown to be down-regulated in a number of human and murine tumours and in in vitro experiments involving oncogene transformations, including experiments involving c-fos-mediated transformation ((Lohmann et al., 1996) and references within). This reduction in MHC class I levels is speculated to allow transformed cells to escape immune surveillance.
The MHC class I heavy chain gene contains AP-1 sites within its promoter region and c-Jun has been shown to negatively regulate the expression of this gene (Howcroft et al., 1993). There is also a cis-acting regulatory element called site $\alpha$ within the MHC class I promoter region. This site contains a CRE site and is constitutively occupied by various DNA binding proteins, including ATF-1, CREB-1, CREM-1, JunB and JunD. Another regulatory region of the MHC class I promoter is the enhancer A site which is bound by a Mod-1 complex. This Mod-1 complex has been shown to consist of the NF-$\kappa$B p50 subunit and Fra-2 proteins (Giuliani et al., 1995). This Mod-1 complex has been shown to enhance the transcription of the MHC class I gene. This analysis by Giuliani et al. did not find any association with either the NF-$\kappa$B p65 subunit or c-Jun with this enhancer site.

Taking all this information together, there are a number of ways that increased Fra-2 could up-regulate the levels of MHC class I heavy chain mRNA. Firstly, by directly acting on the MHC class I heavy chain gene promoter to enhance transcription. Secondly, Fra-2 may sequester c-Jun and therefore reduce the levels of c-Jun homodimers that are available to repress the expression of the gene. Finally, Fra-2 may interact with a protein that binds the $\alpha$ site (eg. JunB or JunD) to enhance transcriptional activation from this CRE regulatory element. Future investigations are required to determine if the increased MHC class I heavy chain mRNA levels are reflected in an increase in the cell surface expression of the MHC class I molecules. If so, then this may protect the animal from cellular transformation, by eliminating those cells that do become transformed due to an increase in the level of Fra-2 expression.

The expression of the MIF mRNA was shown to be up-regulated in the fra-2 transfected Swiss 3T3 fibroblasts compared to the non-transfected cells. In the fra-2 transgenic eye there was no significant difference in the expression levels of the MIF protein compared to the littermate control eyes. Interestingly, MIF is
known to be expressed in both the lens and cornea of the eye (Wistow et al., 1993).

The MIF protein was originally identified as a protein that inhibited macrophage migration and activated them at inflammatory loci. MIF is constitutively expressed in many cells and enhances the production of inflammatory cytokines such as TNF-α and IL-1. It may also play a role in cellular proliferation and differentiation (reviewed in Nishihira, 1998). A literature search did not reveal any evidence that Fra-2/AP-1 plays a role in the regulation of the MIF gene.

The cyclin D1-related clone mRNA was up-regulated in both the fra-2 transfected Swiss 3T3 fibroblasts and the fra-2 transgenic mouse eyes compared to the non-transfected cells and littermate control eyes, respectively, as shown by Northern and Virtual Northern analysis. This clone may be cyclin D1 or a cyclin D1-related gene. Cyclin D1 is an important regulator of cell cycle progression through the G1 phase (Sherr, 1993). Cyclin D1 expression is elevated in many human tumours including breast, prostrate and colon adenocarcinoma. Transgenic mice over-expressing cyclin D1 do show increased cell proliferation ((Mueller et al., 1997) and references within).

Interestingly, c-myc/TGFα transgenic mice show a strong induction of cyclin D1 mRNA and protein and have increased levels of cellular proliferation (Santoni-Rugiu et al., 1998). Increased cyclin D1 expression is also observed following activation of the Ras, MEK, ERK and SAPK pathways (Lui et al., 1995; Watanabe et al., 1996). The first three of these pathways are known to lead to increased Fra-2 expression (Mechta et al., 1997; Treines et al., 1999). The cyclin D1 promoter does contain an AP-1 site, which can induce cyclin D1 expression if bound by c-Jun (Albanese et al., 1995; Watanabe et al., 1996). In contrast, over-expression of c-Fos leads to a decrease in cyclin D1 levels (Albanese et al., 1995; Miao and Curran, 1994; Phuchareon and Tokuhisa, 1995). Recently, sustained MAPK activation has been shown to induce expression of Fra-1, Fra-2, c-Jun and
JunB (Cook et al., 1999). Sustained MAPK activation also leads to increased cyclin D1 expression, therefore combinations of these AP-1 proteins might regulate cyclin D1.

Taking all the above information together with the results obtained in this work, showing that Fra-2 is induced by TGFα and a cyclin D1-related mRNA was identified as an up-regulated species in cells over-expressing Fra-2, it seems likely that Fra-2 may positively regulate cyclin D1-related gene expression.

Whether this increased cyclin D1 expression had any physiological impact on the cells, particularly in the transgenic mice, still remains to be determined. In the fra-2 transfected cells there did not appear to be any gross increased proliferation rate, but this was not determined quantitatively. The cells were harvested too early following transfection to observe qualitative signs of increased proliferation. In the transgenic mice, development of tumours, which might indicate increased proliferation, has not occurred to date. Indeed, there does not appear to be any gross evidence of increased proliferation in these mice; in fact the opposite appears true, with a decrease in proliferation seen, for example, in the developing eyelid buds.

There are several plausible explanations for why this may be the case. Firstly, increased cyclin D1 mRNA levels may not actually correspond to an increase in cyclin D1 protein in all circumstances. Secondly, the increased levels of cyclin D1 in the transgenic mouse eye may have been localised and may not have been significant enough to cause an effect. Thirdly, the effects of Fra-2 on the expression of other proteins within the cells may have countered the effects of the increased cyclin D1 expression.

The A10 cDNA showed an increase in expression in the fra-2 transfected cells compared to the non-transfected cells. In contrast, A10 cDNA levels were decreased in the fra-2 transgenic eyes compared to the littermate control eyes. The
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cDNA clones showed homology to the murine A10 gene and to the rat phosphoglycerate dehydrogenase. Therefore, it may be that A10 and the clones identified here are the mouse homologue of the phosphoglycerate dehydrogenase mRNA. Interestingly, the A10 cDNA was identified by subtractive cloning of the murine erthyroleukemia DNA (Miller and Bieker, 1997).

Phosphoglycerate dehydrogenase is an enzyme that catalyses the first step in the pathway of serine formation from glycolytic intermediates (referenced in Achouri et al., 1997). This enzyme is expressed in most tissues and in many different organisms. Phosphoglycerate dehydrogenase is regulated at the level of transcription (Achouri et al., 1997). A search of the literature did not reveal any evidence to support or rule out a role for Fra-2/AP-1 in the regulation of this gene.

If Fra-2/AP-1 does not directly regulate the expression of the phosphoglycerate dehydrogenase gene, then the altered expression of this gene may be an indirect effect of changes in cellular processes occurring in the cells due to over-expression of Fra-2. Importantly, the changes in gene expression in the fra-2 transgenic eyes and the fra-2 transfected fibroblasts reflect different cellular processes taking place in the different circumstances. In the fra-2 transgenic eyes there was a switch from proliferation to differentiation. While in contrast, the fra-2 transfected fibroblasts were proliferating. Thus, the increased A10 expression in transfected cells and decreased A10 expression in transgenic eyes is further confirmation that different cellular programs were taking place in the eyes and the fibroblasts.

A similar expression pattern was observed for the ST2/T1 mRNA as for the A10 mRNA: increased levels in the fra-2 transfected cells and decreased levels in the fra-2 transgenic eyes. ST2/T1 is a receptor that is highly homologous to the IL-1 receptor (Mitcham et al., 1996) and is thought to play a role in inflammation (Kumar et al., 1997). It is a delayed early gene whose expression is induced by serum, cytokines and over-expression of v-mos and Ha-ras oncogenes ((Kumar et
al., 1997) and references within). ST2/T1 is the same gene as the *fos* responsive gene (Fit-1) (Bergers et al., 1994). The gene produces two forms of ST2/T1, a soluble (ST2) and membrane bound form (ST2L), which are produced by the use of alternative promoters (Bergers et al., 1994). The ST2/T1 gene contains an AP-1 site within its promoter region (Kalousek et al., 1994) that regulates the expression of ST2/T1 in murine non-hematopoietic cells in response to proliferation (Thomassen et al., 1995).

Despite ST2/T1 (or Fit-1) being identified as a *fos* responsive gene, there is no effect on ST2/T1 expression in c-*fos* knockout mice, indicating that other AP-1 members can also regulate the expression of this gene (Bergers et al., 1994). Analysis of the AP-1 complexes that can bind and activate transcription of the ST2/T1 gene showed that c-Fos and FosB were able to bind and that Fra-1 was not (Bergers et al., 1994). In this experiment they did not analyse the ability of Fra-2 to bind to the ST2/T1 promoter. However, a recent report using NIH3T3 fibroblasts with sustained expression of a ras oncogene led to decreased ST2/T1 activity (Kessler et al., 1999). These cells also showed repressed levels of c-Fos, FosB and Fra-2. Therefore Fra-2 may also regulate the expression of this gene.

Interestingly, the murine ST2/T1 gene is only expressed during organogenesis and neoplasia in non-hematopoietic tissue. Fra-2 is also expressed during organogenesis (Carrasco and Bravo, 1995). Therefore Fra-2 may be involved in the regulation of the expression of the ST2/T1 gene. Considering that ST2/T1 levels are increased in response to proliferation, the reduced levels observed in the eye may again reflect the lack of eyelid proliferation. The increased ST2/T1 levels in the *fra-2* transfected cells once again raises the question as to whether the *fra-2* transfected Swiss 3T3 cells actually had an increased proliferation rate. This requires further investigation.

SCD2 mRNA was up-regulated in the *fra-2* transfected cells and slightly down-regulated in the *fra-2* transgenic mouse eyes. SCD2 is an enzyme involved in the
synthesis of unsaturated fatty acids. It catalyses \( \Delta^\text{9} \)-cis desaturation of fatty acyl-CoA esterified with stearoyl CoA to oleic acid (Ntambi, 1995). There is another isoform of SCD called SCD1 and together these enzymes are important in regulating the ratio of monounsaturated to saturated fatty acids in the cell. Alterations in the ratio can lead to various diseases including cancer, diabetes, obesity and vascular disease (Ntambi, 1995).

Recently, the bHLH-ZIP protein sterol regulatory element-binding protein (SREBP) was shown to activate the transcription of SCD1 and SCD2 genes (Shimomura et al., 1998; Tabor et al., 1998). As mentioned in chapter 1, members of the AP-1 transcription family have been shown to interact with members of the bHLH-ZIP family eg c-Fos with FIP (Blanar and Rutter, 1992) and Fra-1 with USF (Pognonec et al., 1997). Therefore, Fra-2 could potentially interact with SREBP to regulate the expression of the SCD2 gene.

There are two other possibilities as to how Fra-2 could affect SCD2 expression. Firstly, Fra-2/AP-1 may directly regulate the expression of the SCD2 gene, although to date no AP-1 sites have been identified within the promoter of this gene. The second possibility is that alterations in SCD2 expression are an indirect effect of cellular changes that occur in the cells due to the over-expression of Fra-2. The decreased SCD2 levels in the transgenic mice may have led to reduced levels of fat stored in the cell and may therefore have contributed to the reduced weight observed in these mice.

G3BP mRNA was down-regulated in both the fra-2 transfected cells and the fra-2 transgenic mice eyes compared to their appropriate controls. G3BP is a cytosolic protein that binds the Ras-GTPase-activating protein (GAP), which is essential for Ras signalling (Parker et al., 1996). G3BP only associates with GAP when cells are proliferating (Parker et al., 1996). Recently G3PB has also been identified as the human DNA helicase VIII protein which can unwind RNA/DNA and RNA/RNA duplexes (Costa et al., 1999).
To date there is very little information known about the regulation of G3PB apart from its post-translational modification. AP-1 family members are downstream target genes of the Ras signalling pathway. Therefore, Fra-2/AP-1 could potentially regulate the expression of this gene via a negative feedback mechanism. However, further investigation into G3PB regulation is required to understand how changes in the expression of this gene can impact on the cell and what role Fra-2 may play in this.

Complement component C3 mRNA was down-regulated in both the fra-2 transfected cells and the fra-2 transgenic mouse eyes compared to their respective controls. The complement system is an immunological process involved in lysing microorganisms and infected cells. The complement system comprises a coordinated cascade of reactions in which the cleavage of the complement component C3 is a key event (Stryer, 1988). Complement component C3 is positively transcriptionally regulated by IL-β1 and TNFα in human epithelial cells and inhibited by IFN-γ (Moon et al., 1997). Recently the IL-β1 induction of Complement component C3 was shown to be regulated by NF-κB (Moon et al., 1999). Interestingly, AP-1 proteins, in complexes with other transcription factors, have been shown to regulate the expression of TNF-α and IFN-γ genes (reviewed in Foletta et al., 1998). For example, c-Jun can interact with NF-κB proteins to enhance transcription of the TNF-α gene. There are also NFAT sites within the promoters of both TNF-α and IFN-γ genes, which are regulated, at least in part, by AP-1 complexes.

Therefore increased Fra-2 levels may result in decreased complement component C3 mRNA levels by any or all of the following mechanisms:

(i) Fra-2 may directly negatively regulate the expression of the complement component C3 gene by binding to a site within the C3 gene promoter region or by antagonising the activity of the NF-κB protein.
(ii) Fra-2 may sequester an AP-1 protein, such as c-Jun, and decrease its availability to complex with other proteins, thereby reducing the activation of TNF-α and the subsequent induction in complement component C3.

(iii) Fra-2 may be involved in the AP-1 complex that activates the IFN-γ promoter leading to increased IFN-γ expression, which then leads to a reduction in complement component C3 levels.

The PCR-select differential screens described in this chapter have identified a number of potential target genes of Fra-2. The results also highlighted aspects of cellular function in which Fra-2 may play a role, particularly the regulation of proliferation and differentiation. Further investigations are required to determine how, or if, Fra-2 regulates the expression of these genes under physiological conditions.
Chapter 6

General Discussion
6.1 General Discussion

The studies presented in this thesis have highlighted several functional roles of Fra-2. A number of potential "downstream target genes" of Fra-2 have been identified and evidence has been presented which suggests that Fra-2 is regulated at both the level of transcription and translation. This chapter will focus on the in vivo significance of these findings and discuss the role of Fra-2 as a member of the AP-1 family of transcription factors. In addition, future experimental directions will be discussed.

One of the major findings of the studies that were undertaken concerns the regulation of Fra-2 itself. The amount of functional Fra-2 protein present in the cell may be regulated at all levels, namely transcription, post-transcription, translation and post-translation. Two of these forms of regulation were observed in analyses presented in this thesis and these will be discussed in more detail below.

At the level of transcription, fra-2 expression is controlled by many regulatory elements within its promoter region, including the AP-1 site identified within the mouse fra-2 gene promoter region (Foletta et al., 1994). The work presented here has shown that, at least under some circumstances, Fra-2 can negatively regulate the activity of its own promoter. This was evident in the transgenic stomach samples, which showed a decrease in the endogenous fra-2 mRNA levels coincident with the expression of the fra-2 transgene. This phenomenon was not observed in all tissues where the transgene and endogenous gene were shown to be co-expressed by Northern blotting of total RNA. As discussed in chapter 4, the apparent lack of down-regulation of the endogenous gene in some circumstances may reflect one of two possibilities. Firstly, the transgene and the endogenous gene may not have been expressed in the same sub-population of cells within the tissue. Secondly, even if the two genes were expressed in the same cell populations, there may not have been an appropriate protein partner for Fra-2
available within those cells to result in down-regulation of the endogenous gene. Negative auto-regulation by Fra-2 has been observed previously with the chicken Fra-2 protein (Sonobe et al., 1995).

The work presented in this thesis also suggested, for the first time, that Fra-2 might be regulated at the level of translation. The level of transgenic fra-2 RNA observed in many of the tissues of the fra-2 transgenic mice was not reflected in the level of Fra-2 protein detected in that tissue. This phenomenon appears to also occur with the endogenous fra-2 mRNA. For example, in the ovary high levels of fra-2 mRNA do not result in high levels of Fra-2 protein (Foletta, 1995). The precise mechanism for this control is yet to be identified, however, as discussed in the literature review in chapter 1, translational control generally occurs at the initiation stage of translation through the selection of the mRNA by the ribosomal complex and/or identification of the translation initiation codon. Future experiments may involve investigations into the role of initiation factors, such as eIF-2, in regulating the level of Fra-2 protein. This factor is known to be involved in the regulation of GCN4 translation (Hinnebusch, 1994); GCN4 is a yeast protein that is related to the mammalian AP-1 proteins.

In light of this high level of control on Fra-2 expression, it is not surprising that tumours were not observed in the fra-2 transgenic mice. In these animals the level of fra-2 mRNA was significantly increased in many tissues, but the level of Fra-2 protein remained mostly unchanged; therefore significant over-expression of Fra-2 was not, in fact, obtained. These results leave unanswered the question of whether Fra-2 is an oncogene or not. To be able to answer this question, future experiments need to bypass this translational control and achieve high levels of Fra-2 protein expression. Interestingly, increased Fra-2 levels have been observed in some tumours (Matsui et al., 1990). To date it is unclear as to whether increased Fra-2 levels are a cause or an effect of those tumours. If over-expression of Fra-2 is a cause of the tumour, then the analysis presented here has revealed that a key step in the oncogenic pathway must be the disabling of the translational
control mechanism acting upon *fra-2* mRNA to allow high Fra-2 protein expression. Thus, placing a high level promoter in front of a potential oncogene does not always result in oncogenic consequence; for some genes additional changes are required. Fra-2 is not the only AP-1 protein for which this is the case. The c-Fos protein requires the disabling of the post-transcriptional control mechanism that destabilises the *c-fos* mRNA. Hence, *c-fos* transgenic mice had the 3’ destabilising sequences removed to allow significant over-expression of the c-Fos protein (Grigoriadis et al., 1993; Rüther et al., 1987; Rüther et al., 1989).

Considering the multiple levels of regulatory mechanisms acting upon Fra-2, it seems that the cell expends considerable energy to keep the levels of Fra-2 within tight limits. This suggests that the cell cannot tolerate major fluctuations in the amount of Fra-2 protein. Furthermore, similar phenotypic consequences have been observed when *fra-2* levels are either reduced or increased. This was evident from comparisons between the results obtained with the *fra-2* transgenic mice reported here and the results obtained with *fra-2* anti-sense transgenic mice (Foletta, 1995). In the testes, a similar phenotype was observed in both types of transgenic animals at approximately 6 months of age (see chapter 4). The results from the analysis on IBD tissue samples presented in chapter 2 also showed that tissues from some diseased patients had increased *fra-2* levels, while tissues from other diseased patients had decreased *fra-2* levels.

How can an increase and a decrease in Fra-2 levels both produce the same outcome for a cell? A scheme for how such an effect is possible was discussed in chapter 4 and shown in figure 4.27. Such a scheme is plausible because Fra-2 is a member of a transcription factor family in which the members compete for binding to each other and the resulting complexes compete for binding to the same sites. Therefore, the many forms of regulation on the expression of the *fra-2* gene may play an important role in maintaining the correct level of Fra-2 relative to other family members. Under normal circumstances the negative auto-regulation and translational control mechanisms are activated in response to
increasing Fra-2 levels and deactivated in response to decreasing Fra-2 levels. In the case of the fra-2 transgenic mice, an increase in fra-2 mRNA may have led to an increase in Fra-2 protein, which then activated the negative auto-regulation and translational control mechanisms. The auto-regulation mechanism was unable to function on the fra-2 transgene, as the transgene was under the control of the constitutive CMV promoter, not the fra-2 promoter. The negative auto-regulation mechanism may have functioned on the endogenous fra-2 gene, as seen in the stomach, but this could only occur in those cells where the transgene and endogenous fra-2 genes were co-expressed, as discussed above. With the failure of the transcriptional control mechanism, the translational control mechanism provided the necessary backup required, keeping the levels of Fra-2 protein within an acceptable limit.

Despite this high level of control on the expression of Fra-2, there were some phenotypes observed in the transgenic mice over-expressing fra-2. Phenotypes were observed in the testes and eyes and there was a reduction in overall body weight. In these circumstances, the translational control mechanism must have been absent or bypassed by the cell.

In the testes of male transgenic mice aged approximately 6 months, a degeneration of the cells within the seminiferous tubules was observed. The tubules from transgenic mice showed a reduction in the total number of cells present, but not in the relative number of spermatogenic cell types, when compared to littermate control mice (refer to chapter 4). As mentioned above, a similar phenotype was observed in the anti-sense fra-2 transgenic mice (Foletta, 1995).

The disruption of spermatogenesis in transgenic mice did not occur until approximately 6 months of age. The reason for the latency in phenotype development may be attributed to a gradual build up in transgene expression, so that with time the levels (of either sense or anti-sense fra-2 RNA) may have become significant enough to produce an effect in the tissue. The effects on the
testes may have resulted from disrupted regulation of the expression of some testes specific genes. For example, Fra-2/AP-1 could potentially be involved in the regulation of testosterone gene expression in Leydig cells. Fra-2/AP-1 may also be involved in regulating other genes involved in the signalling pathways controlling spermatogenesis, such as CRISP-1 and Tesk1.

The major observable phenotype in the transgenic mice was an eye abnormality, anterior chamber dysgenesis. Anterior chamber dysgenesis is characterised by the lack of endothelial and Descemet’s membranes of the cornea and the absence of an anterior chamber of the eye. Histological analysis of fra-2 transgenic embryos showed that one of the early steps in the development of the eye abnormality was the failure of the eyelid to fuse. This was first evident at embryonic day 15.5. The eyelids of the transgenic mice never developed and the transgenic mice were born with their eyes open.

Interestingly, unlike the testis and the IBD examples discussed above, where increased and decreased Fra-2 levels produced the same phenotype, the fra-2 anti-sense mice did not show the eye abnormality that was present in the transgenic mice over-expressing fra-2. There are two possibilities why this may be the case. Firstly, the anti-sense fra-2 transgene may not have been expressed in the same cells in the eye as the sense fra-2 transgene. Secondly, it would have taken some time for the anti-sense fra-2 levels to become high enough to significantly decrease the level of Fra-2 protein (Foletta, 1995); thus, the decreased Fra-2 level would have occurred too late to have any effect on the development of the eye. Thus, the absence of the eye abnormality in the anti-sense transgenic mice does not contradict the hypothesis being developed to explain how increases and decreases in Fra-2 expression can both produce the same phenotype.

A search of the literature revealed that TGFα transgenic (Decsi et al., 1994; Reneker et al., 1995) and knockout (Luetteke et al., 1993; Mann et al., 1993) mice also developed anterior chamber dysgenesis. This information suggested a link
between Fra-2 and TGFα. *In vitro* studies showed that *fra-2* expression could be induced in response to TGFα stimulation (see chapter 5). Thus, the anterior chamber dysgenesis observed in TGFα transgenic and knockout mice may have resulted from alterations in the level of Fra-2 protein, such that over-expression TGFα led to increased Fra-2 levels and complete ablation of functional TGFα led to reduced Fra-2 levels. Thus, the same phenotype being observed with both increased and decreased TGFα expression can be explained by the same mechanism as applies to Fra-2 – namely, disruption of the balance of AP-1 family members – since TGFα signalling appears to be mediated by Fra-2.

As mentioned above, the eyelids of the transgenic mice failed to fuse. This appeared to result from a lack of proliferation of the eyelid buds (see chapter 4). Therefore, it was postulated that Fra-2 might play an anti-proliferative role in this circumstance. The reduced weight and generalised reduced size of the *fra-2* transgenic mice also supports this hypothesis. Interestingly, the pattern of gene expression changes determined by the PCR-select differential screen performed on the E15.5 mouse eyes from transgenic and littermate control animals suggested that Fra-2 might induce differentiation. An increase in globin gene expression and a decrease in EF-Tu gene expression were observed and these changes are associated with differentiation (Ganguly and Skoultchi, 1985; Roth et al., 1987). Decreases in the levels of cyclophilin, tubulin and LLRep3 (or S2 ribosomal protein) mRNAs may be a consequence of this induction of differentiation. Thus, the altered levels of Fra-2 in the eye may have switched the signal from a proliferation signal to a differentiation signal.

In support of the idea that Fra-2 function may be linked to the cellular switch from proliferation to differentiation, *fra-2* is known to be expressed during monocyte-macrophage differentiation (Matsui et al., 1990). AP-1 proteins, including Fra-2 have also been shown to regulate differentiation of human keratinocytes (Gandarillas and Watt, 1995; Rutberg et al., 1997; Rutberg et al., 1996; Welter
and Eckert, 1995). Furthermore, Fra-2 and JunB are known to be expressed at the onset of keratinocyte differentiation (Gandarillas and Watt, 1995) and this complex predominates in the granular layer of the skin (Rutberg et al., 1996). Interestingly, Fra-2 has been shown to induce keratinocyte differentiation by inhibiting transcription of certain genes (Rutberg et al., 1997). Thus, Fra-2 may act as a negative transcriptional regulator to induce differentiation. Other reports have also shown that Fra-2-JunB heterodimers can inhibit transcriptional activation (Sonobe et al., 1995; Suzuki et al., 1991). Expression of Fra-1 and Fra-2 proteins is also induced by phenolic anti-oxidants and results in anti-tumour promotion activity by repressing AP-1 activity (Yoshioka et al., 1995).

The PCR-select differential screen procedures identified some potential target genes of Fra-2 that were up-regulated and other potential target genes that were down-regulated. Northern and Virtual Northern blots were used to independently confirm the results of the differential screens. In some cases, opposite effects were observed for the expression levels of the potential target genes in the fra-2 transfected fibroblasts and fra-2 transgenic eyes, e.g. A10 and ST2. These genes were both up-regulated in the fra-2 transfected fibroblasts and down-regulated in the fra-2 transgenic eyes. As discussed in chapter 5, the expression of most genes is regulated by many transcription factors. Different combinations of transcription factors binding to a promoter can produce different transcriptional outcomes for that gene. Under different cellular circumstances, the array of transcription factors present and functionally available for regulating the expression of any given gene changes. Therefore, the opposing effects observed in the fra-2 transgenic eyes and fra-2 transfected fibroblasts may be attributed to the different array of transcription factors present and acting upon the promoters of the specific genes in these circumstances.

In support of the idea that different arrays of transcription factors were present and functioning within the two populations of cells, the results obtained with the two PCR-select differential screens suggested that the cells were undergoing different
cellular programs. As mentioned above, the majority of changes in gene expression in the E15.5 mouse eyes were involved in differentiation. In contrast, the results from the PCR-select differential screen with the fra-2 transfected and non-transfected Swiss 3T3 fibroblasts showed that the majority of changes in gene expression were involved with proliferation. The proliferation rate of the fra-2 transfected cells compared to the non-transfected cells was not determined due to the nature of the transfection experimental conditions and there were no obvious phenotypic signs of increased proliferation observed. Future experiments allowing the cells to grow for longer periods of time might help to determine if any changes in proliferation rates were occurring in the fra-2 transfected cells.

In support of the idea that Fra-2 does lead to increased proliferation in fibroblasts, both mouse and chicken Fra-2 have been shown to transform CEF (Foletta et al., 1994; Nishina et al., 1990). Fra-2 levels are also elevated or Fra-2 protein is hyperphosphorylated in a number of tumours and tumour cell lines (Matsui et al., 1990; Urakami et al., 1997) and in cells transformed by other oncogenes (eg. ras, v-src, raf) (Mechta et al., 1997; Murakami et al., 1997; Suzuki et al., 1994). In fact, in v-src and activated c-raf cellular transformation, the predominant AP-1 complex comprises c-Jun and Fra-2 (Suzuki et al., 1994).

In contrast, there are also cases where cellular transformation has been shown to have reduced Fra-2 expression levels, for example cellular transformation by the Rel family of proteins (Kralova et al., 1998). However, in these cells c-Fos levels are elevated. In Ha-ras and Ki-ras transformed NIH3T3 clones the predominant AP-1 DNA binding activity is from c-Jun-Fra-1 complexes (Mechta et al., 1997). This suggests that different oncogenes use different mechanisms to alter different AP-1 members, but all ultimately result in cellular transformation. This also supports the idea that it is the balance and interplay between all the AP-1 proteins that is vital for normal cellular function.
The corollary of this hypothesis – i.e. that it is the relative levels of Fra-2 compared to the other Fos family members which is important, rather than the absolute level of Fra-2 – is that the same principle should apply to other AP-1 proteins. Thus, it is the correct balance of the various AP-1 complexes that ultimately results in the correct gene expression. In this context, it is important to remember that under different circumstances the repertoire of AP-1 proteins expressed differs and results in different permutations of AP-1 complexes. Therefore, it depends on which repertoire of AP-1 proteins is being expressed as to whether changes in the level of expression of a particular AP-1 member will have any cellular effect. Changes in the relative levels of some AP-1 members may be compensated for by other family members, for example c-Fos knockout mice did not show any phenotypic consequences in the majority of tissues despite the complete ablation of c-Fos expression (Hu et al., 1994; Jain et al., 1994; Johnson et al., 1992; Wang et al., 1992). In this case, other AP-1 members must have been able to compensate for the lack of c-Fos expression.

In support of the idea that distinct patterns of AP-1 complexes function in particular circumstances, many systems have been shown to have ordered and precise patterns of AP-1 family member expression. For example, in the cell cycle c-Jun, JunD, and Fra-2 are expressed at high levels in cycling cells and are only weakly induced by serum. Jun B, c-Fos, FosB and FosB-SF are expressed at low levels in cycling cells and are strongly induced following serum stimulation. Fra-1 is absent from cycling cells and acts as a delayed early response protein in response to serum stimulation (Lallemand et al., 1997). This analysis by Lallemand et al. demonstrated that different AP-1 complexes are present throughout different stages of the cell cycle. Kovary and Bravo have also shown that inhibiting individual members of both the Fos and Jun proteins in fibroblasts disrupts the progression through the cell cycle, also suggesting a coordinated role for the AP-1 proteins (Kovary and Bravo, 1991).
Consequently, it might not be appropriate to consider the function of Fra-2—and its ability to act as either a tumour promoter or a tumour suppressor—in isolation. Depending upon the available protein partners and the relative levels of other competing and cooperating transcription factors, Fra-2 may produce a range of gene expression (and therefore, phenotypic) consequences for the cell.

The AP-1 transcription factor family are known to be regulators of immune development and function (reviewed in Foletta et al., 1998). AP-1 has roles in T-cell and B-cell activation, cytokine regulation, and immunoglobulin production. Fra-2 has previously been shown to contribute to the signalling pathways in some of these systems. The analyses presented in chapter 5 identified three genes that have roles in immune responses and whose expression was altered by increased Fra-2 expression, namely MHC class I heavy chain, complement component C3 and MIF. These molecules are involved in the cytotoxic immune response, the innate immune response and inflammatory responses, respectively. Thus, Fra-2 might be a regulator of immune responses.

One of the first steps in T-cell activation is the presentation of foreign antigens to the T-cell by the MHC class I molecules. The cytotoxic T-cell response signals the T-cells to die. The death of the cell can signal, via MIF, to recruit macrophages to the site of death, which assist in removing the dead cell. The role of Fra-2 in regulating the expression of the MHC class I molecule and MIF proteins, proteins associated with T-cell activation, may act as a protective mechanism for the cell by activating the adaptive immune responses. Furthermore, the fact that Fra-2 down-regulated the C3 complement component suggests that Fra-2 may switch off the non-specific innate immune responses. Future experiments will involve determining if the levels of MHC molecules are increased on the cell surface and whether this does lead to increased cytotoxic T-cell activity. The role of Fra-2/AP-1 complexes in the innate immune system also requires further investigation.
In addition to the genes already mentioned, the work presented here suggests Fra-2 may be involved in the regulation of crystallin (particularly the γ-crystallins), G3BP and SCD2 gene expression. Future experiments will involve investigation into how Fra-2 regulates expression of these genes and the *in vivo* significance of this regulation.

The experimental studies reported in this thesis have highlighted a number of interesting aspects of Fra-2 function. Two controls on Fra-2 expression, namely negative auto-regulation of transcription and translational regulation, were identified. The high level of control acting upon Fra-2 meant that the *fra-2* transgenic mice were unable to achieve high levels of Fra-2 protein expression. Hence, the question of the oncogenic potential of Fra-2 remains unanswered. In addition, this work has shown, at least *in vitro*, that Fra-2 is downstream of TGFα in the signalling pathway. A number of putative downstream target genes for Fra-2 were also identified, including cyclin D1, MHC class I heavy chain, EF-Tu and β-globin. The alterations in gene expression of the potential target genes have indicated areas where Fra-2 may play a functional role. Fra-2 may be involved in regulating the signals that control proliferation and differentiation and may also function as a regulator of immune responses. Finally, a consistent theme throughout this thesis has been that for the AP-1 transcription factor family, the *relative* levels of each AP-1 protein may be more important than the *absolute* levels of the individual family members.
APPENDICES
APPENDIX A: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>A</td>
<td>Adenine</td>
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<tr>
<td>ala</td>
<td>alanine</td>
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<tr>
<td>AF-2</td>
<td>activation function 2</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>ATF-2</td>
<td>activating transcription factor-2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>bZIP</td>
<td>basic DNA binding-leucine zipper</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>°C</td>
<td>degrees centigrade</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
</tr>
<tr>
<td>CBF</td>
<td>CCAAT-binding transcription factor</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy deoxyribonucleic acid</td>
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<tr>
<td>CEF</td>
<td>chicken embryo fibroblasts</td>
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<tr>
<td>CI</td>
<td>chloroform:isoamyl alcohol</td>
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<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
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<tr>
<td>CKII</td>
<td>casein kinase II</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CRD</td>
<td>cytoplasmic retention domains</td>
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<tr>
<td>CRE</td>
<td>cAMP responsive element</td>
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<tr>
<td>CREB/ATF</td>
<td>CRE binding protein/ activating transcription factor</td>
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CREM  CRE motif
CTD  carboxy-terminal repeat domain
C-terminus  carboxy-terminus
CTF  CCAAT-box-binding factor
CTP  cytosine triphosphate
δ  delta
DBD  DNA binding domain
DMEM  Dulbecco’s modification of Eagle’s medium
DNA  deoxyribonucleic acid
dNTP  deoxyribonucleotidyl phosphate
DPE  downstream promoter element
DSS  dextran sulfate sodium
DTT  dithiothreitol
E14.1  129 ES cells
E8  embryonic day 8
E15.5  embryonic day 15.5
E. coli  Escherichia coli
EDTA  ethylenediaminetetraacetic acid
EF-Tu  elongation factor Tu
EGFR  epidermal growth factor receptor
EGTA  ethylene glycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ER  estrogen receptor
ERK  extracellular signal-regulated kinases
ES  embryonic stem
Exo III  exonuclease III
FBJ-MSV  Finkel-Biskis-Jinkins murine osteosarcoma virus
FBR-MSV  Finkel-Biskis-Reilly murine osteosarcoma virus
FCS  fetal calf serum
FIP  Fos interacting protein
FITC  fluorescein isothiocyanate
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<td>ying yang-l</td>
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APPENDIX B: Solutions

A1 Buffer
(JETSORB, Genomed)
contains the following reagents but refer to manufacturer for absolute amounts
NaClO₄
TBE-stabilizer
Sodium acetate

A2 Buffer
(JETSORB, Genomed)
contains the following reagents but refer to manufacturer for absolute amounts
NaCl
EDTA
Tris-HCl

Acrylamide/bis stock (30%)
(premixed 29:1, BioRad)
29% acrylamide
1 % N-N’-methylene-bis-acrylamide

Acrylamide/bis stock (40%)
(premixed 19:1, BioRad)
38% acrylamide
2 % N-N’-methylene-bis-acrylamide

1 X Advantage cDNA polymerase mix
(CLONTECH)
0.1 µg/ml KlenTaq-1 DNA polymerase
1.1 µg/ml TaqStart Antibody
1% glycerol
0.8 mM Tris-HCl, pH 7.5
1 mM KCl
0.5 mM ammonium sulphate
2 µM EDTA
0.1 mM β-mercaptoethanol
0.005% thesit

1 X Advantage PCR reaction buffer
(CLONTECH)
40 mM Tricine-KOH, pH 9.2
15 mM potassium acetate
3.5 mM magnesium acetate
75 µg/ml bovine serum albumin
1 X AMV Reverse transcriptase buffer
(Promega)
50 mM Tris-HCl, pH 8.3
50 mM KCl
10 mM MgCl₂
10 mM DTT
0.5 mM spermidine

BIND solution
(CLONTECH)
Solution contents not available, refer to manufacturer

Blocking solution
10 mg/ml sheared salmon sperm DNA
0.3 mg/ml oligonucleotides corresponding to the nested primers and complementary sequences

Bouin’s solution
75 ml picric acid (saturated aqueous solution)
25 ml formalin (37-40% stock)
5 ml glacial acetic acid

Buffer P1
(QIAGEN)
100 µg/ml RNase A
50 mM Tris-HCl, pH 8.0
10 mM EDTA, pH 8.0

Buffer P2
(QIAGEN)
200 mM NaOH
1 % SDS

Buffer P3
(QIAGEN)
3M potassium acetate

Carnoy’s solution
60% ethanol
30% chloroform
10% glacial acetic acid

Cell lysis solution
(Wizard miniprep kit, Promega)
200 mM NaOH
1% SDS
Cell neutralization solution
(Wizard miniprep kit, Promega)
1.32 M potassium acetate, pH 4.8

Cell resuspension solution
(Wizard miniprep kit, Promega)
50 mM Tris-HCl, pH 7.5
10 mM EDTA
100 μg/ml RNase A

Column wash buffer
(Wizard miniprep kit, Promega)
100 mM NaCl
10 mM Tris-HCl, pH 7.5
2.5 mM EDTA
47.5% ethanol

1 X Cracking buffer
100 mM NaOH
10 mM EDTA, pH 8
1 % (w/v) SDS
0.05 % (w/v) bromocresol green
10 % (v/v) glycerol

Denaturing solution
0.5 M NaOH
1.5 M NaCl

1 X Denhardt’s solution
0.2% ficoll 400 (Sigma)
0.2% polyvinylpyrrolidone (Sigma)
0.2% BSA

Dilution buffer
(CLONTECH)
20 mM HEPES-HCl, pH 8.3
50 mM NaCl
0.2 mM EDTA, pH 8

1 X Dissociation buffer
10% glycerol
50 mM Tris-HCl, pH 6.8
2% SDS
5% 2-β-mercaptoethanol (Sigma)
0.1% bromophenol blue
DMEM medium
2.7 g DMEM (Sigma)
0.4 g Sodium bicarbonate (Sigma)
100 μg/ml penicillin
100 μg/ml streptomycin
200 μg/ml neomycin
make up to 200 ml with distilled water

E14.1 ES media
15% FCS (HYCLONE)
1 mM sodium pyruvate (GibcoBRL)
1% non-essential amino acids (GibcoBRL)
100 μM 2-β-mercaptoethanol (GibcoBRL)
10,000 units penicillin/streptomycin (GibcoBRL)
200 mM L-glutamine (GibcoBRL)
DMEM
2000 units/ml LIF

20 X EDTA/glycogen mix
(CLONTECH)
0.2 M EDTA
1 mg/ml glycogen

Elution buffer
(Pharmacia)
HCl, pH 7.5
1 mM EDTA

End-labeling hybridization buffer
5 X SSC
10 X Denhardt’s solution
0.1% SDS
100 μg/ml salmon sperm DNA

1 X Exonuclease III buffer
(Erase-a-base kit, Promega)
66 mM Tris-HCl, pH 8
0.66 mM MgCl₂

1 X First strand buffer
(CLONTECH)
50 mM Tris-HCl, pH 8.5
8 mM MgCl₂
30 mM KCl
1 mM DTT
Glycogen solution
(Pharmacia)
5-10 mg/ml glycogen in DEPC-treated water

GTE buffer
50 mM glucose
25 mM Tris-HCl, pH 8.0
10 mM EDTA

10X Hanks BSS #1
200 g sodium chloride
5 g potassium chloride
1.25 g magnesium sulphate
1.25 g magnesium chloride
1.75 g calcium chloride
12.5 g glucose
distilled water up to 1 litre.

10X Hanks BSS #2
1 g phenol red
3.9 g disodium hydrogen orthophosphate
3 g potassium dihydrogen orthophosphate

Hanks solution
Mix 10X Hanks BSS #1 with 10X Hanks BSS #2 in a 4:1 ratio,
then dilute 1:2 with distilled water.

HBS buffer
20 mM Hepes, pH 7.4
NaCl

High-Salt buffer
(Pharmacia)
10 mM Tris-HCl, pH 7.5
1 mM EDTA
500 mM NaCl

1X Hybridisation solution
0.75 M NaCl
0.2% SDS
1% glycine
50 mM NaH₂PO₄, pH 7.0
25 mM EDTA
1X Denhardt’s solution
5 X IMF buffer (or Coon’s buffer)
8.5 g disodium hydrogen orthophosphate
87.0 g NaCl
5.4 g potassium dihydrogen orthophosphate
2000 ml distilled water
adjust pH to 7.1-7.3 with NaOH

1 X Injection buffer
10 mM Tris-HCl, pH 7.4
0.1 mM EDTA

8% Instagel
8% acrylamide: acrylamide bis solution (from 40% acrylamide/bis stock)
8 M Urea
1 X TBE buffer

Klenow buffer
(Erase-a-base kit, Promega)
20 mM Tris-HCl, pH 8
100 mM MgCl₂

Laemmli buffer
25 mM Tris-HCl, pH 8.3
250 mM glycine
0.1% SDS

LB (urea broth)
1% bacto-trypstone (Difco)
0.5% bacto-yeast extract (Difco)
1% NaCl

LB Agar
LB
1.5% bacto-agar (Oxoid)
10 mM MgSO₄ diluted in LB

LiCl/ Urea Solution
3 M lithium chloride
6 M Urea

1 X Ligase buffer
(Erase-a-base kit, Promega)
50 mM Tris-HCl, pH 7.6
10 mM MgCl₂
1 mM ATP
Ligase mix (prepared fresh)  
(Erase-a-base kit, Promega)  
1 X ligase buffer  
5% (v/v) polyethylene glycol  
1 mM DTT  
5 units T4 DNA ligase  

1 X Loading dye  
0.04% bromophenol blue  
0.04% xylene cyanol  
5% glycerol diluted in TE buffer, pH 8.0  

Low-Salt buffer  
(Pharmacia)  
10 mM Tris-HCl, pH 7.5  
1 mM EDTA  
100 mM NaCl  

Lysis buffer  
100 mM Tris-HCl, pH 8  
5 mM EDTA  
0.2% SDS  
200 mM NaCl  

Michel’s buffer  
1 M potassium citrate  
0.1 M MgSO4  
0.1 M N-ethyl maleimide  

MLC medium  
DMEM  
2 mM glutamine  
1 mM sodium pyruvate  
10 mM HEPES buffer  
10% FCS  
0.1 mM 2-β-mercaptoethanol (Sigma)  
100 µg/ml penicillin  
100 µg/ml streptomycin  
200 µg/ml neomycin  

1 X MOPS buffer  
20 mM 3-(N-Morpholino)propanesulfonic acid  
1 mM EDTA  
5 mM sodium acetate
10% Neutral buffered formalin
10% formalin (37-40% stock)
4 g sodium phosphate
6.5 g sodium phosphate

Neutralising solution
0.5 M Tris-HCl, pH 8
1.5 M NaCl

1 X Nick translation buffer
(Promega)
50 mM Tris-HCl, pH 7.2
10 mM MgSO₄
0.1 mM DTT

Nick translation enzyme mix
(Promega)
1 unit/μl DNA polymerase I
0.2 ng/μl DNase I
50% glycerol
0.5 mg/ml nuclease-free BSA
1 X NICK translation buffer

1 X One-Phor-All buffer
10 mM Tris-acetate, pH 7.5
10 mM magnesium acetate
50 mM potassium acetate

1 X PCR Reaction buffer IV
(Advanced Biotechnologies)
20 mM ammonium sulphate
75 mM Tris-HCl, pH 8.8
0.01% (v/v) Tween-20

PCR-select stop solution
0.1 M EDTA
500 μg/ml yeast tRNA

PI/RNase solution
40 μg/ml propidium iodide
10 μg/ml RNase A
in PBS
1 X Pre-hybridisation solution
0.5 M NaCl
0.2% SDS
1% glycine
50 mM NaH₂PO₄, pH 7.0
5 mM EDTA
5 X Denhardt’s solution

Protein preparation Buffer A
10 mM Hepes, pH 7.9
10 mM KCl
0.1 mM EDTA
0.1 mM EGTA
1 mM DTT
0.5 mM PMSF

Protein preparation Buffer C
20 mM Hepes, pH 7.9
400 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM DTT
1 mM PMSF

QBT Buffer pH 7
(QIAGEN)
750 mM NaCl
50 mM MOPS
15% ethanol
0.15% Triton X-100

QC Buffer pH 7
(QIAGEN)
1 M NaCl
50 mM MOPS
15% ethanol

QF Buffer pH 8.5
(QIAGEN)
1.25 M NaCl
50 mM Tris-HCl
15% ethanol
Random primer mix
0.9 mg/ml Random nonamers
50 mM Tris-HCl, pH 7.5
10 mM MgCl$_2$
1 mM DTT
50 µg/ml BSA

1 X Reaction buffer (-dCTP)
(CLONTECH)
333 mM Tris-HCl, pH 8
33.3 mM MgCl$_2$
10 mM 2-mercaptoethanol
170 µM dATP
170 µM dGTP
170 µM dTTP

1 X Restriction enzyme buffer #1, pH 7.0
(New England BioLabs)
10 mM Bis Tris Propane-HCL
10 mM MgCl$_2$
1 mM DTT

1 X Restriction enzyme buffer #2, pH 7.9
(New England BioLabs)
10 mM Tris-HCl
10 mM MgCl$_2$
50 mM NaCl
1 mM DTT

1 X Restriction enzyme buffer #3, pH 7.9
(New England BioLabs)
50 mM Tris-HCl
10 mM MgCl$_2$
100 mM NaCl
1 mM DTT

1 X Restriction enzyme buffer #4, pH 7.9
(New England BioLabs)
20 mM Tris-acetate
10 mM magnesium acetate
50 mM potassium acetate
1 mM DTT
RIPA buffer
10 mM Tris-HCl, pH 7.5
150 mM NaCl
1 mM EDTA
1% NP40
0.1% SDS
2.3 µg/ml Aprotinin
100 µg/ml Leupeptin
0.1 mM PMSF

RNA gel
0.8% agarose
1 X MOPS buffer
2.2 M formaldehyde

RNA gel running buffer
1 X MOPS buffer
2.2 M formaldehyde

RNA sample buffer
1 X MOPS buffer
50% formamide
2.2 M formaldehyde

1 X Rsa I restriction buffer
(COLONTECH)
10 mM Bis Tris propane-HCl, pH 7
10 mM MgCl₂
0.1 mM DTT

SALT solution
(COLONTECH)
Solution contents not available, refer to manufacturer

SDS buffer
10 mM sodium acetate pH 4.8
0.5% SDS
1 mM EDTA
0.1% DEPC

SDS polyacrylamide running gel
(X = the % gel required)
X% acrylamide: acrylamide bis solution (from 30% acrylamide/bis stock)
0.125 M Tris-HCl, pH 6.8
0.1 % SDS
0.001% PMSF (Sigma)
SDS polyacrylamide stacking gel
5.5% acrylamide: acrylamide bis solution (from 30% acrylamide/bis stock)
0.375 M Tris-HCl, pH 8.8
0.1 % SDS
0.001% PMSF (Sigma)

20 X Second strand enzyme cocktail
(CLONTECH)
6 units/µl DNA polymerase I
0.25 units/µl RNase H
1.2 units/µl DNA ligase

1 X Second strand buffer
(CLONTECH)
100 mM KCl
10 mM Ammonium sulphate
5 mM MgCl₂
0.15 mM β-NAD
20 mM Tris-HCl, pH 7.5
0.05 mg/ml BSA

Sequencing casting tray gel
8 % instagel
0.5% TEMED (BioRad)
0.124% Ammonium persulphate (BioRad)

Sequencing running gel
8 % instagel
0.038% TEMED (BioRad)
0.054% Ammonium persulphate (BioRad)

1 X SMART first strand buffer
(CLONTECH)
50 mM Tris-HCl, pH 8.3
75 mM KCl
6 mM MgCl₂

7.4 X S1 nuclease buffer
(Erase-a-base kit, Promega)
300 mM potassium acetate, pH 4.6
25 mM NaCl
10 mM ZnSO₄
50% glycerol
20 X SSC (1 litre)  
175.3 g NaCl  
88.2 g Na Citrate  
Adjust pH to 7.0 with NaOH.

**S1 Stop buffer**  
(Erase-a-base kit, Promega)  
300 mM Tris base  
50 mM EDTA

**STET buffer**  
8% sucrose  
0.5% Triton-X 100 (Sigma)  
50 mM EDTA, pH 8.0  
10 mM Tris-HCl, pH 8.0

**1 X SuperTag buffer**  
(P. H. Stehelin and CIE Ag, Basel)  
10 mM Tris-HCl, pH 9.0  
50 mM KCl  
0.01% gelatin  
1.5 mM MgCl₂  
0.1% Triton-X

**1 X TAE buffer**  
40 mM Tris-acetate  
1 mM EDTA

**1 X TBE buffer**  
90 mM Tris-borate  
2 mM EDTA

**TE Buffer**  
10 mM Tris-HCl pH 8.0  
1 mM EDTA

**TEN9 buffer**  
50 mM Tris-HCl, pH 9.0  
100 mM EDTA  
200 mM NaCl  
100 µg/ml RNase A
1 X T4 DNA ligase buffer
50 mM Tris-HCl, pH 7.8
10 mM MgCl₂
10 mM DTT
1 mM ATP
50 μg/ml BSA

1 X T4 DNA Polymerase buffer
33 mM Tris-HCl, pH 8
66 mM Potassium acetate
10 mM Magnesium acetate
0.5 mM DTT

T7 DNA polymerase dilution buffer
(Pharmacia)
20 mM Tris-HCl, pH 7.5
5 mM DTT
100 μg BSA/ml
5% glycerol

1 X TNE buffer
10 mM Tris-HCl, pH 8
10 mM NaCl
0.1 mM EDTA

1 X T4 PNK buffer
(New England BioLabs)
70 mM Tris-HCl, pH 7.6
10 mM MgCl₂
5 mM DTT

1 X Transcription buffer
(Promega)
40 mM Tris-HCl, pH 7.9
6 mM Mg₂Cl₂
10 mM DTT
10 mM NaCl
2 mM Spermidine
0.05% Tween-20

0.025% Trypsin
500 ml PBS
5 ml 2.5% trypsin stock
6.25 ml 0.2% phenol red
1.37 ml 100mM EDTA, pH 7.2
T7 sequencing Annealing buffer
(Pharmacia)
1 M Tris-HCl, pH 7.6
100 mM MgCl₂
160 mM DTT

T7 sequencing dATP short mix
(Pharmacia)
840 µM dCTP
840 µM dGTP
840 µM dTTP
93.5 µM dATP
14 µM ddATP
40 mM Tris-HCl, pH 7.6
50 mM NaCl
Note: Only difference for long mix is 2.1 µM ddATP

T7 sequencing dCTP short mix
(Pharmacia)
840 µM dATP
840 µM dGTP
840 µM dTTP
93.5 µM dCTP
17 µM ddCTP
40 mM Tris-HCl, pH 7.6
50 mM NaCl
Note: Only difference for long mix is 2.8 µM ddCTP

T7 sequencing dGTP short mix
(Pharmacia)
840 µM dCTP
840 µM dATP
840 µM dTTP
93.5 µM dGTP
14 µM ddGTP
40 mM Tris-HCl, pH 7.6
50 mM NaCl
Note: Only difference for long mix is 2.8 µM ddGTP
**T7 sequencing dTTP short mix**  
(Pharmacia)  
840 µM dCTP  
840 µM dGTP  
840 µM dATP  
93.5 µM dTTP  
14 µM ddTTP  
40 mM Tris-HCl, pH 7.6  
50 mM NaCl  
**Note:** Only difference for long mix is 2.8 µM ddTTP

**T7 sequencing labeling Mix-dATP**  
(Pharmacia)  
1.375 µM dCTP  
1.375 µM dGTP  
1.375 µM dTTP  
333.5 mM NaCl

**T7 Sequencing Stop solution**  
(Pharmacia)  
0.3% Bromophenol blue  
0.3% Xylene cyanol FF  
10 mM EDTA, pH 7.5  
97.5% deionized formamide

**WASH solution**  
(COLONTECH)  
Solution contents not available, refer to manufacturer
APPENDIX C: Oligonucleotide primers

Hfra2A  5'-CACGCCGAGTCCTACTCCAGCGGC-3'
Hfra2B  5'-CACCGTGAAGCCGCCCTGCAATGC-3'
Hfra2C  5'-CTCTTTCCAGGGGCTCCTGTTCAC-3'
ActinA  5'-ACACCTTCTACAATGAGC-3'
ActinB  5'-ACGTCACACTTCATGAGT-3'
F2Ex1   5'-GGATTATCCCGGGAACCTTTGACAC-3'
F2Ex3c  5'-GCCTGCAGCTTCTCTGTCAGCTC-3'
F2Ex3c.2 5'-TTTCGACGCTTTCTCCTCCTCCTCAG-3'
F2Ex2   5'-GCTCACAATCCCCTACAGTCCCCTGC-3'
F2Ex2c  5'-TCTCTCTCTCTGCGGCCCACGGTG-3'
NeoP6   5'-GCCAAGTTCTAATTCCATCAAGAC-3'
F2SD3   5'-AGTGGGAACAGTCACAGACAAGAC-3'
SP6     5'-TATTTAGGTGACACTATAG-3'
M13 primer 5'-GTAAACGCGAGGCAGT-3'
T7      5'-TAATACGACTCACTATAGGG-3'
F2int1.1 5'-TGAGGACGGGTTTTGGACTGGGAC-3'
F2int1.2 5'-CTTCTGAACTGCTGGACATGGGAG-3'
F2int2.3 5'-CATGAGGACCAAGCGGACTGCTTG-3'
F2int2.4 5'-TTCGAGCTGCTGGACATGGGAG-3'
Ex2NotI5' 5'-CGGTACCACCGGCGGCCAGAAG-3'
Ex2NotI3' 5'-CTTCTGCGGCCGCCGGTGAGTACCG-3'
NeoP1 5'-GCCGATTGTCTGTTGTGGTGCCCAG-3'
HygroM1 5'-GCTCTCGGAGGGCGAAGAATCTCG-3'
F2KOP1 5'-GAAGGAGAGATGAGCGAGTAAGCC-3'
SMART cDNA synthesis primer
5'-AAGCAGTGTAACAACGCAGTACT(30)N-1N-3'
(where N = A, G, C or T and N-1 = A, G or C)
SMART II oligonucleotide
5’-AAGCAGTGGTAACAACGCAGAGTAGCAGCGGG-3’
SMART PCR primer 5’-AAGCAGTGGTAACAACGCAGAGT-3’
cDNA synthesis primer 5’-TTTTGTACAAGCTT30-3’
Adaptor 1 5’-CTAATACGACTCACTATAGGCTGAGCGCGGCGGCC
GGGCAGGTGGCCCGTCCA-3’
Adaptor 2R 5’-CTAATACGACTCACTATAGGCGTGTCGCGGCGC
CGAGGTGCGCGCTCCA-3’
PCR primer 1 5’-CTAATACGACTCACTATAGGCGGCCCGGCGGCCAGTG-3’
Nested PCR primer 1 5’-TCGAGCGCGCCGGCCGGGCGGCGGCAGT-3’
Nested PCR primer 2R 5’-AGCGTGGGTGCGGGCGGAGGT-3’
Mitfpr1 5’-GTCTAGAGAACCCAGTAAAGCGAG-3’
Mitfpr2 5’-CTCGGCACCATGCGGAGAGCAG-3’
Pax6pr1 5’-GATCCGGAGGCTGCAAACAGACT-3’
Pax6pr2 5’-GTGACTAGGAGTGTTGCTGGCCTG-3’
mTGFpr3 5’-GTCCCCGCGACGAGCTCGGCT-3’
mTGFpr4 5’-GCCCTCGTCTGAGCAGATGGAAG-3’
G3PDH 3’ primer 5’-TCCACCACCCCTGTTGCTGTA-3’
G3PDH 5’ primer 5’-ACCACAGTCCATGCCCATC-3’
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