Mechanisms by which p53 suppresses cell transformation

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I declare that the work presented in this thesis is original research which has been completed independently except as acknowledged in the text.

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Sections of this thesis appear in the following articles:


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Abstract

Through the manipulation of cells in culture, the role of p53 in suppressing tumourigenesis has been demonstrated as an ability to suppress the transformation of cells by a number of viral and activated cellular oncoproteins. The mechanism by which transformation is suppressed probably involves reduced cell growth and/or increased cell death as p53 is known to arrest growth (predominantly in the G1 phase of the cell cycle) and to induce an apoptotic response. However the relationship of these functions to the ability of p53 to suppress oncogenic transformation remains unclear.

While p53 can suppress transformation by various oncoproteins, studies in this thesis have demonstrated that it is unable to suppress the transformation of cells by the SV40 large T antigen (LTag). In the presence of LTag, p53 also failed to inhibit cell growth. Interestingly, the function of LTag which enabled it to overcome both the growth and transformation suppressing effects of p53 was not the binding (and probable inactivation) of p53, but the binding and inactivation of another tumour suppressing protein, pRb. These observations indicate that pRb is required for the mechanism by which p53 suppresses cell growth and transformation.

Although the adenovirus Ela proteins can also bind and inactivate pRb (and consequently overcome p53-dependent growth inhibition), their transformation-related activities were found to be suppressed by p53. Further experiments revealed that cells which express Ela are susceptible to p53-dependent apoptosis. While p53 was also able to suppress transformation involving an activated form of ras (EJ-ras), further experiments showed that this could be attributed to a growth inhibitory rather than apoptotic effect of p53. It can therefore be proposed that p53 suppresses transformation either by causing a growth arrest (which is mediated by pRb) or by inducing apoptosis, and that the mechanism of suppression is determined by the activities of the transforming oncoproteins. Transformation by LTag would remain refractory to suppression due to the abolition of both cellular effects of p53. However since the studies in this thesis have shown that transformation by LTag does not require the binding (and inactivation) of p53, an additional anti-apoptotic function must be associated with this oncoprotein.

It can also be proposed that the process of transformation usually requires cooperation between different oncoproteins (such as Ela and EJ-ras) in order to overcome the activities of endogenous p53. While transformation by EJ-ras is normally prevented due to p53-dependent (pRb-mediated) growth inhibition, Ela can overcome this effect. Furthermore, EJ-ras was shown to inhibit p53-dependent apoptosis which normally prevents transformation by Ela. However, this anti-apoptotic effect is probably limited to low levels of p53, as exogenous p53 can suppress transformation by Ela and EJ-ras.
Through the use of various mutant forms of p53, studies presented in this thesis have shown that within the p53 protein, the regions which are important for growth inhibition are different to those which are important for apoptosis. Thus, inhibition of cell growth and induction of apoptotic cell death are genetically separable functions of p53. Since the studies also demonstrated that p53-dependent growth inhibition is neither sufficient nor necessary for p53-dependent apoptosis, one can suggest that these are alternative rather than sequential functions for suppressing tumourigenesis. This finding is consistent with the observations (as mentioned above) that growth inhibition and apoptosis occur in response to different types of oncoprotein activity.

The activities of various mutant forms of p53 have also revealed that the growth inhibitory effect of p53 is neither sufficient nor necessary for the suppression of transformation by E1a and EJ-ras. However, the ability of a mutant p53 protein to suppress this type of transformation was shown to correlate well with its ability to induce apoptosis. These observations are in agreement with the prediction (as explained above) that transformation by E1a and EJ-ras would be suppressed by an apoptotic rather than growth inhibitory function of p53.

Because the mechanisms involved in growth inhibition and apoptosis are ambiguous, the studies presented in this thesis have examined the relationships between the biochemical and cellular activities of p53. Thus, various mutant forms of p53 were used to demonstrate the regions of the protein which are important for activation and repression of gene transcription as well as inhibition of SV40 origin-dependent DNA replication (as this might reflect a role for p53 in regulating cellular DNA replication).

Although there is considerable evidence that the transactivation of p21WAF1 is involved in p53-dependent growth arrest, studies in this thesis have shown that transcriptional activation is neither sufficient nor necessary for a growth inhibitory effect. It would therefore appear that p53 has more than one biochemical mechanisms for inhibiting cell growth and that only one of these pathways involves the transcriptional activation of p21WAF1. While transcriptional repression and inhibition of DNA replication were also found to be insufficient for growth inhibition, either of these biochemical activities may contribute to this cellular function of p53.

As independent functions, transcriptional activation, transcriptional repression, and inhibition of DNA replication were also found to be insufficient for p53-dependent apoptosis. However, the mechanism by which p53 induces apoptosis may require any of these activities and the combined effects of transcriptional activation and transcriptional repression may even be sufficient for this type of response.

In summary, the growth inhibitory and apoptotic activities of p53 have been shown to be separate and alternative functions which are important for suppressing cell transformation in response to different types of oncoproteins. The mechanisms by which these cellular functions occur appear to involve multiple biochemical activities of p53.
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Chapter 1

Molecular origins of cell transformation
1.1 Cellular changes in carcinogenesis

In a manner which is somewhat reminiscent of our own lifetime, most cell populations undergo an initial period of growth and reproduction then enter a state of rest and eventually die. After a time of proliferation, many cells become committed to a process of differentiation in which they cease to undergo cycles of growth and division, and adapt in various ways to perform a particular function within an organ. While cell proliferation is a prevalent activity in developing tissues, homeostasis must be maintained in adult tissues by the rate of cell proliferation being carefully balanced with the rate of cell differentiation and ultimately, the rate of cell death. On rare occasions when this balance is destroyed, tumours can develop from the unregulated proliferation of cells.

In most cases, the growth of tumours is slow and may cease altogether as there are only minimal changes to the processes which regulate cell proliferation. Because they remain localised to a particular area, these types of tumours are classified as benign. Malignant tumours (ie. cancers) on the other hand, contain rapidly proliferating cells which are able to invade the surrounding tissue. These tumours may also metastasise which means that their cells can break away, enter the bloodstream and / or lymphatic system, and subsequently proliferate in other parts of the body (Darnell et al., 1986). It is at this stage that the cancer becomes fatal. The development of a malignant tumour is an evolutionary process which involves multiple changes within the cells. Cancer cells differ from normal cells in that they have been transformed into a state of rapid and continual proliferation from which the options of cell differentiation and / or cell death are no longer possible.

Due to the obvious difficulties of studying cancers in their natural settings, the propagation of cells in culture has been used to identify the characteristics of cancer cells. The process of cell transformation whereby normal cells are converted into cancer cells, can be induced through various treatments of cells in culture. In contrast to normal cells whose growth patterns are regulated by their environment, transformed cells acquire some unusual growth properties (as explained below) and consequently form very dense clumps called 'foci' (as shown in Figure 1.1A) (reviewed in: Darnell et al., 1986). A focus is considered to be the in vitro equivalent of a tumour. The large number of cells contained in each focus is due not only to the rapid rate at which transformed cells grow and divide, but also to the absence of cell death. While immortalisation is an important part of cell transformation, it is possible for cells to be immortalised and remain untransformed, as is the case for many of the cell lines used in culture. The ability of transformed cells to grow in dense clumps is due in part to a reduced growth factor requirement. In contrast to normal cells, transformed cells do not stop growing when the access of growth factors is blocked. The disordered growth of transformed cells in dense clumps is also explained by the fact that these cells are no longer subject to an arrest of
growth which is induced by contact with other cells. Another factor which allows transformed cells to form foci is the ability of these cells to grow without the normal requirement of adherence to a smooth surface. In addition to the macroscopic characteristics of foci, microscopic examinations (as shown in Figure 1.1B) have revealed that cells within the foci are significantly different in appearance to the surrounding normal cells. The morphological changes which appear to depend upon the agent(s) of transformation, can be explained by alterations to the cytoskeleton of the cell. At the molecular level, other characteristics of transformed cells include changes in proteins on the cell surface and of course, changes in the transcription of genes in the nucleus.

The process of cell transformation occurs when proteins which normally regulate cell growth are functionally disrupted as a result of mutations within and around the genes encoding these proteins. Genetic abnormalities which either hyperactivate growth stimulating proteins or inactivate growth suppressing proteins commonly include: point mutations in DNA sequence; deletions within genes; chromosomal deletions; chromosomal duplications or amplifications (sometimes the result of aneuploidy); chromosomal rearrangements or translocations; and insertions of foreign (usually viral) DNA (reviewed in: Yunis, 1983). While such mutations can be inherited, they may also be somatic in origin as the genomic DNA is susceptible to damage by various environmental influences. These may take the form of: ultraviolet radiation; ionising radiation (such as x-rays and radiation from atomic particles); various chemical carcinogens; and certain viruses (such as DNA tumour viruses and some RNA retroviruses). Commonly known examples of these carcinogenic agents include: the ultraviolet radiation in sunlight which causes skin cancers and melanomas; the chemicals in cigarettes which cause lung cancer; and the human papillomaviruses which cause cervical carcinoma.

The progression of cells towards malignancy involves the accumulation of genetic lesions, as indicated by the numerous chromosomal abnormalities found in cancer cells (reviewed in: Nowell, 1976). While many of these defects would have contributed to the transformed phenotype, many are also the consequence of cell transformation. Failure of cells to respond appropriately to DNA damage, along with an increased rate of proliferation and an extended life span tends to ensure that genetic lesions are selected and therefore an ever-increasing feature of transformed cells.
Figure 1.1: Formation of foci by cell transformation. The transformation of cells in culture allows them to grow in dense clumps called 'foci'. (refer to Section 1.1.) The appearance of the foci (and the cells therein) depends upon the changes induced by the transforming oncoproteins. The foci were produced through the transfection of cells with genes which encode different types of oncoproteins (refer to Chapter 3). The foci marked 'A', 'B', and 'C' were caused by a protein which binds and inactivates p53 along with adenovirus E1a (A) or activated ras (C), while the foci marked 'B' were caused by the combination of adenovirus E1a and activated ras.
1.2 Historical perspective of cancer-related proteins

1.2.1 Oncoprotein cooperation in cell transformation

The concept of cells being transformed by the activities of certain proteins (called oncoproteins) originally came from studies of tumour viruses. Certain oncoproteins encoded by DNA tumour viruses are responsible for transforming the infected cells into a state which is both optimal for virus replication and tumourigenic (refer to Section 1.3.1). In contrast, RNA retroviruses induce transformation by converting some of the normal growth stimulating proteins of infected cells into oncoproteins. Thus, cellular oncoproteins were first discovered as the products of normal genes (called proto-oncogenes) which had undergone retroviral modifications to become oncogenes (reviewed in: Bishop, 1983). These genetic modifications cause cell transformation by increasing either the level or the activity of certain growth stimulating proteins.

Detailed studies of chromosomal amplifications, translocations and rearrangements in tumour cell DNA have since led to the discovery of many more cellular oncogenes. Like viral modifications, these types of chromosomal aberrations can alter proto-oncogenes and consequently cause significant quantitative or qualitative changes in the encoded growth stimulating proteins (reviewed in: Bishop, 1991). Gene amplification or changes within the gene promoter can induce high levels of protein expression, while changes within the coding region can cause structural alterations which result in enhanced protein activity. Although chromosomal aberrations account for the production of many oncoproteins, single point mutations within proto-oncogenes have also been shown to cause oncoprotein activity through structural alterations which constitutively activate the growth stimulating proteins.

Our knowledge of oncoprotein activity has been greatly expanded by the transfection of cloned genes into cells in culture as many oncogenes have been identified through their abilities to transform and consequently produce foci from previously immortalised cells (reviewed in: Cooper, 1982). In contrast to immortal cells, normal (primary) cells which have been taken straight from an animal are rarely transformed by a single oncogene. Primary cells can however, be transformed by the cooperative effects of two different cellular oncogenes (Land et al., 1983). Since oncoproteins which are found predominantly in the nucleus (eg. myc, fos, jun, and adenovirus E1a proteins) tend to cooperate best with oncoproteins which are found predominantly in the cytoplasm (eg. ras, src, and polyomavirus middle T antigen), these two classes of oncoproteins appear to provide complementary tumourigenic activities. In general, 'cytoplasmic' oncoproteins were found to transform immortal cells but not immortalise primary cells, while 'nuclear' oncoproteins were found to immortalise primary cells but not transform immortal cells. For these reasons, the 'nuclear' oncoproteins have been thought to increase cell
proliferation and cause immortalisation, while the 'cytoplasmic' oncoproteins have been thought to provide the phenotypic changes of cell transformation (such as morphological variations, a reduced requirement for growth factors, and an ability to grow while in contact with other cells and without adherence to a smooth surface) (reviewed in: Weinberg, 1985).

In more recent years however, the roles of 'nuclear' and 'cytoplasmic' oncoproteins have been found to be more complicated than originally thought. Both types of oncoproteins are generally able to stimulate cell growth and while this activity is an obvious effect of proteins like myc and adenovirus E1a, it does not necessarily lead to cell immortalisation. Although these oncoproteins increase the rate of cell proliferation they also tend to increase the rate of cell death (Evan et al., 1992; Rao et al., 1992; reviewed in: Harrington et al., 1994) through an active process called 'apoptosis'. In contrast to necrotic cell death, apoptosis (or programmed cell death) is genetically encoded program (reviewed in: Vaux, 1993) which is characterised by condensation of chromatin, intranucleosomal fragmentation of DNA, and certain morphological changes such as cell shrinkage, convolution of the cell membrane, and the rupture of the cell into apoptotic bodies (Kerr et al., 1972; Wyllie et al., 1980). In addition to regulating the size of certain cell populations during tissue development or homeostasis, apoptotic cell death is important for suppressing tumourigenesis. Therefore, in the presence of oncoproteins like myc and E1a, cellular immortalisation requires an additional oncogenic activity which prevents the execution of apoptosis. These observations have led to the discovery of an additional class of oncoproteins (eg. bcl-2 and adenovirus E1b-19K) which inhibit apoptosis and are therefore able to cooperate in the transformation of cells with the 'nuclear' class of oncoproteins (Hockenbery et al., 1990; Bissonnette et al., 1992; Fanidi et al., 1992; Rao et al., 1992) (refer to Section 1.3.5)

Depending upon the combination of oncogenes involved, the transformation of cells entails varying degrees of cell growth stimulation, evasion from cell death, and phenotypic changes which tend to assist the survival and growth of cells under difficult conditions. While the reasons for oncogene cooperation remain somewhat ambiguous, it is quite obvious that tumourigenesis is the result of more than one (and usually several) oncogenic lesions. Further evidence for the cooperative effects of different oncogenes has been provided by studies of tumour formation in transgenic animals. Although the rate of tumourigenesis in a mouse which contains a single type of oncogene is quite low, there is a high incidence of tumour formation in the offspring of a cross between two mice which each harbour a different type of oncogene (reviewed in: Hunter, 1991).
1.2.2 Tumour suppressor proteins

Because the formation of an oncogene through genetic damage causes the encoded protein to gain a tumourigenic function, there is a dominant mode of inheritance as the disruption of one gene allele is sufficient to effect the cell phenotype. With some types of human cancers however, a recessive mode of inheritance has been observed. Statistical analyses of the incidence of retinoblastoma (which occurs in early childhood) led to the proposal of a model in which two successive mutational events are necessary for tumourigenesis (Knudson, 1971). In familial cases, one mutation is inherited (or pre-zygotic) and the other is somatic (or post-zygotic), while in the much rarer sporadic cases, there are two independent somatic mutations. It later became obvious that these mutations were defects in the two alleles of a particular gene locus (as discussed below). The concept of tumourigenesis resulting from the loss of genetic information explained the necessity for two mutational events since the inactivation of only one allele would be compensated by the remaining normal allele. The proteins normally encoded by these genes have been called 'tumour suppressor proteins' as tumourigenic activity is permitted by the loss of their regular functions within the cell.

The first experimental evidence for the existence of tumour suppressor proteins came from the hybridisation of normal cells and cancer cells. In cell hybrid situations, normal cells could suppress the tumourigenic features of the cancer cells, except in cases where the normal cells had lost particular chromosomes. It was therefore suggested that the deleted chromosomes contained genes whose expression could suppress tumourigenesis, and by transferring certain normal chromosomes into cancer cells, this idea was confirmed (reviewed in: Harris, 1988). Thus, these studies implied that in the cancer cells, certain tumour suppressor genes had been lost or inactivated.

Cytogenetic analyses then accounted for some of these genetic defects by revealing the loss of whole chromosomes or large scale deletions within chromosomes in cancer cells. However, in most cases, the chromosomal aberrations have proved to be too small for cytogenetic detection. To detect these smaller aberrations, polymorphisms within genes have been used to distinguish between the alleles inherited by an individual from either parent. While the normal cells of an individual may contain two different types of alleles (ie. exhibit heterozygosity) at a particular gene locus, the cancer cells from this individual may exhibit loss of constitutive heterozygosity (LOCH) due to the loss of a chromosome, a deletion within a chromosome, or a mitotic recombination (Cavenee et al., 1983). The extent of chromosomal damage is indicated by the occurrence of LOCH at various gene loci along the length of the chromosome (reviewed in: Sager, 1989).

By identifying chromosomal regions which are commonly lost in particular types of cancers, these studies have been useful in the localisation and identification of several
tumour suppressor genes (reviewed in: Marshall, 1991; Levine, 1993). These include: the Rb gene which is located in chromosome band 13q14 and lost in retinoblastomas; the WT1 gene which is located at 11p13 and lost in Wilms' tumours; the DCC and APC genes which are located at 18q21 and 5q21 (respectively) and lost in colorectal carcinomas; and the p53 gene which is located at 17p13 and lost in a variety of human cancers. Since the discovery of these tumour suppressor genes, the list of defects which may cause their loss or inactivation, has been expanded to include point mutations, deletions within genes, chromosomal rearrangements, and chromosomal translocations.

With regard to the cellular functions of tumour suppressor proteins, the common feature is cell cycle regulation as disruptions to the control of cell proliferation result in tumourigenesis. Some of these proteins (for example, p53) can even reverse the effects of certain oncoproteins by suppressing the transformation of cells in their presence. The p53 protein is as yet, unique among tumour suppressors as it has at least two activities which may contribute to its anti-tumourigenic effects: an ability to inhibit cell growth and an ability to induce cell death through apoptosis (refer to Sections 1.3.2. and 1.4).

1.3 Cellular activities of oncoproteins and tumour suppressor proteins

1.3.1 Cellular and viral oncoproteins

Cells are equipped with complex systems of biochemical signals which control their fundamental activities such as growth and division, differentiation, and even death. Since these activities usually occur in response to the cellular environment, many of the biochemical signals originate at the cell membrane. To stimulate cell proliferation, an extracellular growth factor binds to a cell surface receptor and initiates a cascade of signals which is transmitted via a series of proteins, through the cytoplasm and into the nucleus. The biochemical signals then alter the transcription of certain genes so that an appropriate cellular response is produced. Because functional disruptions which increase the activity of any of these proteins can augment the growth promoting signals and consequently promote cell transformation, it is not surprising that cellular oncoproteins have been identified at many different parts of the signal transduction system (refer to Figure 1.2) (reviewed in: Cantley et al., 1991; Kazlauskas, 1994; McCormick, 1994). In addition to deregulating cell proliferation, these oncogenic disruptions may induce various phenotypic effects including changes in cell morphology, loss of differentiation potential, and the ability to grow under unfavourable conditions.

With respect to their biochemical activities, cellular oncoproteins may be grouped into various categories, such as: growth factors; protein-tyrosine kinase receptors which receive various growth factors; membrane-associated protein-tyrosine kinases which
Figure 1.2. Oncoproteins and cell signal transduction. Various types of proteins are involved in the transduction of biochemical signals from the cell membrane to the nucleus. The overexpression or constitutive activation of these proteins causes them to become oncoproteins (refer to Section 1.3.1).
transmit signals from cell surface receptors; guanine-nucleotide binding proteins which also transmit signals from cell surface receptors; cytoplasmic protein-serine/threonine kinases which are intermediate signalling molecules; and nuclear transcription factors which regulate gene transcription in response to cytoplasmic signals (refer to Figure 1.2). An additional class of oncoproteins which may or may not be involved in signal transduction pathways, are the proteins which inhibit cell death. Thus, various types of normal cellular proteins can gain the status of an oncoprotein through genetic abnormalities which enhance (and deregulate) their biochemical activities.

A fundamental difference between cellular and viral oncoproteins relates to the mechanisms by which they cause cell transformation. While cellular oncoproteins generally stimulate the transmission of growth promoting signals, viral oncoproteins abrogate the activities of proteins which normally suppress cell growth.

DNA tumour viruses have greatly contributed to an understanding of cell transformation as the functions which are responsible for transforming infected cells are encoded in a short sequence of DNA and contained in only two or three (often alternatively-spliced) proteins. Well known examples of these proteins are: the large and small T antigens (LTag and smTag) encoded by simian virus 40 (SV40); the large, middle and small T antigens (large, middle and small Tags) encoded by polyomavirus; the early region 1A proteins (E1a) and early region 1B 55kDa and 19kDa proteins (E1b-55K and E1b-19K) encoded by human adenoviruses; and the early region 6 and 7 proteins (E6 and E7) encoded by human papillomaviruses (HPV).

These viral oncoproteins have a diverse range of cellular activities some of which are shared by the proteins of different viruses. Functions which appear to be involved in cell transformation include: regulation of signal transduction through the binding of protein-tyrosine kinases (by SV40 small Tag and polyomavirus small and middle Tags (Pallas et al., 1990; Walter et al., 1990)); inhibition of apoptosis (by adenovirus E1b-19K (White et al., 1991; Rao et al., 1992)); and the binding of cellular proteins such as: p53; the retinoblastoma susceptibility protein (pRb) and related proteins, p107 and p130; (as mentioned below); and p300 (by adenovirus E1a and SV40 LTag (Whyte et al., 1989; Yaciuk et al., 1991)). Other protein functions which may or may not contribute to the transformed phenotype include regulation of viral and cellular gene transcription (by many of the viral oncoproteins) and initiation of viral DNA replication (by SV40 LTag (reviewed in: Fanning and Knippers, 1992)).

However, the most important types of viral activity in cell transformation are the binding and apparent inactivation of p53 and pRb. SV40 LTag (Lane and Crawford, 1979; Linzer and Levine, 1979; Bargonetti et al., 1992; Mietz et al., 1992), adenovirus E1b-55K (Sarnow et al., 1982; Yew and Berk, 1992), and HPV E6 (Scheffner et al., 1990; Werness et al., 1990; Lechner et al., 1992; Mietz et al., 1992) can all bind p53, while SV40 LTag (DeCaprio et al., 1988; Dyson et al., 1989a; Ewen et al., 1989),
polyomavirus large Tag (Dyson et al., 1990), adenovirus E1a (Yee and Branton, 1985; Harlow et al., 1986; Whyte et al., 1988), and HPV E7 (Dyson et al., 1989b; Dyson et al., 1992) can all bind the pRb family of proteins. Since the transformation of primary cells generally requires the cooperative effects of viral oncoproteins so that p53 and pRb are both inactivated, these two proteins must play critical roles in the protection of cells from tumourigenic processes.

1.3.2 p53 and suppression of tumourigenesis

The importance of p53 in protecting cells from tumourigenesis is indicated by the fact that deletion or mutation of the p53 gene is the most commonly reported genetic lesion across a wide variety of human cancers (reviewed in: Hollstein et al., 1991; Greenblatt et al., 1994). Occasionally, the mutation of a single p53 allele is sufficient to abrogate the activity of wild-type p53 within the cell. In these cases, the transdominant effect of the mutant p53 protein occurs due to its ability to inactivate wild-type p53 through the formation of oligomeric complexes which force the wild-type protein into a mutant conformation (Milner and Medcalf, 1991; Milner et al., 1991). The necessity of the anti-tumourigenic function(s) of p53 has also been demonstrated by studies of transgenic mice. Although mice which are genetically deficient for p53 are born phenotypically normal, they die very early due to a high incidence of spontaneous tumour formation (Donehower et al., 1992).

Thus, p53 might not be required for normal cell function but may be activated under certain conditions to protect the cell from tumourigenic processes. Although low levels of p53 can be detected in most cell types, high levels of p53 have been observed as a response to the damage of DNA which occurs when cells are subjected to ionising irradiation. Whether it is caused by DNA damage or by the transfection of an exogenous p53 gene, an accumulation of p53 results in an arrest of cell growth (predominantly in the G1 phase of the cell cycle) (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Michalovitz et al., 1990; Kastan et al., 1991; Kuerbitz et al., 1992; Lin et al., 1992) or in some cell types, the induction of apoptosis (Yonish-Rouach et al., 1991; Shaw et al., 1992; Clarke et al., 1993; Lowe et al., 1993b) (refer to Section 4.1 for more details). Because cells which have undergone genetic damage are highly susceptible to oncogenic transformation, the abilities of p53 to prevent the continued proliferation and / or cause the death of these cells would be important for its role in suppressing tumourigenesis. This role has been demonstrated in cell culture as the transformation of cells by a number of viral and cellular oncoproteins can be suppressed by p53 (Eliyahu et al., 1989; Finlay et al., 1989).

The most well known biochemical function of p53 is activation of gene transcription (Fields and Jang, 1990; O'Rourke et al., 1990; Raycroft et al., 1990). This
activity relies on the binding of p53 to specific-sequences of DNA (Kern et al., 1991; El-Deiry et al., 1992; Funk et al., 1992) as the presence of a p53-binding site allows a promoter to be activated by this protein (Farmer et al., 1992; Kern et al., 1992; Zambetti et al., 1992) (refer to Section 5.1 for more details). Although the involvement of transcriptional activation in cellular activities of p53 remains somewhat ambiguous, the p21WAF1 protein has provided a possible link between p53 and the regulation of cell growth. While the gene for p21WAF1 is transcriptionally activated by p53 (El-Deiry et al., 1993), the encoded protein can inhibit the functions of cyclin-dependent kinases which are important for progression through the G1 phase of the cell cycle (Harper et al., 1993; Xiong et al., 1993). Another possible link between p53-dependent transactivation and cell growth inhibition is the GADD45 protein which appears to be induced by p53 when cells undergo growth arrest in response to DNA damage (Kastan et al., 1992). The discovery that another transcriptional target of p53 could be the thrombospondin gene promoter has somewhat broadened the concepts of p53 function (Dameron et al., 1994). Since thrombospondin is a potent inhibitor of angiogenesis, the activation of this protein could prevent the development of new blood vessels and thereby contribute to the role of p53 in suppressing the formation of tumours.

p53 is also known to repress transcriptional activity from various gene promoters (Ginsberg et al., 1991; Subler et al., 1992; Jackson et al., 1993) due to the binding and consequent inhibition of certain transcription factors such as: TATA-binding protein (TBP) (Seto et al., 1992; Mack et al., 1993; Ragimov et al., 1993); CCAAT-binding protein (CBP) (Agoff et al., 1993); and Sp1 (Borellini and Glazer, 1993; Perrem et al., 1995) (refer to Section 5.1 for more details). While the importance of transcriptional repression in the cellular functions of p53 remains ambiguous, there is some suggestion that this activity is involved in the induction of apoptosis (Haldar et al., 1994; Miyashita et al., 1994; Miyashita et al., 1994b; Shen and Shenk, 1994; Sabbatini et al., 1995).

In addition to regulating gene transcription, p53 is known to inhibit the replication of DNA from SV40 origins of replication (Braithwaite et al., 1987). Although this effect may be due to specific inhibition of the activities of LTag in replication of viral DNA (Sturzbecher et al., 1988; Wang et al., 1989), it might also reflect a role for p53 in regulating the replication of genomic DNA (Cox et al., 1995). This function could be accomplished through the ability of p53 to bind and inhibit replication protein A (RPA) (Dutta et al., 1993; Li and Botchan, 1993) which is part of the cellular replication machinery (refer to Section 5.1 for more details).

Another aspect to the biochemical functions of p53 involves the maintenance of genomic integrity through the repair of damaged DNA (Smith et al., 1995). Mechanisms by which p53 might contribute to DNA repair include: the activation of GADD45 which is involved in nucleotide excision repair through the binding of PCNA (Smith et al., 1994); the binding of ERCC3 which is also involved in excision repair mechanisms.
Figure 1.3. A diagrammatic representation of the p53 protein. This nuclear phosphoprotein consists of three structural domains: an N-terminal acidic region, a central hydrophobic region, and a C-terminal basic region. The shaded boxes represent amino acid sequences which are conserved between species. Various biochemical functions which have been mapped to particular domains are indicated (refer to Section 1.3.2). 1(Fields and Jang, 1990); 2(Sang et al., 1994; Subler et al., 1994); 3(Kao et al., 1990; Braithwaite et al., 1991); 4(Liu et al., 1993; Truant et al., 1993); 5(Dutta et al., 1993; Li and Botchan, 1993); 6(Bargonetti et al., 1993; Pavletich et al., 1993; Wang et al., 1993); 7(Tan et al., 1986; Jenkins et al., 1988); 8(Sang et al., 1994; Subler et al., 1994); 9(Shaulian et al., 1992; Sturzbecher et al., 1992); 10(Foord et al., 1991; Wang et al., 1993); 11(Horikoshi et al., 1995); 12(Dutta et al., 1993).
(Wang et al., 1994); or the reassociation of complementary single stranded DNA fragments (Oberosler et al., 1993; Bakalkin et al., 1994). Such activities of p53 may be important for preventing oncogenic mutations which inevitably lead to cell transformation.

Thus, p53 has several functions (as summarised in Figure 1.3) which may be necessary for suppressing tumourigenesis. In addition to reducing the susceptibility of normal cells to transformation, this protein is important for suppressing the growth and / or causing the death of cells which have already started to be transformed.

1.3.3 pRb and control of cell growth

Although known as another tumour suppressor protein, pRb is (in contrast to p53) also involved in normal cellular processes. While deletion or mutation of the gene for pRb leads primarily to the development of retinoblastoma, such defects have also been reported in a subset of other human cancers. The normal functions of pRb are reflected by the fact that re-introduction of this protein into tumour cells in which it has been lost, causes the suppression of cell growth (reviewed in: Wiman, 1993). Thus, pRb is able to inhibit cell proliferation and in specific cell types, this activity may be linked to the withdrawal from the cell cycle which occurs when the process of differentiation begins.

The requirement for pRb in the differentiation of cells during embryonal development has been revealed by the observation that mice which are genetically deficient for pRb die around the fourteenth day of gestation. This effect results from a failure of the erythropoietic system to produce terminally differentiated red blood cells, as well as a massive amount of cell death in central nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In order to study the effects of pRb deficiency in specific cell types, chimeric mice which contain up to 60% pRb-null cells have been produced. Interestingly, these studies have shown that there is a low susceptibility to the development of tumours, including retinoblastoma (Maandag et al., 1994; Williams et al., 1994b). The observation that there is a high incidence of cell death rather than tumour formation among the pRb-deficient cells in the retina, demonstrates that additional genetic defects are required for tumourigenesis (see below).

Since loss or inactivation of the pRb gene contributes to only a subset of human tumours and affects only certain tissues in transgenic mice, it would appear that integrity of pRb function is more important in some cell types than in others. To some extent, this apparent redundancy in pRb function may be explained by the activities of two closely related proteins: p107 and p130. Each of the proteins in the pRb family are: homologous in sequence (Li et al., 1993); targeted by viral oncoproteins (Yee and Branton, 1985; Harlow et al., 1986; Dyson et al., 1989a; Ewen et al., 1989; Dyson et al., 1992) (refer to
Section 1.3.1); and capable of interacting with the cell cycle regulating protein, E2F (Cao et al., 1992; Shirodkar et al., 1992; Cobrinik et al., 1993). Therefore, at least some of the cellular activities which have been attributed to pRb (as described below) may also apply to p107 and p130.

pRb appears to inhibit cell proliferation by preventing the progression of cells from G1 phase into S phase of the cell cycle (DeCaprio et al., 1989; Goodrich et al., 1991). At the beginning of G1, the hypophosphorylation and consequent activation of pRb (Buchkovich et al., 1989; Chen et al., 1989; Mihara et al., 1989) enables it to bind and inactivate the E2F protein (Hiebert et al., 1992; Shirodkar et al., 1992; Schwarz et al., 1993). E2F is a transcriptional regulator which appears to promote the initiation of S phase (Johnson et al., 1993a) by stimulating the expression of proteins which are required for replication of DNA. Transcription of the genes which encode polymerase α, dihydrofolate reductase, ribonucleotide reductase, and thymidine kinase appears to rely on the activity of E2F as each gene contains an E2F binding-site in its promoter (reviewed in: Nevins, 1992). During G1, pRb represses the transcription of these genes by interfering with the binding of transcriptional machinery to E2F (Weintraub et al., 1995). At the end of G1, pRb is inactivated due to hyperphosphorylation (Buchkovich et al., 1989; Chen et al., 1989; Mihara et al., 1989) by complexes of cyclins and cyclin-dependent kinases (cdks) (Hinds et al., 1992; Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993). The consequent release of pRb from E2F allows the expression of proteins which cause the initiation of S phase, as mentioned above (refer to Figure 1.4). Although this account of pRb function is somewhat complicated by the fact that E2F is one of a family of proteins (Ivey-Hoyle et al., 1993; Lees et al., 1993), it demonstrates a mechanism through which pRb can inhibit cell proliferation.

Since the loss of pRb activity within a cell would allow E2F to stimulate a premature onset S phase, it may be expected that defects within the pRb gene would be a common feature of tumourigenesis. However, when pRb is lost due to a genetic deficiency or inactivated by viral oncoproteins, cells become highly susceptible to apoptosis which is mediated by p53 (Howes et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Symonds et al., 1994; Williams et al., 1994a; Almasan et al., 1995). The formation of tumours therefore occurs only when the activities of both p53 and pRb are abrogated. These observations explain both the lack of tumourigenesis and the high incidence of cell death in mice which are genetically deficient for pRb (as mentioned above). In the absence of pRb, the apoptotic response is probably caused by the activity of E2F as this protein is known to induce apoptosis in cooperation with p53 (Qin et al., 1994; Wu and Levine, 1994). As would be expected, the reintroduction of active pRb into cells in which this protein has been previously inactivated prevents apoptotic cell death (Haupt et al., 1995a).
Figure 1.4. Cell cycle progression and cyclin-cdk activity. The progression of cells through the different stages of the cell cycle depends upon the activities of various cyclins and cyclin dependent kinases (cdks) (reviewed in: Sherr, 1993). As an example, cyclin-cdk complexes are responsible for hyperphosphorylating pRb in late G1. The consequent release of pRb from E2F allows the expression of proteins which are required for S phase (refer to Sections 1.3.3 and 1.3.4). Proteins such as p21^{WAF1}, p27^{KIP1}, and p16^{INK4} can inhibit the onset of S phase by inhibiting the activities of cdks (reviewed in: Elledge and Harper, 1994 and Sherr and Roberts, 1995).
Thus, it would appear that cells have evolved multiple coordinated mechanisms for preventing tumourigenesis. Since pRb normally regulates cell cycle progression, the loss of this protein can cause an inappropriate onset of S phase, enhance cell proliferation, and consequently, increase the susceptibility of cells to transformation. Such defects can however be compensated by p53 which prevents cell transformation by inducing an apoptotic response.

1.3.4 Cell cycle regulation by cyclin-cdk

As mentioned in the previous section, cyclins and cyclin dependent kinases (cdks) are necessary for hyperphosphorylation of pRb which then detaches from E2F so that S phase can be initiated (refer also to Figure 1.4). This is one example of the biochemical pathways used by cyclins and cdks to stimulate the progression of cells through the different phases of the cell cycle. The passage of cells through the cell cycle involves various complex intracellular activities which include replication of genomic DNA (during S phase), as well as condensation and separation of chromosomes, breakdown and reformation of the nuclear membrane, and enlargement and division of the cytoplasm and cell membrane (all of which occur during mitosis). The co-ordination of such intracellular events with each other and with extracellular events (such as growth factor stimulation or deprivation) appears to be achieved through the regulation of various cyclins and cdks (Hartwell and Kastan, 1994). When activated, cyclin-cdk complexes are responsible for driving the cells from one phase of the cell cycle to the next. During the G1 phase, cyclin-cdk complexes respond to growth factor-induced cytoplasmic signals by causing the cells to either arrest in G1 and undergo differentiation or proceed into S phase and continue proliferation. After the onset of DNA replication, cyclin-cdk complexes rely entirely upon intrinsic signals to regulate the progression of cells out of S phase, through G2 phase, and into mitosis (Pardee, 1989).

Intracellular activities which are peculiar to certain stages of the cell cycle are thought to be governed by the activation or inactivation of proteins due to phosphorylation by particular cdks. The kinase activities of cdks are activated by association with particular cyclins which are expressed at different stages of the cell cycle. The progression of cells through G1 phase and into S phase is controlled by complexes of D-type cyclins (cyclins D1, D2 or D3) and cdk2, cdk4, cdk5 or cdk6 each of which appear (in response to growth signals) during G1, as well as a complex of cyclin E and cdk2 which appears towards the end of G1. During S phase, a cyclin A-cdk2 complex appears to be important for the proper completion of genomic DNA replication. After this, the passage of cells from S phase, through G2 phase, and into mitosis is thought to be regulated by cdc2 in complexes with cyclin A or cyclin B (reviewed in: Sherr, 1993). This set of events (as illustrated in Figure 1.4) is probably
incomplete as new cyclins and cdks are likely to be discovered and the activities of some
known cyclins (eg. cyclin C, cyclin G) and cdks (eg. cdk3) are yet to be resolved.

Due to the roles of cyclin-cdk complexes in stimulating cell cycle progression, the
activities of these proteins must be carefully regulated. Control of cyclin-cdk activity
occurs through regulation of gene transcription, protein degradation, and
phosphorylation which may either activate or inactivate the protein complex. One protein
which appears to be important for the activation of cyclin-cdks through phosphorylation
is cdk activating kinase (cak) (Draetta, 1994). Another important regulatory mechanism is
provided by cdk inhibitory proteins, such as: p21^WAF1 and p27^KIP1 both of which
inactivate the cyclin-cdk complex and p16^INK4 which displaces the cyclin from the cdk.
p21^WAF1 inhibits A, D, and E type cyclins in complex with cdk2 or cdk4, and p27^KIP1
inhibits D and E type cyclins in complex with cdk2 or cdk4, while p16^INK4 specifically
inhibits D type cyclins in complex with cdk4 or cdk6 (reviewed in: Elledge and Harper,
1994; Sherr and Roberts, 1995). One example of the effects of these proteins is the
inhibition of pRb phosphorylation (refer to Figure 1.4) so that cells are prevented from
entering S phase. Our knowledge of cdk inhibition in cell cycle regulation is likely to
expand, particularly since new cdk inhibitors and new functions may yet be discovered.

While positive regulation of cyclin-cdks is important for the stimulation of cell
proliferation by growth factors, negative regulation would be necessary to prevent cells
from prematurely entering the next stage of the cell cycle. Inhibition of cyclin-cdk activity
would also be required to arrest the cell cycle at the onset of cell differentiation or when
damage to DNA (or other important cellular components) is detected. Certain biochemical
pathways (called cell cycle checkpoints) which involve cdk inhibitors are therefore
necessary to ensure that cell proliferation does not continue unless the replication of DNA
or mitotic events are completed and genomic integrity is maintained (reviewed in:
Hartwell and Kastan, 1994; Murray, 1994).

Defects in cyclin-cdk complexes or in cell cycle checkpoints can enhance cell
proliferation and allow it to continue in spite of damage to the DNA. These conditions
greatly increase the susceptibility of cells to the process of transformation. While any
protein involved in cell cycle regulation has the potential to become oncogenic,
tumourgenic defects in the transition from G1 to S phase appear to be more prevalent
than those in the G2 to M phase transition. While mutations causing the loss or
inactivation of one cdk inhibitor, p16^INK4 have been demonstrated in various types of
human cancers (Kamb et al., 1994; Nobori et al., 1994), another cdk-inhibitor, p21^WAF1
is transcriptionally activated by the tumour suppressor protein, p53 (El-Deiry et al.,
1993) (refer to Section 1.3.2). Thus, p16^INK4 and p21^WAF1 have also been classed as
tumour suppressor proteins. Additionally, cyclins and cdks have the potential to become
oncoproteins as is the case for cyclin D1 which is overexpressed in some cancers
(Motokura and Arnold, 1993) and capable of cooperating with other oncoproteins to
cause cell transformation (Hinds et al., 1994; Lovec et al., 1994). Defects in other cell cycle related proteins are also likely to be found in human cancers.

Since cyclin-cdk complexes are necessary for stimulating cell cycle progression, their activities must be carefully regulated to ensure that appropriate levels of cell proliferation occur under certain conditions. Defects either in cyclin-cdk complexes or in the proteins which regulate them pre-dispose cells to tumourigenic processes.

1.3.5 Three classes of oncoproteins

All viral and cellular oncoproteins may be broadly classed into three categories. The first class occurs due to the overexpression or enhanced activation of proteins which are involved in the transduction of biochemical signals from the cell surface to the nucleus (refer to Figure 1.2 and Section 1.3.1). These include: extracellular growth factors (e.g. epidermal growth factor (EGF), platelet-derived growth factor (PDGF)); protein-tyrosine kinase receptors (e.g. EGF receptor, PDGF receptor) which receive various growth factors; membrane-associated protein-tyrosine kinases (e.g. src-related proteins, abl-related proteins) which transmit signals from cell surface receptors; guanine-nucleotide binding proteins (e.g. ras) which also transmit signals from cell surface receptors; cytoplasmic protein-serine/threonine kinases (e.g. raf, mek, erk, ie. mitogen-activated protein (MAP) kinases) which are intermediate signalling molecules; and possibly, nuclear transcription factors (e.g. fos, jun) which regulate gene transcription in response to cytoplasmic signals. Class I oncoproteins cause many varied effects on the cell phenotype (such as those described in Section 1.1) as each would induce different cascades of biochemical signalling pathways in the cytoplasm. While unable to transform cells alone, these oncoproteins cooperate in the process of transformation with either class II or class III oncoproteins (Land et al., 1983; Ruley, 1983; Reed et al., 1990). However, the precise reasons for these cooperative effects remain unclear.

The second class of oncoproteins is defined by the ability to deregulate cell cycle progression through the activities caused by cyclin-cdk complexes. This class therefore includes the viral oncoproteins such as SV40 LTg, adenovirus E1a, and HPV E7 each of which selectively bind hypophosphorylated (ie. active) forms of pRb (refer to Section 1.3.1). The consequent disassociation of pRb from E2F (Chellappan et al., 1992) stimulates the entry of cells into S phase (reviewed in: Nevins, 1994) (refer also to Section 1.3.3). The same cellular effect is caused by the overexpression of cyclin D1 which complexes with cdk5 and hyperphosphorylates pRb (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993) so that E2F is released and S phase is induced (Jiang et al., 1993b). Another member of this class of oncoproteins is the cellular myc protein which also stimulates the replication of genomic DNA (Heikkila et al., 1987; Eilers et al., 1991). While the actual
targets of this transcriptional regulator are yet to be defined, myc may act in the same biochemical pathway as (but downstream of) E2F. In addition to activating the expression of proteins which are directly involved in DNA replication, E2F can activate the expression of c-myc (Hiebert et al., 1989; Thalmeier et al., 1989). The common effect of all these class II oncoproteins is enhanced cellular proliferation due to a shortened G1 phase and early onset of genomic DNA replication. However, a premature onset of S phase is not always beneficial for tumourigenesis, as the other common effect of class II oncoproteins (as well as the loss of pRb or overexpression of E2F both of which also induce S phase - refer to Section 1.3.3.) is an enhanced susceptibility to cell death through apoptosis (Evan et al., 1992; Rao et al., 1992; reviewed in: Harrington et al., 1994).

Proteins in the third class of oncoproteins are able to cooperate with class II oncoproteins by virtue of the fact that they can inhibit apoptosis (Bissonnette et al., 1992; Fanidi et al., 1992; Rao et al., 1992). The survival of cells is obviously an important factor in the process of transformation (refer to Section 1.2.1). While apoptosis inhibiting proteins are known to include adenovirus E1b-19K (White et al., 1991; Rao et al., 1992) and bcl-2 (which is part of a family of related cellular proteins) (Hockenbery et al., 1990), the biochemical mechanisms through which these proteins function remain ambiguous. Since an apoptotic response (which is caused by premature entry into S phase and inhibited by bcl-2 and E1b-19K) is mediated by p53 (Debbas and White, 1993; Lowe and Ruley, 1993; Chiou et al., 1994; Hermeking and Eick, 1994; Howes et al., 1994; Morgenbesser et al., 1994), dominant negative p53 mutants which abrogate the activity of wild-type p53 (Milner and Medcalf, 1991; Milner et al., 1991) (refer to Section 1.3.2) may be additional members of this third class of oncoproteins.

The effects of the three classes of oncoproteins on the proliferation and phenotype of cells and how they contribute to transformation are illustrated in Figure 1.5. While the categorisation of oncoproteins into three classes is generally convenient, there may be examples where the effects of an oncoprotein overlap that of two different classes. In theory, tumourigenesis can result from mutations which deregulate the activities of any proteins involved in promoting the survival or growth of cells.
Oncoproteins may generally be classed into three broad categories. This figure illustrates how different types of oncoproteins contribute to the process of transformation through their effects on the phenotype, survival, and proliferation of cells (refer to Section 1.3.5). The anti-tumourigenic effects of p53 are also indicated (refer to Section 1.3.2).
1.4 Mechanisms by which p53 suppresses cell transformation

The studies presented in this thesis concern the cellular mechanisms through which p53 exerts its anti-tumourigenic effects. The ability of p53 to suppress the formation of tumours in various cell types, is reflected by the observations that this protein suppresses cell transformation by a number of viral and activated cellular oncoproteins. Consistent with a role in tumour suppression, the phenotypic effects of induced p53 expression are cell growth arrest and apoptotic cell death (refer to Section 1.3.2).

Studies described in Chapter 3 indicate that growth inhibition and apoptosis are caused by p53 under different intracellular conditions. This concept of p53 having alternative means of suppressing tumourigenesis is reinforced in Chapter 4 where the evidence shows that growth inhibition and apoptosis are completely separable functions of p53. Thus, an arrest of cell growth is not a necessary prerequisite for the induction of apoptosis.

The intracellular conditions which determine the phenotypic effect of p53 can be influenced by the presence of certain oncoproteins. In studies on the one cell type, p53 is shown to inhibit cell growth in the presence of activated ras, but cause apoptosis in the presence of adenovirus E1a. The observations of Chapter 3 together with those of other recent studies, suggest that p53-mediated apoptosis is an alternative response which occurs when pRb is inactivated (refer to Section 1.3.3) and when p53 fails to arrest cell growth. These findings also imply that the mechanism by which p53 suppresses cell transformation depends upon the activities of the transforming oncoprotein(s).

From the above conclusions, it may be predicted that transformation involving E1a would be suppressed by p53-mediated apoptosis as the ability of E1a to inactivate pRb causes an enhanced susceptibility to this type of cell death. In agreement with this, studies presented in Chapter 4 demonstrate that the transformation of cells by E1a and ras is suppressed through an apoptotic rather than growth inhibitory function of p53.

The findings of Chapter 3 also provide an indication of a biochemical pathway through which p53 may inhibit cell growth. While these studies show that p53-dependent growth inhibition requires the presence of active pRb, other studies have shown that p53 activates p21WAF1 whose inhibitory effect on cyclin dependent kinases ensures that pRb is maintained in an active state (refer to Section 1.3.4). Thus, pRb would mediate the effects of p53 by repressing the activities of E2F so that cell growth is arrested in the late G1 phase of the cell cycle (refer to Section 1.3.3).

The biochemical activities through which p53 may inhibit cell growth or induce apoptosis are addressed in greater detail in Chapter 5. Interestingly, these studies demonstrated that p53 can inhibit the growth of cells without necessarily activating gene transcription. Thus, the transcriptional activation of p21WAF1 cannot completely account
for the growth inhibitory effects of p53. Along with the transcriptional activation function, the abilities of p53 to repress gene transcription and to inhibit viral DNA replication were also examined for possible roles in either the growth inhibitory or apoptotic effects of this protein. The concept of there being multiple biochemical mechanisms for the cellular activities of p53 is further emphasised by the findings presented in this chapter.

To conclude, the studies presented in this thesis have indicated that p53 has various strategies for suppressing oncogenic transformation. The two cellular effects of this protein (growth inhibition and apoptosis) are independent and alternative functions which may be achieved through multiple biochemical pathways.
Chapter 2

Methods used for investigating activities of p53
2.1 Plasmids

2.1.1 Descriptions of plasmids

Many of the plasmids used in the studies in this thesis contain genes whose transcription is controlled by the immediate-early enhancer-promoter of the human cytomegalovirus (CMV). In the experiments evaluating cell transformation, protein expression, and transcriptional regulation, CMVneo was used as the control plasmid. CMVneo was also used for evaluating cell survival and cell growth, as it contains the Tn5 gene which encodes for resistance to the neomycin / kanamycin family of antibiotics and therefore allows the selection of transfected cells with the neomycin analogue, G418 (Southern and Berg, 1982) (refer to Section 2.4). In these experiments, pBR322 was used as the control plasmid.

Wild-type mouse p53 is encoded by CMVNc9, while CMVc5 encodes mouse p53 with mutations at amino acids 168 (R to G) and 234 (S to I) (Eliyahu et al., 1989). CMVmp53 encodes mouse p53 with mutations at amino acids 45 (R to Q), 76 (P to Q) and 78 (A to W); CMVdl163 encodes mouse p53 with a deletion of amino acids 14 to 66; and CMVdl518 encodes mouse p53 with a deletion of amino acids 133 to 148 (Jenkins et al., 1985). Both CMV281EG and CMV281EK encode mouse p53 proteins with mutations at amino acid 281: E to G for CMV281EG and E to K for CMV281EK (Braithwaite et al., 1987). CMVdl1.1 encodes mouse p53 with a deletion of amino acids 304 to 323, and CMVdl2.3 encodes mouse p53 with a deletion of amino acids 304 to 304. These two plasmids were constructed by reverse PCR with CMVmp53 as the template. (Mark Griffiths is thanked for having constructed CMVdl1.1 and CMVdl2.3.)

Each of the p53 expression plasmids described above (as well as CMVneo) contain an SV40 origin of replication. MSV300-390 encodes only the C-terminal region of mouse p53 (from amino acids 280 to 390) under the transcriptional control of the Moloney sarcoma virus (MSV) promoter (Reed et al., 1993).

In each of the plasmids encoding SV40 LTag, transcription is driven by the Rous sarcoma virus (RSV) 3' long terminal repeat. RSV-T encodes wt LTag (Sakamoto et al., 1993), while RSV-TΔRBBS encodes LTag with a deletion of amino acids 92-124 ie. ΔRBBS (DeCaprio et al., 1988; Maclean et al., 1994); RSV-TK1 encodes LTag with an E to K mutation at amino acid 107 ie. T-K1 (Sakamoto et al., 1993); and RSV-T402DE encodes LTag with a D to E mutation at amino acid 402 ie. 402DE (Lin and Simmons, 1991b; Maclean et al., 1994). Each of the LTag expression plasmids also direct the expression of the SV40 small t antigen (Sakamoto et al., 1993; Maclean et al., 1994).

CMVE1a contains the adenovirus early region 1 which encodes all of the E1a proteins (Morris and Mathews, 1991), while CMV19K encodes the adenovirus E1b-19K protein. To construct this plasmid, the E1b-19K gene region was amplified using PCR
from Adenovirus-2 DNA and inserted in the RC-CMV vector at HindIII and XbaI restriction sites. (Steve Loof is thanked for having constructed CMV19K.) CMVbcl-2 which encodes the human bcl-2 protein was constructed by inserting a bcl-2 cDNA (Cleary et al., 1986) in the RC-CMV vector at a HindIII restriction site. T24EJ6.6 allows the expression of an activated ras protein (EJ-ras - as derived from T24 bladder carcinoma cells) under the transcriptional control of its own promoter (Reddy et al., 1982).

pETCAT encodes chloramphenicol acetyl transferase (CAT) under the transcriptional control of the mouse muscle creatinine kinase (MCK) promoter (Weintraub et al., 1991), while pSV2CAT encodes CAT under the transcriptional control of the SV40 promoter (Gorman et al., 1982).

Each of the plasmids described above contain the gene which encodes for resistance to ampicillin in bacterial cells.

The following people are thanked for having provided some of the plasmids described above: Moshe Oren (for CMVNc9 and CMVc5); Michael Reed (for MSV280-390); Roger Reddel (for RSV-T, RSV-ΔRBBS, RSV-TK1, and RSV402DE); Gill Morris (for pCMVE1a); Eric Blair (for pT24EJ6.6); Stephen Hauschka (for pETCAT); and Merilyn Sleigh (for pSV2CAT).

2.1.2 Preparation of plasmid DNA

The methods used for transforming bacterial cells with plasmids and for preparing the plasmid DNA from those cells were adapted from the methods described in: Sambrook et al., 1989.

To make bacterial cells competent for transformation with plasmid DNA:
500μl from a 5ml overnight culture of DH5α (E.coli.) cells was used to inoculate 200ml SOB medium (2% bacto-tryptone; 0.5% bacto-yeast extract; 20mM MgSO4; 10mM MgCl2; 10mM NaCl; and 2.5mM KCl). This culture was then incubated at 37°C until the optical density (at 550nm) of the culture reached 0.45-0.55. The cells were then chilled, centrifuged at 3000rpm for 10 min, and resuspended in 64ml cold transformation buffer (TFB) (10mM Mes.KOH; 100mM KCl; 45mM MnCl2; 10mM CaCl2; and 3mM [Co(NH3)6]Cl3). After being incubated on ice for 10 min, the cells were centrifuged once more at 3000 rpm for 10 min and resuspended in 16ml TFB. To this was added 560μl dimethyl sulfoxide (DMSO), 1.26ml 1M dithiothreitol (DTT), and another 560μl DMSO, with 5 min intervals of incubation on ice between each addition. The cells (which were then competent for transformation) were aliquoted into lots of 1ml and stored at -70°C.

To transform bacterial cells with plasmid DNA:
200μl thawed competent DH5α cells were mixed with approximately 10ng plasmid DNA and incubated on ice for 30 min. The cells were then heat shocked at 42°C for 75 sec and
returned immediately to ice for about 2 min. 800μl SPTMG medium (1% bacto-tryptone; 0.5% bacto-yeast extract; 171mM NaCl; 20mM glucose; 10mM MgCl2; 10mM MgSO4; and 2.5mM KCl) was then added and the cells were incubated at 37°C for 1 hour. The cells were then centrifuged at 6000 rpm for 6 min, resuspended in approximately 100μl SPTMG medium, and plated out on Luria Bertani (LB) agar (1% bacto-tryptone; 0.5% bacto-yeast extract; 171mM NaCl; and 1.5% bacto-agar) containing 100μg/ml ampicillin. After the plates were incubated at 37°C overnight, colonies of transformed cells appeared at an approximate rate of 1 x 10^6 colonies / μg plasmid DNA. 10ml LB medium (1% bacto-tryptone; 0.5% bacto-yeast extract; and 171mM NaCl) containing 100μg/ml ampicillin was then inoculated with a single colony and incubated at 37°C for overnight. To store the transformed cells, 850μl overnight culture was mixed with 150μl glycerol and frozen at -70°C.

To prepare plasmid DNA (on a large scale) from bacterial cells:
400ml LB medium containing 100μg/ml ampicillin was inoculated with approximately 100μl of thawed transformed cells and incubated at 37°C overnight. The cells were then chilled, centrifuged at 6000rpm for 15 min at 4°C, and resuspended in 30ml solution 1 (25mM Tris.HCl pH8; 10mM EDTA; and 50mM glucose). To this was added, 60ml fresh solution 2 (200mM NaOH and 1%SDS), followed by 45ml solution 3 (3M KAc pH4.8). The mixture was incubated on ice for 15 min and then centrifuged at 9000 rpm for 15 min at 4°C. The supernatant was then mixed with 81ml isopropanol, and incubated at room temperature for 10min. Centrifugation of the mixture at 9000rpm for 20 min at 20°C produced a DNA pellet which was resuspended in 7ml TE (10mM Tris.HCl and 1mM EDTA - pH8). To isolate supercoiled plasmid DNA with a CsCl gradient, 8.6g CsCl and 300μl EtBr were added to the DNA solution. After cell debris was removed by centrifugation at 9000 rpm for 5 min at 20°C, the supernatant was centrifuged at 35000 rpm for about 44 hours at 20°C. The band of supercoiled plasmid DNA was then removed and extractions with butanol were performed 4-5 times to remove all traces of EtBr. The DNA was then precipitated with a 2x volume of cold ethanol and resuspended in 500μl TE, and this was repeated several times until all the CsCl was removed from the DNA pellet. After being left to dry, the DNA was resuspended in an appropriate volume TE. The concentration and purity of the DNA preparation was then determined by its UV light absorbency at 260nm and 280nm. The actual plasmid contained in the DNA preparation was checked by observing characteristic fragments after digestion of the DNA with certain restriction enzymes (refer to Section 2.1.3).
2.1.3 Construction of recombinant plasmid DNA

The methods used for preparing and inserting a DNA fragment in a plasmid vector and for preparing and examining the recombinant plasmid were adapted from those described in: Sambrook et al., 1989.

To cleave plasmid DNA at particular sites:
1-20µg plasmid DNA was mixed with 1-10µl 1x One-Phor-All buffer (Pharmacia) and 5-20U appropriate restriction enzyme (Pharmacia) in a total volume of 10-50µl. The restriction enzyme digest reactions were allowed to proceed at 37°C for 1-2 hours.

To separate and purify fragments of plasmid DNA:
Products of restriction enzyme digest reactions were mixed with a 0.1x volume of 10x electrophoresis dye (24.5% Ficoll type 400; 100mM EDTA; 10mM Tris.HCl; and 0.24% bromophenol blue). Differently-sized fragments of DNA were separated by electrophoresis in a 1% agarose gel (made with 1x TE and 0.05µl/ml EtBr) and observed under UV light. For isolating DNA fragments, a 1% low-melting point agarose gel was used. A slice of gel containing the DNA fragment was then mixed with a 400µl solution of 20mM Tris.HCl and 1mM EDTA, and melted at 68°C for 10 min. Extractions with phenol and chloroform at a ratio of 4:1 and then 1:4 (phenol : chloroform) were performed to remove all traces of agarose. The DNA solution of approximately 500µl was then mixed with 1ml cold ethanol and 30µl 3M NaAc so that DNA fragments could be precipitated by centrifugation at 13000 rpm for 15 min at 4°C. The DNA pellet was resuspended in 10-20µl TE.

To recombine fragments of plasmid DNA:
Vector and insert fragments of DNA were mixed together at molar ratios of 1:3, 1:10, or 1:30 (vector : insert) and heated to 45°C for 5 min to break any previously reannealed fragment ends. 1µl 10x ligase buffer (Promega) and 1.5U T4 DNA ligase (Promega) was then added to the DNA in a total volume of 10µl. The ligation reactions were allowed to proceed at 16°C overnight.

To isolate, prepare, and examine recombinant plasmids:
Products of ligation reactions were used to transform bacterial cells as described in Section 2.1.2. 10ml LB medium containing 100µg/ml ampicillin was then inoculated with a colony of transformed cells and incubated at 37°C overnight. The cells were then chilled, centrifuged at 13000rpm for 5 min and resuspended in 200µl solution 1 (25mM Tris.HCl pH8; 10mM EDTA; and 50mM glucose). To this was added, 400µl fresh solution 2 (200mM NaOH and 1%SDS), followed by 300µl solution 3 (3M KAc pH4.8). The mixture was incubated on ice for 10 min and then centrifuged at 13000 rpm for 5 min. The supernatant was mixed with 540µl isopropanol and incubated at room temperature for 10min. Centrifugation of the mixture at 13000rpm for 5 min produced a DNA pellet which was resuspended in 200µl TE. An extraction with phenol and
chloroform (at a ratio of 1:1) was performed to remove any remaining cell debris. The DNA solution of approximately 200μl was then mixed with 400μl cold ethanol and 15μl 3M NaAc, so that plasmid DNA could be precipitated by centrifugation at 13000 rpm for 5 min at 4°C. The DNA pellet was resuspended in 50μl TE. The actual plasmid contained in the DNA preparation was then checked by observing characteristic fragments after digestion of the DNA with certain restriction enzymes.

2.2 Cell culture and DNA transfections

2.2.1 Cell culture

Rat embryo fibroblasts (REFs) were extracted from 15 to 17 day-old Wistar rat embryos by a method adapted from that described by Bellett and Younghusband, 1979. To prepare REFs, the embryos were decapitated and delimbed, repeatedly washed in phosphate buffered saline (PBS), and then minced. After the cells were treated with 0.25% trypsin in PBS for 30 min, the suspended cells were separated from any clumps and then washed and maintained in DMEM (Dulbecco's modified eagle medium (Gibco-BRL) - containing 4.5mg/ml glucose) with 10% foetal bovine serum (FBS) (CSL).

Immortalised human fibroblasts (LFSFs) were derived from a patient with Li-Fraumeni Syndrome (LFS) (Rogan et al., 1995) and were maintained in RPMI1640 (Gibco-BRL) with 10% FBS. (Eileen Rogan is thanked for having provided the LFSF cell line.) HeLa cells (which were derived from a human cervical carcinoma - ATCC CCL2) and CV1 cells (which were derived from the kidney of an African green monkey (AGM) - ATCC CRL1650) were both maintained in MEM (minimal essential medium) (Gibco-BRL) with 10% FBS. COS cells (which were derived from the kidney of an AGM and transformed by simian virus 40 - ATCC CCL70) were maintained in MEM with 5% FBS. All cells were incubated at 37°C with 5% CO₂.

2.2.2 Lipofectamine transfections

REFs and LFSFs were seeded at a density of 1.2 x 10⁵ cells per 35 mm dish and transfected with plasmid DNA after 24 hours. The method of transfection involved the use of lipofectamine and was adapted from that recommended by the manufacturer (Gibco-BRL). 2μg of total plasmid DNA (usually 667ng of each plasmid type) and 3μl of lipofectamine were combined in 200μl Opti-MEM (Gibco-BRL) and incubated at room temperature for 45 min. 800μl antibiotic-free DMEM with 10% FBS was then added, and the mixture was placed onto the cells which had been rinsed in antibiotic-free DMEM. After incubating the cells at 37°C for 5 hours, the usual medium (DMEM with 10% FBS)
was replaced. The cells were incubated again and the medium was changed after 24 hours, and again after 48 hours.

2.2.3 Calcium phosphate transfections

REFs were seeded at a density of \(5 \times 10^5\) cells per 90mm dish and transfected with plasmid DNA after 24 hours. The method of transfection involved the use of calcium phosphate and was adapted from that described by Chen and Okayama, 1987. 20\(\mu\)g of total plasmid DNA (usually 10\(\mu\)g of each plasmid type) was mixed with 500\(\mu\)l 0.25M CaCl\(_2\) and added to 500\(\mu\)l 2x BES-buffered saline (BBS) (50mM BES pH6.95; 280mM NaCl; and 1.5mM Na\(_2\)HPO\(_4\)). The mixture was incubated at room temperature for 20 min, mixed, and placed dropwise onto the cells in 10ml of DMEM with 10% FBS. After incubating the cells at 35°C with 3% CO\(_2\) for approximately 24 hours, the medium was removed, and the cells were washed twice in PBS. The usual medium (DMEM with 10% FBS) was then replaced and the cells were incubated at 37°C with 5% CO\(_2\) once more.

HeLa and CV1 cells were seeded at a density of \(1 \times 10^6\) cells per 90mm dish and transfected with plasmid DNA after 24 hours. The method of transfection involved the use of a calcium phosphate transfection system and was adapted from that recommended by the manufacturer (Gibco-BRL). 20\(\mu\)g of total plasmid DNA (usually 10\(\mu\)g of each plasmid type) was diluted in 430\(\mu\)l ddH\(_2\)O and mixed with 10\(\mu\)l and then another 50\(\mu\)l calcium solution. 500\(\mu\)l 1x HBS (Hepes buffered solution) with 10\(\mu\)l phosphate solution was then bubbled with air and mixed with the DNA / calcium solution. The mixture was incubated at room temperature for 20 min, mixed, and placed dropwise onto the cells in 10ml of MEM with 10% FBS. After incubating the cells at 37°C for approximately 24 hours, the medium was removed and the cells were washed twice in PBS. The usual medium (MEM with 10% FBS) was then replaced and the cells were incubated again at 37°C.

2.2.4 DEAE-dextran transfections

COS cells were seeded at a density of \(2.5 \times 10^6\) cells per 90mm dish and transfected with plasmid DNA after 2 hours. The method of transfection involved the use of DEAE-dextran and was adapted from that described by Sturzbecher et al., 1987. 10\(\mu\)g of plasmid DNA was combined with 750\(\mu\)g DEAE-dextran (molecular weight: \(2 \times 10^6\)-Sigma) in 3ml FBS-free DMEM, and the mixture was placed onto the cells which had been rinsed twice in FBS-free MEM. After incubating the cells at 35°C with 3% CO\(_2\) for 4 hours, the medium was removed and the cells were shocked for 2 min in Hanks' basic salt solution (1.71M NaCl; 67mM KCl; 12mM CaCl\(_2\); 6mM MgCl\(_2\); 5mM MgSO\(_4\); and
75 mM glucose) containing 10% DMSO. The cells were then washed twice with FBS-
free MEM, the usual medium (MEM with 5% FBS) was replaced, and the cells were
incubated again at 37°C with 5% CO₂.

2.3 Evaluation of cell transformation

A focus forming assay was used to evaluate the transformation of cells under
certain conditions. For this purpose, 2nd or 3rd passage REFs were transfected with
plasmid DNA (including plasmids which encode certain oncoproteins) through the use of
lipofectamine (as described in Section 2.2.2). The cells were then maintained in normal
medium for 22-24 days. After this time, the cells were washed in PBS, then fixed and
stained in 0.1% crystal-violet in 20% ethanol for 5 min, and washed once more in
double-distilled (dd) H₂O. Once the cells had dried, the foci of transformed cells were
counted under a microscope at 10x magnification.

2.4 Evaluation of cell survival and cell growth

To investigate the survival and growth of cells under certain conditions, LFSFs
and 2nd or 3rd passage REFs were transfected with plasmid DNA through the use of
lipofectamine (as described in Section 2.2.2). In order to observe the effects of
exogenous proteins on cell survival and growth, the cells which actually received plasmid
DNA during transfection had to be selected from the surrounding cells. CMVneo was
therefore included as one of the transfected plasmids as it contains the Tn5 gene which
codes for resistance to the neomycin/kanamycin family of antibiotics. This selectively
permits the survival of transfected cells in the presence of the neomycin analogue, G418
(Southern and Berg, 1982). Thus, LFSFs were maintained in normal medium containing
250 μg/ml Geneticin G-418 (Gibco-BRL) for 12-14 days, while REFs were maintained in
normal medium containing 200 μg/ml Geneticin G-418 for 18-20 days. (The Geneticin-
G418 was prepared as stock solution at a concentration of 10 mg/ml in DMEM with 10%
FBS.) The cells were then fixed and stained in 0.1% crystal violet in 20% ethanol as
described in Section 2.3.

To measure cell survival, LFSFs were treated as described above and the colonies
of neomycin-resistant cells were counted.

To measure cell growth, REFs were treated as described above but not fixed and
stained. Instead the cells were lysed so that the total protein concentrations of the lysates
could be measured as a reflection of overall cell number. For this purpose, the cells were
washed twice in cold PBS and harvested in PBS. After one more wash in PBS, the cells
were resuspended in 200 μl 0.25 M Tris.HCl pH 7.8, and then lysed by three cycles of
freezing in dry ice and thawing at 37°C. Cell debris was removed by centrifuging the
lysates at 13000rpm for 15 min at 4°C. To measure the protein concentrations of cell lysates, a Coomassie blue G-250 protein dye was used in a manner recommended by the manufacturer (Biorad). Thus, the 200μl of each cell lysate was diluted in 600μl of ddH₂O and mixed with 200μl of Coomassie blue G-250 protein dye. Standard concentrations of bovine serum albumin (BSA) were also prepared in 200μl 0.25M Tris.HCl pH7.8, diluted in 600μl of ddH₂O, and mixed with 200μl of the Coomassie blue G-250 protein dye. The total protein concentration of each sample was determined by measuring its optical density at 595nm and comparing the reading to those of the protein standards.

2.5 Evaluation of protein expression

The method used for observing the expression of exogenous (and endogenous) proteins was adapted from that described by Zhang et al., 1990. For this purpose, REFs and LFSFs were transfected with plasmid DNA through the use of lipofectamine (as described in Section 2.2.2), and HeLa cells and CV1 cells were transfected with plasmid DNA through the use of CaPO₄ (as described in Section 2.2.3). 72 hours post-transfection, newly-synthesised proteins were radiolabelled by incubating the cells with 100μCi of [³⁵S]methionine in 1ml methionine-free DMEM with 2% FBS at 37°C for 2 hours. The cells were then rinsed in cold PBS and harvested in lysis buffer (50mM Tris.HCl pH8; 120mM NaCl; 5mM EDTA; 0.5% NP40; and 0.001% PMSF). Cell debris was removed by centrifuging the lysates at 36000rpm for 30 min at 4°C and the supernatants were stored overnight at -20°C.

The cell lysates were pre-immune cleared with normal rabbit serum and protein A sepharose beads (CL-4B - Pharmacia) or protein G sepharose beads (4 - Pharmacia) for 1 hour at 4°C. The lysates were then pre-immune cleared again with only protein A or G sepharose beads for 30 min at 4°C. Immunoprecipitations were then performed with normal rabbit serum (as a control) or the appropriate antibody, and protein A or G sepharose beads for 16 hours at 4°C. The sepharose beads were then washed several times in lysis buffer and boiled in Laemmli buffer (2% SDS; 10% glycerol; 100mM DTT; 60mM Tris pH6.8; and 0.001% bromophenol blue) to release the precipitated proteins. Samples were run on a 10% SDS-polyacrylamide gel, then fixed in 47% methanol and 7% acetic acid, and treated with Amplify fluorographic reagent (Amersham). The gel was then dried and the labelled proteins were visualised by fluorography.

The antibody used for detecting expression of the p53 proteins in different cells was pAb200-47 (which recognises rodent p53 (Dippold et al., 1981)). The antibodies used for determining the conformation of the p53 proteins were pAb246 (which specifically recognises conformations similar to wild-type mouse p53 (Yewdell et al., 1986)) and pAb240 (which specifically recognises mutant conformations (Gannon et al., 1990)). pAb200-47 was also used for immunoprecipitating complexes of LTag and p53,
while pAb419 (which recognises LTag (Harlow et al., 1981)) was used for immunoprecipitating complexes of LTag and pRb.

2.6 Evaluation of transcriptional regulation

The method used for measuring the regulation of gene transcription was adapted from that described by Sleigh, 1986. For this purpose, REFs were transfected with plasmid DNA (including a plasmid encoding chloramphenicol acetyl transferase (CAT)) through the use of CaPO$_4$ (as described in Section 2.2.3). 72 hours post-transfection, the cells were washed twice and harvested in cold PBS. After one more wash in PBS, the cells were resuspended in 200µl 250mM Tris.HCl pH7.8, and then lysed by three cycles of freezing in dry ice and thawing at 37°C. Cell debris was removed by centrifuging the lysates at 13000rpm for 15 min at 4°C, and the supernatants were stored overnight at -20°C.

To account for variations in the number of cells contributing to different samples, the total protein concentration of each cell lysate was estimated by its UV light absorbency at 280nm. The volume of lysate to be used in determining CAT activity was then adjusted so that the measurements could be based on an equal cell number. The cell lysates were then heated to 65°C to inactivate inhibitors of the CAT enzyme (Sleigh, 1986). CAT activity was determined by measuring the transfer of $^{14}$C]acetyl groups from $^{14}$C]acetyl CoA into chloramphenicol. The reaction therefore included: 20µl chloramphenicol; 20µl $^{14}$C]acetyl CoA mixture (0.05µCi $^{14}$C]acetyl CoA in 0.45mM cold acetyl CoA); 20-60µl cell lysate; and 0-40µl 250mM Tris.HCl pH7.5, so that the final volume was 100µl. After allowing the reaction to proceed for 2-6 hours at 37°C, the labelled chloramphenicol was extracted twice with 100µl cold ethyl acetate each time. To ensure that no labelled acetyl CoA was included, 100µl 250mM Tris.HCl pH7.5 was used for a back extraction on the combined organic phases. 100µl of the organic phase was then added to 4ml scintillation fluid and the amount of radioactivity was measured as the average number of counts per min from 5 min of scintillation counting.

2.7 Evaluation of SV40 origin-dependent DNA replication

The method used for evaluating the replication of DNA from SV40 origins of replication was adapted from that described by Braithwaite et al., 1987. For this purpose, COS cells were transfected with plasmid DNA (using plasmids which each contain an SV40 origin of replication) through the use of DEAE-dextran (as described in Section 2.2.4) and HeLa cells were transfected with plasmid DNA (using plasmids which each contain an SV40 origin as well as a plasmid which encodes LTag) through the use of CaPO$_4$ (as described in Section 2.2.3). 72 hours post-transfection, plasmid DNA was
extracted from the cells through the use of a modified small-scale plasmid preparation procedure. This involved the cells being washed twice in PBS and incubated at room temperature for 3 min in 500μl fresh solution 2 (200mM NaOH and 1% SDS). The lysed cells were then harvested and 360μl solution 3 (3M KAc pH4.8) was added to the cell lysate. From this point the preparation of plasmid DNA was continued as described in Section 2.1.3. The final DNA pellet was resuspended in 20μl TE.

The amount of replicated plasmid DNA (as opposed to the original transfected plasmid DNA) in each extract was determined through the use of a methylation-dependent restriction enzyme, DpnI. While the transfected plasmids (having been prepared in bacterial cells) were methylated and therefore sensitive to digestion by DpnI, the replicated plasmids (which were synthesised in COS cells) were unmethylated and therefore resistant to digestion by DpnI. Therefore, 9μl of DNA extract was mixed with 1.2μl 10x DpnI-specific buffer (Promega) and 0.5U DpnI (Promega) in a total volume of 12μl. The DpnI digest reactions were allowed to proceed at 37°C for 1 hour. The quantity of undigested replicated plasmid DNA was then determined by transformation of bacterial cells. The 12μl digest reaction was used to transform 200μl of thawed competent DH5 cells as described in Section 2.1.2. After plating out the cells and incubating the plates at 37°C overnight, the colonies of transformed cells were counted. The number of colonies of transformed cells is proportional to the number of intact (replicated) plasmids in the DNA sample.
Chapter 3

p53-dependent suppression of transformation: mechanisms are determined by the transforming oncoproteins
3.1 Introduction

As discussed in Section 1.3.2, p53 is induced in response to DNA damage to prevent the continued proliferation of cells which are genetically abnormal and consequently, highly susceptible to transformation. This role in suppressing tumourigenesis is reflected by the fact that high levels of p53 can suppress transformation by a number of viral and activated cellular oncoproteins (Eliyahu et al., 1989; Finlay et al., 1989). The mechanism by which transformation is suppressed probably involves a decrease in cell growth and/or an increase in cell death since p53 is known to arrest growth predominantly in the G1 phase of the cell cycle (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Michalovitz et al., 1990; Kastan et al., 1991) and under certain cellular conditions, induce an apoptotic response (Yonish-Rouach et al., 1991; Shaw et al., 1992; Clarke et al., 1993; Lowe et al., 1993b). However, the relationship between these cellular effects of p53 and the ability to suppress cell transformation remains unclear.

To investigate the cellular activities by which p53 suppresses transformation, different pathways of transformation have been examined for their susceptibility to suppression by p53. This study involved the transformation of cells with different combinations of oncoproteins including: wild-type and mutant forms of the SV40 large T antigen (LTag), the adenovirus E1a proteins (E1a), and an activated form of the ras protein (EJ-ras).

As a viral protein, LTag is critical for both the replication of SV40 DNA and for the transformation of SV40 infected cells (reviewed in: Fanning and Knippers, 1992). While most oncoproteins require the cooperative effects of a second oncoprotein to cause transformation, LTag is able to transform normal cells alone. This process appears to involve the binding and consequent inactivation of certain proteins which negatively regulate cell growth (refer to Section 1.3.1). Various studies (DeCaprio et al., 1988; Ewen et al., 1989; Peden et al., 1989; Srinivasan et al., 1989; Lin and Simmons, 1991a; Sompayrac and Danna, 1991; 1992; Yaciuk et al., 1991; Zhu et al., 1992) have shown that the functions of LTag which are important for the initiation and maintenance of the transformed phenotype are: the binding of the retinoblastoma susceptibility protein, pRb and two other closely related proteins, p107 and p130; the binding of p53; and possibly, the binding of other cellular proteins such as p300. However, there is some uncertainty about the necessity of binding to p53 for the stimulation of cell growth and transformation (Peden et al., 1989; Srinivasan et al., 1989; Sompayrac and Danna, 1991; 1992).

The E1a proteins are encoded by five differently spliced transcripts from the early region of human adenoviruses and are important for the transformation of adenovirus infected cells (reviewed in: Shenk and Flint, 1991). Like LTag, E1a contributes to the
process of transformation by binding and inactivating certain cellular proteins including pRb, p107, p130, and p300 (Whyte et al., 1988; 1989; Egan et al., 1989; Howe et al., 1990) (refer to Section 1.3.1). Although these functions enable E1a to stimulate cell growth by inducing the onset of S phase (Braithwaite et al., 1983; Bellett et al., 1985; Stabel et al., 1985), the cooperative effects of other oncoproteins are required for cells to be fully transformed (Ruley, 1983). Such oncoproteins include the adenovirus-encoded partners of E1a: E1b-55K and E1b-19K. Because E1a (in addition to stimulating cell growth) causes a susceptibility to apoptosis (Rao et al., 1992) which is mediated by p53 (Debbas and White, 1993; Lowe and Ruley, 1993), E1b-19K would be necessary for cell transformation as it is able to prevent this type of cell death (White et al., 1991; Rao et al., 1992) (refer to Sections 1.2.1 and 1.3.5). Since E1b-55K is known to bind and at least partially inactivate p53 (Sarnow et al., 1982; Yew and Berk, 1992), this protein might also be important for inhibiting the apoptotic response.

EJ-ras is similar to E1a in that it can stimulate cell growth (and various other transformation-related cellular activities) but is unable to transform normal cells alone (Newbold and Overall, 1983). However, since it will transform in cooperation with E1a (Ruley, 1983), EJ-ras represents a separate class of oncoproteins (refer to Section 1.3.5). The normal cellular ras protein has an important role in the transduction of biochemical signals from cell surface growth factor receptors to the nucleus (refer to Figure 1.2) through its ability to bind GDP and GTP, and act as a GTPase (reviewed in: Lowy and Willumsen, 1993). When certain mutations lock the protein into an active state, ras is converted into an oncoprotein which provides the cell with a continual growth signal.

LTag, E1a, and EJ-ras are distinguished by their differing abilities to bind p53 and pRb. LTag binds to both p53 and pRb, while E1a binds only to pRb, and EJ-ras binds to neither of these proteins. For this reason, the studies presented in this chapter have examined the effects of p53 on transformation involving each of these different oncoproteins. Through the use of mutant forms of LTag, the p53 and pRb binding properties of this protein were also investigated with respect to their roles in cell transformation. Furthermore, each of the above oncoproteins was examined for their effects on the survival and growth of cells and their responses to the growth inhibitory and apoptotic effects of p53.
3.2 Results

3.2.1 Transformation of REFs by LTag is refractory to suppression by p53

To explore the mechanisms by which p53 suppresses cell transformation, different pathways of transformation have been examined for their susceptibility to the suppressive effects of p53. This study was performed by transfecting REFs with plasmids encoding different transforming oncoproteins along with a plasmid encoding wild-type mouse p53. After 22-24 days, the frequency of cell transformation was assessed by counting the foci of transformed cells under a microscope (at 10x magnification).

In agreement with other reports (Eliyahu et al., 1989; Finlay et al., 1989), this study showed that wild-type but not mutant p53 reduced the frequency of foci generated by adenovirus E1a in cooperation with EJ-ras (Figures 3.1 and 3.2). In contrast, wild-type p53 did not reduce the frequency of focus formation by LTag (Figures 3.1 and 3.2). Since the failure of p53 to suppress transformation by LTag was observed in several independent experiments, one can conclude that transformation of REFs by LTag is refractory to suppression by p53.

3.2.2 Transformation of REFs by LTag requires the binding of pRb but not p53

Since transformation of REFs by LTag is refractory to suppression by p53, the process by which cells are transformed by this oncoprotein was investigated. Because this process is thought to involve the binding and consequent inactivation of both p53 and pRb (DeCaprio et al., 1988; Ewen et al., 1989; Peden et al., 1989; Srinivasan et al., 1989; Lin and Simmons, 1991a; Sompayrac and Danna, 1991; 1992), some mutant forms of LTag were examined for their ability to transform. The proteins studied were: 402DE (Figure 3.3A) which is defective for binding p53 (Figure 3.3B; Lin and Simmons, 1991b), and ΔRBBS and T-K1 (Figure 3.3A) both of which are defective for binding pRb (Figure 3.3C; DeCaprio et al., 1988).

This study showed that both ΔRBBS and T-K1 are defective for transformation of REFs. In comparison to an average of 24 foci (per dish) produced by wild-type LTag, ΔRBBS produced an average of only 2 foci, while T-K1 produced an average of only 5 foci (Figure 3.4). These data demonstrate that the binding (and presumed inactivation) of pRb is critical for transformation by LTag.
Figure 3.1. Transformation by LTag is refractory to suppression by p53.

To determine the frequency of focus formation, REFs were transfected (through the use of lipofectamine) with: 670ng CMVE1a (encodes E1a) and 670ng T24EJ6.6 (encodes EJ-ras), or 670ng RSV-T (encodes LTag) and 670ng CMVneo (control). In each case, the effects of p53 were assessed by co-transfection with either: 670ng CMVneo (control), 670ng CMVNC9 (encodes wild-type mouse p53), or 670ng CMVc5 (encodes mutant mouse p53). 22-24 days post-transfection, the cells were fixed and stained (with 0.1% crystal-violet in 20% ethanol), and the foci of transformed cells were counted. The average numbers of foci were determined from at least three independent experiments.
Figure 3.2. Examples of focus formation experiments which show that p53 suppresses transformation by various combinations of oncoproteins, but not by LTag. REFs were transfected with combinations of plasmids including: CMVE1a (encodes E1a), T24EJ6.6 (encodes EJ-ras), RSV-T (encodes wild-type LTag), and RSV-TΔRBBS (encodes LTag defective for binding pRb). In each case, the effects of p53 were assessed by co-transfection with CMVneo (control) or CMVnc9 (encodes p53). 22-24 days post-transfection, the cells were fixed and stained (refer to Figure 3.1 for more details), and the foci of transformed cells were counted under a microscope at 10x magnification (refer to figure 1.1 for photographs of magnified foci).
Figure 3.3. Mutant forms of LTag and their abilities to bind p53 or pRb.

(A) A diagrammatic representation of LTag. The binding sites for pRb and p53 are represented by the shaded regions. The mutant forms of LTag used in this study are indicated: ΔRBBS has a deletion of the pRb binding site, T-K1 has a point mutation within the pRb binding site, and 402DE has a point mutation within the p53 binding site. 

(B) Immunoprecipitations of LTag and p53 complexes. HeLa cells (which contain endogenous p53) were transfected (through the use of CaPO₄) with: 10μg CMVNc9 (encodes p53), along with 10μg of either CMVneo (control), RSV-T (encodes wild-type LTag), or RSV-T402DE (encodes mutant LTag). Proteins were immunoprecipitated with normal rabbit serum (NRS) or pAb200-47 (recognises p53). Complexes with p53 were observed with wild-type LTag, but were significantly reduced with 402DE. 

(C) Immunoprecipitations of LTag and pRb complexes. CV1 cells (which contain endogenous pRb) were transfected (through the use of CaPO₄) with: 20μg CMVneo (control) or 10μg CMVneo (control) and 10μg of either RSV-T (encodes wild-type LTag), RSV-TΔRBBS (encodes mutant LTag), or RSV-TK1 (encodes mutant LTag). Proteins were immunoprecipitated with normal rabbit serum (NRS) or pAb419 (recognises LTag). Complexes with pRb were observed with wild-type LTag, but not with either ΔRBBS or TK1.

NB: Due to low transfection efficiencies, REFs were unsuitable for demonstrating the formation of complexes between LTag and p53 or pRb. Because the abilities of wild-type and mutant forms of LTag to bind p53 and pRb are not expected to vary between different cell types, HeLa and CV1 cells were used for these experiments.
In the same experiments however, it was observed that the frequency of focus formation by 402DE was not significantly different to that caused by LTag (Figure 3.4). These data indicate that the binding of p53 is not required for LTag to transform REFs in this type of experiment. The possibility that any residual capacity of 402DE to bind (and inactivate) endogenous p53 (Figure 3.3B; Lin and Simmons, 1991b) might be sufficient for it to transform (by overcoming the suppressive effects of this protein) was then considered. This is not the case however, as the ability of 402DE to transform was retained even when the levels of p53 within the cell were greatly increased by the addition of exogenous p53 (Figure 3.4). These data therefore demonstrate that the binding and inactivation of p53 is not necessary for the transformation of REFs by LTag.

3.2.3 The pRb binding-defective forms of LTag transform in cooperation with either E1a or EJ-ras

Because E1a is known to bind pRb (Whyte et al., 1988), the defects in ARBBS and T-K1 should be complemented by the use of E1a in combination with each of these mutant forms of LTag. Although unable to transform REFs alone, E1a (as expected) could transform in cooperation with either ARBBS or T-K1 (Figures 3.2 and 3.5). Additionally, the presence of E1a was observed to enhance the frequency of focus formation by both LTag (by an average factor of 3.6 as compared to LTag alone) and 402DE (by an average factor of 2.6 as compared to 402DE alone) (Figure 3.5).

Surprisingly, EJ-ras (which does not bind pRb) was also found to transform REFs in cooperation with either ARBBS or T-K1 (Figures 3.2 and 3.5). Like E1a, EJ-ras also enhanced the frequency of focus formation by both LTag (by an average factor of 1.9 as compared to LTag alone) and 402DE (by an average factor of 1.7 as compared to 402DE alone) (Figure 3.5).

These data show that both E1a and EJ-ras transform in cooperation with either ARBBS or T-K1. However, since E1a and EJ-ras are known to cooperate with each other (Figures 3.1 and 3.2), these proteins must contribute to the process of transformation in different ways. Because ARBBS and T-K1 cooperate with both E1a and EJ-ras, it would appear that these mutant forms of LTag (though unable to transform alone) can complement two different pathways of transformation.

3.2.4 Binding of pRb but not p53 is necessary for LTag to overcome growth suppression by p53

The experiments described in Section 3.2.1 showed that p53 is unable to suppress transformation by LTag (Figures 3.1 and 3.2). Since the ability of a protein to induce or suppress cell transformation is closely linked with its ability to induce or
Figure 3.4. Transformation by LTag requires the binding of pRb but not p53. REFs were transfected with: RSV-T (encodes wild-type LTag), RSV-TΔRBBS (encodes LTag defective for binding pRb), RSV-TK1 (encodes LTag defective for binding pRb), or RSV-T402DE (encodes LTag defective for binding p53). In each case, the effects of p53 were assessed by co-transfection with CMVneo (control) or CMVnc9 (encodes p53). 22-24 days post-transfection, the average numbers of foci were determined from at least three independent experiments (refer to Figure 3.1 for more details).
Figure 3.5. Both E1a and EJ-ras transform cells in cooperation with wild-type or mutant forms of LTag. REFs were transfected with: CMVneo (control), RSV-T (encodes wild-type LTag), RSV-TΔRBBS (encodes LTag defective for binding pRb), RSV-TK1 (encodes LTag defective for binding pRb), or RSV-T402DE (encodes LTag defective for binding p53), along with: CMVneo (control), CMVE1a (encodes E1a), or T24EJ6.6 (encodes EJ-ras). 22-24 days post-transfection, the average numbers of foci were determined from at least three independent experiments (refer to Figure 3.1 for more details).
suppress cell growth, LTag was examined for its ability to stimulate cell growth and overcome any growth suppressing effects of p53. To observe the effects of exogenous proteins on the survival and growth of cells, REFs were transfected with the appropriate plasmids (including a plasmid containing the Tn5 gene which encodes for resistance to the neomycin family of antibiotics (Southern and Berg, 1982)) and the transfected cells were selected with the neomycin analog, G418. 18-20 days post-transfection, the cells were stained and the extent of cell growth was assessed by observing the size of colonies which arise from the transfected cells.

Thus, it was observed that in the dishes containing control cells, there were many colonies of G418-resistant cells. While in the presence of exogenous p53, the number of colonies was similar to that of the control cells, the overall cell population was significantly reduced as the individual colonies were much smaller in size (Figure 3.6). This study therefore demonstrated that the growth of REFs is suppressed by high levels of wild-type p53.

This study also showed that the presence of LTag provides cells with a strong growth signal as their average colony size of cells with LTag was much larger than that of the control cells. It was also found that co-expression of exogenous p53 with LTag made no significant difference to the size of the colonies (Figure 3.6). Thus, one can conclude that stimulation of cell growth by LTag overcomes the growth inhibitory effects of p53.

Because the transformation of REFs by LTag requires the binding of pRb but not p53 (Figure 3.4), these functions were investigated for their importance in the ability of LTag to stimulate cell growth and overcome p53-dependent growth inhibition. For this purpose, the growth of cells in the presence of the mutant forms of LTag (ΔRBBS, T-K1, and 402DE, as described in Section 3.2.2) was examined.

This study showed that in both the presence and absence of exogenous p53, the average colony size of cells with 402DE was very similar to that with LTag (Figure 3.6). Thus, it would appear that the ability of LTag to stimulate cell growth and overcome the growth inhibitory effects of p53 does not depend upon the binding of this protein. This study also demonstrated that in the presence of either ΔRBBS or T-K1, cells exhibited slower growth (than that observed in the presence of LTag) and remained susceptible to the growth suppressing effects of exogenous p53 (Figure 3.6). These observations indicate that the ability of LTag to overcome the growth inhibitory effects of p53 requires the binding of pRb.
Figure 3.6. Binding of pRb but not p53 is necessary for LTag to overcome growth suppression by p53. To evaluate the survival and growth of cells, REFs were transfected (through the use of lipofectamine) with: 670ng CMVneo (encodes for resistance to neomycin) along with 670ng of either: pBR322 (control), RSV-T (encodes wild-type LTag), RSV-TΔRBBS (encodes LTag defective for binding pRb), RSV-TK1 (encodes LTag defective for binding pRb), or RSV-T402DE (encodes LTag defective for binding p53). In each case, the effects of p53 were assessed by co-transfection with 670ng of pBR322 (control) or CMVNe9 (encodes p53). The transfected cells were selected with media containing 200μg/ml Geneticin G-418 (a neomycin analogue). 18-20 days post-transfection, the surviving cells were fixed and stained (with 0.1% crystal-violet in 20% ethanol). The observed patterns of cell survival and growth were found to be reproducible in at least three independent experiments.
3.2.5 Binding of pRb but not p53 is necessary for LTag to overcome transformation suppression by p53

Since the ability of LTag to overcome growth suppression by p53 requires the binding of pRb but not p53, these functions were investigated for their importance in the ability of LTag to overcome the transformation suppressing effects of p53. It was previously observed that both wild-type LTag and 402DE cause transformation which is completely refractory to suppression by high levels of p53 (Figure 3.4). Thus, one can conclude that the binding and inactivation of p53 is not necessary for LTag to overcome the suppressive effects of p53 on cell transformation.

To determine whether the binding of pRb is necessary for LTag to overcome the transformation suppressing effects of p53, a cooperating oncoprotein was required as neither ARBBS nor T-K1 could transform REFs alone. As shown in an earlier experiment, both E1a and EJ-ras are able to cooperate with these mutant forms of LTag (Figure 3.5).

The effects of exogenous p53 on transformation by E1a with the wild-type or mutant forms of LTag were therefore investigated. This study showed that when the frequency of transformation by LTag or 402DE was enhanced by E1a, there was a slight susceptibility to suppression by p53 (~20% reduction in focus formation - Figure 3.7A). Thus, it would appear that in the presence of E1a, LTag is not completely capable of overcoming the suppressive effects of p53 on transformation. In comparison to transformation by E1a with LTag or 402DE, it was observed that transformation by E1a with either ARBBS or T-K1 was considerably more susceptible to suppression by p53 (~50% reduction in focus formation - Figures 3.2 and 3.7A). While these data suggest that the binding of pRb might be more important than the binding of p53 for LTag to overcome the transformation suppressing effects of p53, they are complicated by the fact that E1a also binds to pRb. Even without this complication, the results are not conclusive as LTag cannot completely overcome suppression by p53 in the presence of E1a.

For these reasons, the effects of exogenous p53 on transformation by EJ-ras with the wild-type or mutant forms of LTag were investigated. This study showed that (as with transformation by LTag or 402DE alone) the transformation of REFs by EJ-ras with either LTag or 402DE remained refractory to suppression by p53 (on average, no reduction in focus formation - Figure 3.7B). In contrast, p53 was able to reduce the frequency of transformation by EJ-ras with either ARBBS or T-K1 (~55% reduction in focus formation - Figures 3.2 and 3.7B). These data clearly demonstrate that the ability of LTag to overcome the transformation suppressing effects of exogenous p53 requires the binding of pRb but not the binding of p53.
Figure 3.7. Binding of pRb but not p53 is necessary for LTag to overcome transformation suppression by p53. (A) REFs were transfected with CMVE1a (encodes E1a) along with: RSV-T (encodes wild-type LTag), RSV-TΔRBBS (encodes LTag defective for binding pRb), RSV-TK1 (encodes LTag defective for binding pRb), or RSV-T402DE (encodes LTag defective for binding p53). In each case, the effects of p53 were assessed by co-transfection with CMVneo (control) or CMVNe9 (encodes p53). 22-24 days post-transfection, the average numbers of foci were determined from at least three independent experiments (refer to Figure 3.1 for more details). In the presence of E1a, LTag cannot completely overcome the transformationsuppressing effects of p53. (B) REFs were transfected and treated as described above except that T24EJ6.6 (encodes EJ-ras) was used instead of CMVE1a. In the presence of EJ-ras, LTag requires the binding of pRb but not p53 to overcome the transformation-suppressing effects of p53.
3.2.6 Transformation involving E1a is susceptible to suppression by p53

In an earlier experiment, it was observed that E1a cannot transform alone, but will cooperate with the wild-type or mutant forms of LTag (Figure 3.5). Since LTag and 402DE can already transform, E1a would probably enhance the frequency of foci formation by ensuring that a critical level of transforming activity is reached in a larger number of cells. However, E1a can also cooperate with ΔRBBS or T-K1 both of which are unable to transform REFs alone. This indicates that although these mutant forms of LTag are defective for binding pRb, they must retain a function which cooperates with E1a in the process of transformation. Since both ΔRBBS and T-K1 remain capable of binding (and presumably inactivating) p53 (Figure 3.3A), it is possible that inactivation of endogenous p53 is the activity which allows E1a to transform. Although it has been shown that transformation of REFs by LTag does not require the binding of p53 (Figure 3.4), this activity might still be important for transformation by E1a.

To investigate this possibility, E1a had to be examined for its ability to transform cells in the absence of wild-type p53. It is well established that some mutant p53 proteins have a transdominant effect by oligomerization and consequent inactivation of wild-type p53 proteins (Milner and Medcalf, 1991; Milner et al., 1991). A C-terminal fragment of p53 (p53ct) which encompasses the oligomerization domain of the protein (Shaulian et al., 1992; Reed et al., 1993) was therefore used to inactivate wild-type p53 within the cells.

This study showed that p53ct is unable to transform alone, but will cooperate with E1a in the transformation of REFs (Figure 3.8A). Additionally, it was demonstrated that neither ΔRBBS nor T-K1 are capable of cooperating with p53ct. This observation supports the argument that each of these proteins contributes to transformation through a similar function, that is the binding and consequent inactivation of endogenous p53. Since E1a can cooperate with two different types of proteins both of which bind and inactivate wild-type p53, it would appear that E1a is capable of transforming cells in which the endogenous p53 has been functionally disrupted.

This study also showed that the introduction of exogenous p53 reduced the frequency of transformation by E1a in cooperation with ΔRBBS, T-K1, or p53ct (~45% reduction in focus formation - Figure 3.8A). With high levels of p53 within the cells, it would appear that at least some of the wild-type p53 is not bound by ΔRBBS, T-K1, or p53ct and therefore remains capable of suppressing transformation. These data demonstrate that the transformation stimulating effects of E1a (in contrast to those of LTag) are susceptible to suppression by p53.
Figure 3.8. (A) Transformation involving E1a is suppressed by p53. REFs were transfected with combinations of plasmids including: CMVneo (control), CMVE1a (encodes E1a), RSV-TΔRBBS (encodes LTag defective for binding pRb), RSV-TK1 (encodes LTag defective for binding pRb), and MSV280-390 (encodes the C-terminal region of p53, p53ct). In each case, the effects of p53 were assessed by co-transfection with CMVneo (control) or CMVNc9 (encodes p53). 22-24 days post-transfection, the average numbers of foci were determined from at least three independent experiments (refer to Figure 3.1 for more details). (B) Expression of E1a induces cell death. REFs were transfected with: CMVneo (encodes for neomycin resistance) along with pBR322 (control) or CMVE1a (encodes E1a). In each case, the effects of p53 were assessed by co-transfection with pBR322 (control) or CMVNc9 (encodes p53). Transfected cells were selected with G418. The patterns of cell survival and growth were observed 18-20 days post-transfection and found to be reproducible in at least three independent experiments (refer to Figure 3.6 for more details).
**A**

![Bar chart showing number of foci per dish for different conditions: Ela, Ela + ΔRBBS, Ela + T-K1, ΔRBBS + p53ct, T-K1 + p53ct, Ela + p53ct.]

- **control**
- **p53**

**B**

![Images illustrating the effect of control and p53 on colony formation.]

- **control**
- **p53**

---

*Ela control*
3.2.7 E1a reduces the survival of cells by p53-mediated apoptosis

To investigate why the transformation-related activities of E1a are ineffective in the presence of active p53, E1a was examined for its effects on the survival and growth of cells (in the absence and presence of exogenous p53) by using the selection of transfected cells, as described in Section 3.2.4. In the dishes containing the control cells, there were many colonies of G418-resistant cells. However, in the presence of E1a, the number of colonies arising from the transfected cells was dramatically reduced (Figure 3.8B). Unlike p53 whose growth inhibitory effects were observed as a reduction in the size of the colonies (refer to Section 3.2.4), E1a caused a reduction in the survival of REFs. This massive decline in cell survival might be explained by a significant increase in the rate of cell death since E1a is known to induce apoptosis (Rao et al., 1992) which is mediated by endogenous p53 (Debbas and White, 1993; Lowe and Ruley, 1993).

If the observed effects of E1a are due to a p53-mediated apoptotic response, they should be inhibited by the presence of either p53ct which can abrogate the functions of the endogenous wild-type p53 (refer to Section 3.2.6) or bcl-2 or E1b-19K both of which are known to prevent apoptosis (Hockenbery et al., 1990; White et al., 1991; Rao et al., 1992). The survival and growth of cells in the presence of E1a along with either bcl-2, E1b-19K or p53ct was therefore examined. This experiment showed that bcl-2, E1b-19K and p53ct can all reverse the effects of E1a as each protein promoted the survival and growth of REFs (Figure 3.9). Thus, one can conclude that E1a reduces the number of colonies by causing the cells to die through p53-mediated apoptosis. This study has therefore indicated that p53 suppresses or prevents transformation involving E1a by mediating an apoptotic response.

3.2.8 EJ-ras overcomes E1a-induced apoptosis

While neither E1a nor EJ-ras can transform alone, the cooperative effects of these two proteins allows the transformation of REFs (Figures 3.1 and 3.2). Since transformation by E1a alone is prevented by p53-mediated apoptosis, EJ-ras may cooperate with E1a by overcoming the apoptotic response. This possibility was investigated by examining the survival and growth of REFs which express both E1a and EJ-ras. Since this study showed that EJ-ras promoted cell survival and growth in the presence of E1a (Figure 3.10), one can conclude that EJ-ras can inhibit apoptosis which is induced by E1a and mediated by endogenous p53.
Figure 3.9. E1a-induced cell death occurs through p53-mediated apoptosis. REFs were transfected with: CMVneo (encodes for neomycin resistance) along with pBR322 (control) or CMVE1a (encodes E1a). Inhibition of E1a-induced cell death was attempted by co-transfection with: CMVbcl-2 (encodes bcl-2), CMV19K (encodes E1b-19K), or MSV280-390 (encodes the C-terminal region of p53, p53ct). Transfected cells were selected with G418. The patterns of cell survival and growth were observed 18-20 days post-transfection and found to be reproducible in at least three independent experiments (refer to Figure 3.6 for more details). Since the cytotoxic effect of E1a can be inhibited by either bcl-2, E1b-19K (both of which are known to inhibit apoptosis), or p53ct (which abrogates the activity of endogenous p53), it can be attributed to p53-mediated apoptosis.
Figure 3.10. **EJ-ras overcomes E1a-induced apoptosis.** REFs were transfected with: CMVneo (encodes for neomycin resistance) along with pBR322 (control) or CMVE1a (encodes E1a). The effect of EJ-ras on E1a-induced cell death was assessed by co-transfection with T24EJ6.6 (encodes EJ-ras). Transfected cells were selected with G418. The patterns of cell survival and growth were observed 18-20 days post-transfection and found to be reproducible in at least three independent experiments (refer to Figure 3.6 for more details).
3.2.9 Transformation involving EJ-ras is susceptible to suppression by p53

In an earlier experiment it was observed that EJ-ras cannot transform alone but will cooperate with the wild-type or mutant forms of LTag (Figure 3.5). As is the case for E1a (refer to Section 3.2.6), EJ-ras would probably enhance the frequency of focus formation with LTag or 402DE by ensuring that a critical level of transforming activity is reached in a larger number of cells. However, EJ-ras is also capable of cooperating in the transformation of REFs with ΔRBBS or T-K1 both of which are unable to transform alone, but remain capable of binding (and presumably inactivating) p53.

The possibility that EJ-ras is similar to E1a in that it requires the inactivation of endogenous p53 for transformation was therefore investigated. For this purpose, EJ-ras was examined for an ability to transform cells in which the functions of wild-type p53 have been disrupted by p53ct. Since this study showed that REFs can be transformed by the cooperative effects of p53ct and EJ-ras (Figure 3.11A), one can conclude that EJ-ras is capable of transforming cells in which the endogenous p53 has been inactivated.

This study also showed that the introduction of exogenous p53 reduces the frequency of transformation by EJ-ras in cooperation with either ΔRBBS, T-K1, or p53ct (~55% reduction in focus formation - Figure 3.11A). As noted in Section 3.2.6, it would appear that with high levels of p53, some of the wild-type p53 is not bound by ΔRBBS, T-K1, or p53ct and therefore remains capable of suppressing cell transformation. These data demonstrate that the transformation stimulating effects of EJ-ras (like those of E1a) are susceptible to suppression by p53.

3.2.10 Stimulation of cell growth by EJ-ras is suppressed by p53

To investigate why the transformation-related activities of EJ-ras are ineffective in the presence of active p53, EJ-ras was examined for its effects on cell growth (in the absence and presence of exogenous p53) by using the selection of transfected cells, as described in Section 3.2.4. While there were many colonies of G418-resistant cells in the control cell populations, the growth inhibitory effects of exogenous p53 were indicated by the reduction in the size (but not the number) of colonies. The presence of EJ-ras also did not affect the number of colonies, but caused a significant increase in colony size. Thus, EJ-ras was shown to stimulate the growth of REFs. However, the co-expression of exogenous p53 with EJ-ras still reduced the overall cell population as the colonies were generally smaller in size (Figure 3.11B). One can therefore conclude that cells which express EJ-ras remain susceptible to the growth suppressing effects of p53. This study has therefore indicated that p53 suppresses or prevents transformation involving EJ-ras by inhibiting cell growth rather than inducing cell death.
Figure 3.11. (A) Transformation involving EJ-ras is suppressed by p53. REFs were transfected with combinations of plasmids including: CMVneo (control), T24EJ6.6 (encodes EJ-ras), RSV-TΔRBBS (encodes LTag defective for binding pRb), RSV-TK1 (encodes LTag defective for binding pRb), and MSV268-590 (encodes the C-terminal region of p53, p53ct). In each case, the effects of p53 were assessed by co-transfection with CMVneo (control) or CMVNe9 (encodes p53). 22-24 days post-transfection, the average numbers of foci were determined from at least three independent experiments (refer to Figure 3.1 for more details).

(B) Stimulation of cell growth by EJ-ras is suppressed by p53. REFs were transfected with: CMVneo (encodes for neomycin resistance) along with pBR322 (control) or T24EJ6.6 (encodes EJ-ras). In each case, the effects of p53 were assessed by co-transfection with pBR322 (control) or CMVNe9 (encodes p53). Transfected cells were selected with G418. The patterns of cell survival and growth were observed 18-20 days post-transfection and found to be reproducible in at least three independent experiments (refer to Figure 3.6 for more details).
A

number of foci/dish

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<td>EJ-ras + p53ct</td>
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B

control

p53

control EJ-ras
3.3 Discussion

3.3.1 Binding of p53 is not necessary for LTag to transform and overcome suppression by p53

Transformation of cells by LTag is thought to involve the binding of p53 as some of the functions of this transformation suppressing protein are abrogated by this interaction (Bargonetti et al., 1992; Mietz et al., 1992; Jiang et al., 1993a; Segawa et al., 1993). However, the studies described in this chapter have demonstrated that the binding (and at least partial inactivation) of endogenous p53 is not required for transformation of REFs by LTag (Figure 3.4). While this finding is in conflict with the results of some studies (Peden et al., 1989; Srinivasan et al., 1989; Lin and Simmons, 1991a; Zhu et al., 1992), it is nonetheless in agreement with others (Peden et al., 1989; Srinivasan et al., 1989; Sompayrac and Danna, 1991; 1992). Such inconsistencies in the literature may occur due to differences in the types of cells and/or quantities of mutant forms of LTag used in these studies.

The results of this chapter have also shown that in contrast to transformation by other oncoproteins, transformation by LTag is refractory to suppression by exogenous p53 (Figures 3.1, 3.2, and 3.4). Thus, the transformation suppressing effects of p53 are overcome by LTag, even in the presence of EJ-ras (Figure 3.7B) whose transformation-related activities are normally susceptible to suppression by p53 (Figure 3.11A). Whether it acts alone or in combination with EJ-ras, a p53 binding-defective form of LTag (402DE) is able to transform as effectively as wild-type LTag (Figures 3.4 and 3.7B). These observations demonstrate that LTag does not need to bind p53 in order to overcome its anti-proliferative effects. This finding complements other reports which have shown that mutation or deletion of the p53 binding site can reduce but certainly not eliminate the capacity of LTag to overcome the growth inhibitory effects of p53 (Michael-Michalovitz et al., 1991; Quartin et al., 1994).

Since both of these studies and the study presented in this chapter all involved REFs and mouse p53, the observations might be specific to the cell type and/or species from which the p53 was derived. Alternatively, the results could indicate that the binding (and probable inactivation) of p53 is redundant in the normal functioning of LTag. This is in agreement with other reports which suggest that several independent functions of LTag contribute to its complete growth stimulatory effect, and that some of these functions are dispensable as the loss of one activity can be compensated by another (Dobbelstein et al., 1992; Dickmanns et al., 1994). An alternative but not incompatible hypothesis is that the binding and inactivation of p53 is a reserve mechanism by which LTag allows the continued growth of cells with very high levels of p53. Such a mechanism may be useful as p53 is known to accumulate in SV40 transformed cells (Oren et al., 1981; Duthu et al.,...
1985). Since a common characteristic of transformation by LTag is the presence of chromosomal aberrations (Ray et al., 1990; Stewart and Bacchetti, 1991) which would be accompanied by DNA damage, an accumulation of p53 could be expected. The continued survival and growth of these transformed cells might therefore require an additional mechanism for overcoming the anti-proliferative effects of p53.

### 3.3.2 Binding of pRb is necessary for LTag to transform and overcome suppression by p53

In agreement with other reports (DeCaprio et al., 1988; Ewen et al., 1989), the studies in this chapter have shown that transformation of REFs by LTag requires a functional pRb binding site (Figure 3.4). Because the segment of LTag which binds pRb can also interact with either p107 or p130 (Dyson et al., 1989a; Ewen et al., 1989), the binding of these proteins might also be critical in the process of transformation. p107, p130 and pRb are all homologous in sequence (Li et al., 1993) and each is able to interact with the cell cycle regulating protein, E2F (Cao et al., 1992; Shirodkar et al., 1992; Cobrinik et al., 1993). Therefore, at least some of the activities which have been attributed to pRb may also apply to p107 and p130.

As discussed in Section 1.3.3, pRb regulates cell growth by inhibiting their progression from G1 phase into S phase of the cell cycle (DeCaprio et al., 1989; Goodrich et al., 1991). At the beginning of G1, pRb is activated due to hypophosphorylation (Buchkovich et al., 1989; Chen et al., 1989; Mihara et al., 1989) so that it can bind and inactivate E2F (Hiebert et al., 1992; Shirodkar et al., 1992; Schwarz et al., 1993). At the end of G1, hyperphosphorylation and consequent inactivation of pRb (Buchkovich et al., 1989; Chen et al., 1989; Mihara et al., 1989) allows the release of E2F which initiates S phase (Johnson et al., 1993a) by stimulating the expression of proteins required for DNA replication (reviewed in: Nevins, 1992).

This account of pRb function may be over simplified as pRb is known to bind several proteins including other members of the E2F family (Ivey-Hoyle et al., 1993; Lees et al., 1993). However, the described series of events is able to explain why transformation by LTag requires the binding of pRb. At the beginning of G1 phase, the selective binding of LTag with hypophosphorylated forms of pRb (Ludlow et al., 1989; 1990) causes the immediate release of E2F (Chellappan et al., 1992). While this response would benefit the replication of viral DNA, it would also stimulate the growth of cells by allowing them to bypass regulatory events which occur during G1. This effect is indicated by cell cycle analyses which have shown that the proportion of SV40-infected cells in G1 phase is greatly reduced in comparison to that of normal cells (Gershey, 1979; Hiscott and Defendi, 1980).
The studies described in this chapter have demonstrated that LTag requires the binding of pRb not only for cell transformation but also for overcoming the anti-proliferative effects of exogenous p53. pRb binding-defective forms of LTag (ARBBS and T-K1) have limited abilities to stimulate cell growth and are therefore unable to overcome p53-dependent growth inhibition (Figure 3.6). When transforming in cooperation with EJ-ras, ARBBS and T-K1 (in contrast to wild-type LTag) are also incapable of overcoming the suppressive effects of p53 on transformation (Figures 3.2 and 3.7B). These results suggest that p53 can only suppress cell growth and transformation when pRb is not bound by LTag. Thus, it would appear that pRb actually mediates the growth inhibitory effects of p53.

This finding might be explained by the observations that growth arrest by p53 in G1 phase (Mercer et al., 1990; Michalovitz et al., 1990; Kastan et al., 1991; Martinez et al., 1991) is associated with the ability of p53 to activate the expression of p21WAF1 (El-Deiry et al., 1993; 1994) which inhibits the activities of cyclin-dependent kinases, cdk2 or cdk4 in complexes with the A, D1, D2, or E-type cyclins (Harper et al., 1993; Xiong et al., 1993; Dulic et al., 1994) (refer also to Sections 1.3.2 and 1.3.4). Since these complexes are responsible for the hyperphosphorylation of pRb in late G1 (Hinds et al., 1992; Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993), overexpression of p21WAF1 causes pRb to remain hypophosphorylated (Harper et al., 1993). High levels of p53 can therefore maintain pRb in an active state (Slebos et al., 1994), so that E2F is not released and the cells are prevented from entering S phase. Thus, the requirement of pRb in a p53-dependent G1 arrest is able to explain why the binding and inactivation of this protein by LTag can overcome the growth inhibitory effects of exogenous p53 (refer to Figure 3.12).

3.3.3 Transformation involving E1a is suppressed by p53-dependent apoptosis

Although the adenovirus E1a proteins are also known to bind pRb (Whyte et al., 1988), their transformation-related activities remain susceptible to suppression by p53. The studies described in this chapter have shown that E1a cannot transform REFs alone, but will cooperate with proteins which bind and inactivate endogenous p53. However, when high levels of exogenous p53 are introduced, transformation is suppressed (Figure 3.8A). These results are consistent with the observation that in contrast to their normal counterparts, mouse embryo fibroblasts (MEFs) which are genetically deficient for p53, can be transformed by E1a alone (Lowe et al., 1994).

A p53-induced, pRb-mediated growth arrest is unable to explain why the presence of active p53 prevents the transformation of cells by E1a. Like LTag, E1a stimulates cell proliferation by binding to pRb (Egan et al., 1989; Whyte et al., 1989;
pRb is hypophosphorylated and therefore able to repress the activities of E2F. At the end of G1, the hyperphosphorylation of pRb allows the release of E2F so that S phase is induced. Growth arrest by p53 is mediated by pRb and may involve the activation of p21WAF1 which inhibits cyclin dependent kinases (cdk). This would prevent the hyperphosphorylation of pRb so that E2F is not released and cells are prevented from entering S phase. LTag is able to overcome the growth inhibitory effects of p53 by binding and inactivating pRb (refer to Section 3.3.2 for more details).

Figure 3.12. Cells which express LTag are refractory to p53-dependent growth inhibition due to inactivation of pRb. During G1 phase, pRb is hypophosphorylated and therefore able to repress the activities of E2F. At the end of G1, the hyperphosphorylation of pRb allows the release of E2F so that S phase is induced. Growth arrest by p53 is mediated by pRb and may involve the activation of p21WAF1 which inhibits cyclin dependent kinases (cdk). This would prevent the hyperphosphorylation of pRb so that E2F is not released and cells are prevented from entering S phase. LTag is able to overcome the growth inhibitory effects of p53 by binding and inactivating pRb (refer to Section 3.3.2 for more details).
Howe et al., 1990) and releasing E2F (Chellappan et al., 1992; Zamanian and La Thangue, 1992). This causes an early onset of S phase as reflected by cell cycle analyses which show that in comparison to normal cells, the proportion of adenovirus E1a-infected cells in G1 phase is significantly reduced (Braithwaite et al., 1983; Bellett et al., 1985). Thus, E1a should overcome any p53-dependent growth inhibitory effects which rely upon the maintenance of active pRb in late G1. In support of this hypothesis, E1a has been shown to prevent an arrest of cell growth in the presence of p53 (Lowe et al., 1993a; Vousden et al., 1993).

An explanation then for the ability of p53 to suppress the transformation-related activities of E1a, could be that p53 causes cells to die through apoptosis (refer to Figure 3.13). In addition to stimulating cell growth, E1a is known to induce a pathway of apoptosis (Rao et al., 1992) which is mediated by p53 (Debbas and White, 1993; Lowe and Ruley, 1993). In p53-deficient MEFs, abrogation of this apoptotic response permits transformation by E1a alone (Lowe et al., 1994). In the studies on the survival and growth of REFs described in this chapter, the apoptotic activity of E1a was reflected by a massive decline in the number of colonies arising from surviving cells (Figure 3.8B). The cause of this cell death must be an apoptotic response as the cells were rescued by co-expression of either bcl-2 or E1b-19K both of which are known to inhibit apoptosis (Hockenbery et al., 1990; White et al., 1991; Rao et al., 1992) (Figure 3.9). Additionally, this effect of E1a must be mediated by endogenous p53 as it was also reversed by co-expression of p53ct which binds and inactivates wild-type p53 (refer to Section 3.2.6) (Figure 3.9).

3.3.4 Inhibition of apoptosis is important for transformation involving E1a or LTag

The studies described in this chapter have also demonstrated that E1a enhances the frequency of transformation by both LTag and 402DE (Figure 3.5) and that these transformation events are relatively refractory to suppression by p53 (Figure 3.7A). If transformation involving E1a is suppressed by p53-mediated apoptosis, then these observations indicate that apoptosis must be inhibited in the presence of LTag. An anti-apoptotic activity of LTag has actually been demonstrated and attributed to the binding and inactivation of p53 (Hermeking et al., 1994; Symonds et al., 1994). However, the studies in this chapter suggest that binding of p53 is not the only SV40-encoded function which inhibits apoptosis as transformation by E1a and the p53-binding defective form of LTag (402DE) is also relatively refractory to suppression by p53 (Figure 3.7A).

A probable explanation for an anti-apoptotic function being associated with LTag is that a separate function of this oncoprotein causes a susceptibility to apoptosis which must be overcome for transformation to occur. Increased susceptibility to apoptosis in
Figure 3.13. Cells which express E1a are refractory to p53-dependent growth inhibition but susceptible to p53-dependent apoptosis. Like LTag, E1a is able to bind and inactivate pRb and consequently overcome growth arrest by p53. However, a susceptibility to the apoptotic effects of p53 is caused by the inactivation of pRb (refer to Figure 3.12 and Section 3.3.3 for more details).
cells expressing either E1a or LTag is consistent with reports which show that an apoptotic response is induced by the functional inactivation of pRb (Howes et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Symonds et al., 1994; Williams et al., 1994a; Almasan et al., 1995) (refer also to Section 1.3.3). This response would probably be caused by the consequent increased activity of E2F as this protein is known to mediate apoptosis in cooperation with p53 (Qin et al., 1994; Wu and Levine, 1994). Accordingly, the re-introduction of active pRb (and repression of E2F activity) in cells where it had been previously inactivated is able to protect cells from p53-mediated apoptosis (Haupt et al., 1995a).

While the binding of pRb allows both LTag and E1a to overcome the growth inhibitory effects of p53 (refer to Sections 3.3.2 and 3.3.3), this function also causes a susceptibility to p53-mediated apoptosis. Thus, it would appear that when p53 fails to induce a pRb-mediated growth arrest, apoptosis is induced as a reserve mechanism for suppressing the transformation of cells. Transformation involving E1a therefore requires an additional oncoprotein to overcome apoptosis by endogenous p53, and there remains a susceptibility to apoptosis by high levels of exogenous p53. However, since both the growth inhibitory and apoptotic effects of p53 are abrogated by the activities of LTag, transformation occurs by LTag alone and remains refractory to suppression by high levels of p53 (refer to Figure 3.15).

3.3.5 Transformation involving EJ-ras is suppressed by p53-dependent growth inhibition

The studies described in this chapter have demonstrated that the transformation-related activities of EJ-ras are similar to those of E1a in that they are also susceptible to suppression by p53. While EJ-ras is unable to transform REFs alone, it will cooperate with proteins which can bind and inactivate endogenous p53. However, when high levels of exogenous p53 are introduced, transformation is suppressed (Figure 3.11 A). Furthermore, the studies have shown that EJ-ras differs to E1a in that it can stimulate the growth of REFs without increasing the rate of cell death. However, since this growth stimulus is suppressed by the addition of exogenous p53 (Figure 3.11B), one can suggest that p53 suppresses the transformation-related activities of EJ-ras by inhibiting cell growth (refer to Figure 3.14). These findings are in agreement with a report which indicated that abrogation of a growth arrest induced by endogenous p53 was necessary for transdominant mutant p53 proteins to transform cells in cooperation with EJ-ras (Hicks et al., 1991). Also in agreement with the results of this study are the observations that the growth of cells which have been transformed by activated ras and a temperature sensitive (and transdominantly-acting) mutant form of p53 is suppressed when shifted to a temperature at which the mutant p53 becomes wild-type (Michalovitz et
Figure 3.14. Cells which express EJ-ras are susceptible to p53-dependent growth inhibition. Although EJ-ras can stimulate cell growth, it cannot overcome growth arrest by p53 (refer to Figure 3.12 and Section 3.3.5 for more details).
al., 1990; Martinez et al., 1991). Although the activated ras protein is a GTPase, which provides cells with a continual growth signal through cytoplasmic signal transduction pathways (reviewed in: Lowy and Willumsen, 1993), the evidence suggests that cells expressing EJ-ras remain susceptible to the growth inhibitory effects of p53.

3.3.6 E1a and EJ-ras cooperate by overcoming functions of p53

Since transformation by E1a is prevented by p53-mediated apoptosis and transformation by EJ-ras is prevented by p53-mediated growth inhibition, the cooperative effects of E1a and EJ-ras may be explained by each of these proteins overcoming the suppressive effects of endogenous p53 on the other protein. As discussed in Section 3.3.3, E1a is able to prevent p53-induced growth arrest by binding and inactivating pRb (Lowe et al., 1993a; Vousden et al., 1993). In turn, EJ-ras would need to inhibit the apoptotic response caused by endogenous p53 in the presence of E1a. However, since transformation by E1a and EJ-ras is suppressed by exogenous p53, it could be expected that high levels of p53 would cause apoptosis despite the presence of EJ-ras (refer to Figure 3.15).

The studies described in this chapter have demonstrated EJ-ras is capable of suppressing apoptosis which is induced by E1a and mediated by endogenous p53 (Figure 3.10). Although some reports have indicated that activated ras has no anti-apoptotic activity (Lowe and Ruley, 1993; Lowe et al., 1994), the findings of this chapter are consistent with those of a recent study which showed that activated ras is able to prevent cells from undergoing p53-mediated apoptosis, while the presence of E1a is required for overcoming p53-dependent growth inhibition (Lin et al., 1995).

3.3.7 p53 suppresses cell transformation either by growth inhibition or by apoptosis

The studies described in this chapter have demonstrated that the binding of p53 is not necessary for LTag to transform REFs and resist p53-dependent suppression of cell growth and transformation. The binding of pRb however, is critical for these activities of LTag. Thus, one can conclude that pRb mediates a suppressive effect of p53 on the growth and transformation of cells. While E1a can also bind pRb and prevent growth inhibition by p53, the transformation-related activities of E1a are suppressed by p53-mediated apoptosis. Although EJ-ras can overcome apoptosis which is induced by E1a and mediated by endogenous p53, the transformation-related activities of EJ-ras are suppressed by p53-dependent growth inhibition. The studies in this chapter have therefore contributed in defining the requirements for cooperation between different oncoproteins. Thus, p53 suppresses cell transformation either by inducing a growth
arrest (which is mediated by pRb in the late G1 phase of the cell cycle) or by causing apoptosis (refer to Figure 3.15). The mechanism used by p53 to suppress transformation appears to be determined by the activities of the transforming oncoprotein(s) and in particular, the status of pRb within the cell.
Figure 3.15. The mechanism by which p53 suppresses cell transformation (pRb-mediated growth arrest or apoptosis) is determined by the transforming oncoproteins. p53 can suppress oncogenic transformation either by arresting cell growth (in the presence of active pRb) or by inducing apoptosis (in the absence of active pRb). Ela overcomes p53-dependent growth inhibition but causes a susceptibility to p53-dependent apoptosis, while EJ-ras prevents apoptosis (by low-levels of p53) but cannot overcome p53-dependent growth inhibition. LTag is able to overcome both of the anti-proliferative effects of p53 (refer to Figures 3.12 to 3.14 and Sections 3.3.2 to 3.3.7 for more details).
Chapter 4

Inhibition of cell growth and induction of apoptosis: genetically separable functions of p53
4.1 Introduction

High levels of exogenous p53 are known to suppress the growth of cells derived from various types of cancers including colorectal carcinoma, osteosarcoma, breast carcinoma, lung adenocarcinoma, glioblastoma and chronic myelogenous leukaemia (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Casey et al., 1991; Pietenpol et al., 1994; Zhang et al., 1994b). While the endogenous p53 in most of these cells has been either lost or inactivated, the growth suppressive effects of exogenous of p53 are not confined to cells which are normally deficient for the expression of this protein (Baker et al., 1990; Mercer et al., 1990). Such is the case with normal rat embryo fibroblasts which have been transformed by activated ras and a temperature sensitive (and transdominantly-acting) mutant form of p53. When shifted to a temperature at which the mutant p53 becomes wild-type, these cells are subject to a suppression of growth (Michalovitz et al., 1990; Martinez et al., 1991). Additionally, the growth inhibitory effects of endogenous p53 can be observed (in normal fibroblasts, in haematopoietic cells, and in various cancer cells) when there is an accumulation of this protein in response to the damage of DNA caused by ionising irradiation (Kastan et al., 1991; Kuertbitz et al., 1992; Di Leonardo et al., 1994; Dulic et al., 1994; El-Deiry et al., 1994). Thus, p53-dependent growth inhibition appears to be important for suppressing tumourigenesis by preventing the continued proliferation of genetically damaged cells.

While there is some evidence for p53 causing a growth arrest in the G2 phase of the cell cycle (Michalovitz et al., 1990; Stewart et al., 1995), the growth suppressive effects of this protein occur predominantly in G1 phase. Cells with high levels of p53 have been shown to accumulate in G1 phase (Mercer et al., 1990; Michalovitz et al., 1990; Kastan et al., 1991; Martinez et al., 1991), with the point of arrest occurring before the cells become committed to replicating their DNA (Lin et al., 1992). In studies which have used exogenous p53, the withdrawal of this protein allows the growth arrest to be reversed and the cells proceed into S phase (Mercer et al., 1990; Michalovitz et al., 1990; Lin et al., 1992). However, the G1 arrest which is induced by endogenous p53 in response to ionising irradiation (though originally thought to be temporary (Kastan et al., 1991)) appears to be prolonged and probably irreversible (Di Leonardo et al., 1994).

In addition to inhibiting cell growth, high levels of exogenous p53 have been shown to cause apoptosis in cells derived from various types of haematopoietic malignancies including myeloid leukaemia, erythroleukaemia, and Burkitt lymphoma (Yonish-Rouach et al., 1991; Ramqvist et al., 1993; Ryan et al., 1993). Cells from other types of cancers (including cervical carcinoma and colon carcinoma) which have previously lost endogenous p53 are also susceptible to this type of cell death in the presence of exogenous p53 (Shaw et al., 1992; Haupt et al., 1995a). While p53 appears to have a role in the elimination of cells through apoptosis, mice which are genetically
deficient for p53 are born phenotypically normal (Donehower et al., 1992) despite the fact that apoptotic cell death is required for normal embryonal development. This observation indicates that p53 may be important for apoptosis only in specific circumstances which (as discussed below) tend to involve a requirement for the suppression of tumourigenesis.

p53 appears to be necessary for an apoptotic response which occurs after ionising irradiation as thymocytes and intestinal epithelial cells which are deficient for p53 are resistant to this type of apoptosis (Clarke et al., 1993; Lowe et al., 1993b; Clarke et al., 1994). Thus, the activation of p53 in response to DNA damage may prevent the proliferation of genetically abnormal cells through growth inhibition and / or apoptotic cell death. An apoptotic function of p53 may also be important for regulating the size of particular cell populations in response to a limitation in the supply of survival factors (Oren, 1994). Evidence for this has come from various types of haematopoietic cells whose survival depends upon the presence of certain interleukins. In the absence of these survival factors, the cells undergo apoptosis which is mediated by p53 (Yonish-Rouach et al., 1991; Johnson et al., 1993b; Levy et al., 1993; Gottlieb et al., 1994; Canman et al., 1995). Another type of apoptotic response which depends upon p53 is that which occurs when there are viral or genetic disruptions to the functions of pRb (Howes et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Symonds et al., 1994; Williams et al., 1994a; Almasan et al., 1995) and substantial increases in the activity of E2F (Qin et al., 1994; Wu and Levine, 1994) (refer to Sections 1.3.3 and 3.3.4). Thus, p53 may also be responsible for the elimination of cells whose growth is deregulated by a premature onset of S phase in the cell cycle.

Since the tumour suppressing activities of p53 involve both cell growth inhibition and apoptotic cell death, it is of interest to determine the relationship between these two functions. It has been suggested that in response to DNA damage, p53 accumulates and causes a G1 arrest so that the DNA can be repaired before it is replicated in S phase. If at this time, the DNA damage is beyond repair, p53 eliminates the cell through apoptosis (Lane, 1992). While this hypothesis suggests that p53-mediated apoptosis is preceded by growth arrest, the arguments presented in Chapter 3 (refer to Sections 3.3.2 to 3.3.4) imply that this is not the case. The findings that p53-dependent growth inhibition is mediated by pRb (refer to Section 3.3.2) (Slebos et al., 1994) and that the loss or inactivation of pRb causes a susceptibility to p53-dependent apoptosis (refer to references in preceding paragraph) indicate that apoptosis may be a reserve mechanism which occurs when p53 fails to arrest cell growth.

Thus, the studies presented in this chapter were designed to determine more precisely the relationship between the growth inhibitory and apoptotic activities of p53. For these purposes, various mutant forms of p53 were used in an attempt to determine whether these two functions have similar or separate genetic requirements. The same
approach was also used to determine whether inhibition of cell growth or induction of apoptosis are linked with the ability of p53 to suppress the transformation of cells (which occurs due to the cooperative effects of E1a and EJ-ras).
4.2 Results

4.2.1 p53 inhibits the growth of REFs

The cellular response to high levels of p53 (cell growth inhibition or apoptotic cell death) may depend upon the type of cell in which this protein is expressed. To identify the effects of exogenous p53 on normal rat embryo fibroblasts (REFs), these cells were co-transfected with a control plasmid or a plasmid encoding wild-type mouse p53, along with a plasmid containing the Tn5 gene which encodes for resistance to the neomycin family of antibiotics (Southern and Berg, 1982). The transfected cells were then selected with the neomycin analogue, G418. 18-20 days post-transfection the cells were stained and the extent of cell survival and cell growth was assessed by observing the number and size (respectively) of colonies which arise from the transfected cells.

As observed in Figure 4.1, numerous colonies of G418-resistant cells arose in the control cell populations. While in the presence of exogenous p53, the number of colonies was similar to that of the control cells, the overall cell number was significantly reduced as the colonies were generally much smaller in size (Figure 4.1). Thus, high levels of p53 were shown to inhibit the growth (but not the survival) of REFs.

4.2.2 p53 causes the death of LFSFs

Having observed that p53 inhibits the growth of normal (rat embryo) fibroblasts, immortalised (human) fibroblasts (LFSFs) were next examined for their response to the expression of exogenous p53. These fibroblasts were derived from a patient with Li-Fraumeni Syndrome (LFS) and were spontaneously immortalised during cell culture. They have been found to be deficient for the expression of both p53 and p16INK4 (Rogan et al., 1995). To examine the effects of p53, LFSFs were transfected with the appropriate plasmids and those which received plasmid DNA were selected as described in the preceding section. The extent of cell survival and cell growth was then assessed by observing the number and size (respectively) of colonies which arise from the transfected cells.

As observed in Figure 4.1, numerous colonies of G418-resistant cells arose in the control cell populations. However, in the presence of exogenous p53, the number of colonies was significantly reduced as very few surviving cells remained. Although it is not seen in Figure 4.1, replicate experiments showed that the few cells which did survive in the presence of p53 developed into colonies which were similar in size to the colonies of control cells (Figure 4.2). Thus, the effect of p53 on LFSFs can be attributed to induction of cell death rather than inhibition of cell growth.
Figure 4.1. p53 inhibits the growth of REFs and causes the death of LFSFs. To evaluate the survival and growth of different cell types, REFs and LFSFs were transfected (through the use of lipofectamine) with: 670ng CMVneo (encodes for resistance to neomycin) along with either: 1340ng pBR322 (control) or 670ng pBR322 and 670ng CMVNc9 (encodes wild-type mouse p53). The transfected cells were selected with media containing 200μg/ml Geneticin G-418 (a neomycin analogue). 18-20 days (for REFs) or 12-14 days (for LFSFs) post-transfection, the surviving cells were fixed and stained (with 0.1% crystal-violet in 20% ethanol). The observed patterns of cell survival and growth were found to be reproducible in at least three independent experiments.
To further investigate this cytotoxic effect of p53, the possibility that p53 causes apoptotic cell death in LFSFs was considered. If this is the case, one could expect that the effects of p53 to be overcome by either bcl-2 or E1b-19K as both of these proteins are known to inhibit apoptosis which is mediated by p53 (Debbas and White, 1993; Chiou et al., 1994). Plasmids which encode either of these proteins were therefore co-transfected with a plasmid which encodes p53 in an experiment as described in the previous section. This study showed that both bcl-2 and E1b-19K can partially reverse the effects of p53 as the presence of either protein promoted the survival and growth of LFSFs (Figure 4.2). These findings have provided further support for the conclusion that exogenous p53 causes the death of LFSFs and have indicated that this outcome occurs through an apoptotic response.

To further distinguish the characteristics of REFs and LFSFs, these cells were examined (through the procedure described above) for their response to expression of the adenovirus E1a proteins. In the studies presented in Chapter 3, E1a was observed to cause the death of REFs and this effect was attributed to apoptosis which is mediated by endogenous p53 (refer to Section 3.3.3). This study has now shown that E1a is unable to induce cell death in LFSFs (which contain no endogenous p53). However, the death of LFSFs does occur in the presence of E1a and exogenous p53, as it does with exogenous p53 alone (Figure 4.3). Thus, the failure of E1a (alone) to cause the death of LFSFs reflects the deficiency of p53 in these cells. When induction of apoptotic cell death is prevented, E1a would be expected to stimulate cell growth (refer to Section 3.3.3 and Figure 3.9). However, in LFSFs, the colonies which arose in the presence of E1a were very similar in size to those which arose from the control cells (Figure 4.3). Thus, E1a was unable to enhance the growth of LFSFs. Since this observation suggests that these cells have already undergone oncogenic disruptions similar to those caused by E1a, it may be expected that LFSFs would be susceptible to p53-mediated apoptosis.

### 4.2.3 Inhibition of cell growth and induction of cell death are genetically separable functions of p53

To investigate the relationship between p53-dependent growth inhibition and p53-dependent cell death, various mutant forms of p53 were used in an attempt to determine whether these two functions have similar or separate genetic requirements. Because wild-type p53 was observed to inhibit the growth of REFs and cause the death of LFSFs, these cells were used to examine the abilities of the mutant p53 proteins to perform either of these cellular activities. Diagrammatic representations of the mutant p53 proteins used in these studies are shown in Figure 4.4.

Qualitatively, the ability of each p53 protein to inhibit the growth of REFs was investigated as described in section 4.2.1. To measure the growth of REFs in the
Figure 4.2. Anti-apoptotic proteins (bcl-2 and E1b-19K) partially rescue LFSFs from the cytotoxic effects of p53. LFSFs were transfected with CMVneo (encodes for neomycin resistance) along with either pBR322 (control) alone or pBR322 and CMVNe9 (encodes p53). Inhibition of p53-induced cell death was attempted by co-transfection with CMVbcl-2 (encodes bcl-2) or CMV19K (encodes E1b-19K). Transfected cells were selected with G418. The patterns of cell survival and growth were observed 12-14 days post-transfection and found to be reproducible in at least three independent experiments (refer to Figure 4.1 for more details).
Adenovirus E1a does not stimulate cell growth nor induce cell death in LFSFs. REFs and LFSFs were transfected with CMVneo (encodes for neomycin resistance) along with either pBR322 (control) alone or pBR322 and CMVNc9 (encodes p53). The effects of E1a were examined by co-transfection with CMVE1a (encodes E1a). Transfected cells were selected with G418. The patterns of cell survival and growth were observed 12-14 days post-transfection and found to be reproducible in at least three independent experiments (refer to Figure 4.1 for more details).
Figure 4.4. Wild-type and mutant forms of the mouse p53 protein. Amino acids which are mutated are indicated by a single line, while amino acids which are deleted are indicated by a shaded region. Refer to Figure 1.3 for more details on the p53 protein.

NB: The numbering of amino acids in mouse p53 assumes that the second in-frame AUG is the translation start site. (An alternative numbering system which assumes that the first in-frame AUG is the translation start site suggests that mouse p53 has 390 amino acids.)
presence of wild-type or mutant p53, the cells were treated as described in section 4.2.1 except that instead of staining the cells, lysates were prepared. For an indication of overall cell number, the total protein concentration of each cell lysate was measured through a colourimetric assay.

This study showed that the growth of REFs is effectively inhibited by wild-type p53 (Nc9) and a mutant p53 protein, dll63. Another protein, c5 retained some ability to inhibit cell growth, but the remaining mutant forms of p53 were completely defective for this activity (Figure 4.5A).

The ability of each p53 protein to cause the death of LFSFs was investigated by transfecting LFSFs with the appropriate gene expression plasmids and selecting the transfected cells as described in section 4.2.2. After staining the LFSFs, the extent of cell survival in the presence of wild-type or mutant p53 was measured by counting the number of colonies which arise from the surviving cells.

This study showed that wild-type p53 (Nc9), and two mutant proteins, mp53 and 281EG can induce cell death in LFSFs, while the remaining mutant forms of p53 were defective for this activity (Figure 4.5B).

The studies described above have demonstrated that dll63 inhibits cell growth yet remains defective for inducing cell death, while mp53 and 281EG induce cell death yet completely fail to inhibit cell growth (Figure 4.5A&B). These results indicate that within the p53 protein, the regions which are important for growth inhibition are different to those which are important for cell death. Thus, inhibition of cell growth and induction of cell death are genetically separable functions of p53.

**4.2.4 Mutant p53 proteins are adequately expressed in REFs and LFSFs**

The results described in the previous section rely on there being adequate levels of protein expression for each of the mutant forms of p53 in both types of cells. Figures 4.6 and 4.7 show the expression of the p53 proteins in REFs and LFSFs (respectively) as determined from immunoprecipitations of radiolabelled cellular proteins with a p53-specific antibody. Also shown in Figure 4.6B, is the conformational status of each protein, as determined by its interactions with certain monoclonal antibodies. Proteins whose conformations remain similar to wild-type p53 bind to pAb246, but not pAb240, while proteins whose conformations are significantly disrupted bind to pAb240, but not pAb246 (Yewdell et al., 1986; Gannon et al., 1990). Thus, proteins with 'wild-type' conformations are Nc9, mp53, dll163, and 281EG, while proteins with 'mutant' conformations are dll518 and c5. While 281EK was not detected, this protein has also been shown to have a 'mutant' conformation (Antony Braithwaite - personal communication).

Although undetected in REFs, 281EK, dll1.1, and dll2.3 were found to be expressed in LFSFs (Figures 4.6A and 4.7). Thus, the inability to detect these proteins in
Figure 4.5. Mutant p53 proteins vary in their abilities to inhibit cell growth, induce cell death, and suppress cell transformation. (A) To determine cell growth: REFs were transfected with CMVneo (encodes for neomycin resistance) along with either pBR322 (control) alone or pBR322 and a plasmid encoding wild-type (Nc9) or mutant p53. Transfected cells were then selected with G418 (refer to Figure 4.1 for more details). 18-20 days post-transfection, the total protein concentrations of cell lysates were measured for an indication of overall cell number. (B) To determine cell survival: LFSFs were transfected and treated as described above. 12-14 days post-transfection, the cells were stained and the colonies of G418-resistant cells were counted. (C) To determine cell transformation: REFs were transfected with CMVE1a (encodes E1a) and T24EJ6.6 (encodes EJ-ras) along with either pBR322 (control) or a plasmid encoding wild-type (Nc9) or mutant p53. 22-24 days post-transfection, the cells were stained and the foci of transformed cells were counted. For each cellular activity, the represented data were derived from at least three independent experiments.
Figure 4.6. Wild-type and mutant p53 proteins: expression in REFs and conformational status. (A) To determine the expression of proteins: REFs were transfected (through the use of lipofectamine) with either 2μg CMVneo alone (control) or 1μg CMVneo and 1μg of a plasmid encoding wild-type (Nc9) or mutant p53. Three days post-transfection, the cellular proteins were radiolabelled, cell lysates were prepared, and immunoprecipitations were performed with normal rabbit serum (NRS) or pAb200-47 (recognises p53). (B) To determine the conformation of proteins: REFs were treated as described above, except that immunoprecipitations were performed with pAb240 (specifically recognises conformations similar to wild-type p53) or pAb246 (specifically recognises disrupted conformations of p53).
Figure 4.7. Wild-type and mutant p53 proteins: expression in LFSFs. To determine the expression of proteins: LFSFs were transfected (through the use of lipofectamine) with either 2μg CMVneo alone (control) or 1μg CMVneo and 1μg of a plasmid encoding wild-type (Nc9) or mutant p53. Three days post-transfection, the cellular proteins were radiolabelled, cell lysates were prepared, and immunoprecipitations were performed with normal rabbit serum (NRS) or pAb200-47 (recognises p53).
REFs is probably not because they fail to be expressed, but because they are expressed at levels below that required for detection by this method. This argument is supported by the observations that dl1.1 and dl2.3 are able to regulate gene transcription in REFs (refer to Sections 5.2.1 and 5.2.2 and Figures 5.2 and 5.3). Since the assays for gene transcription involve the protein acting on promoters which are exogenous and therefore present in high quantities, while the assays for cell growth involve the protein acting on endogenous target molecules, more protein would be required for an effect on transcription than for an effect on cell growth. Thus, the lack of effect of dl1.1 and dl2.3 (and probably, 281EK) on cell growth cannot be attributed to insufficient quantities of protein.

While Nc9, mp53, and 281EG are expressed in REFs, they fail to be detected in LFSFs (Figures 4.6A and 4.7). The failure to detect these proteins in LFSFs can be explained by the abilities of Nc9, mp53, and 281EG to cause the death of the cells in which they are expressed (Figure 4.5B). Thus, the conclusion that Nc9, mp53, and 281EG can each cause the death of LFSFs is further supported by these findings.

4.2.5 p53-dependent suppression of transformation (by E1a and EJ-ras) is separable from growth inhibition but not separable from cell death

Although p53 is known to suppress the transformation of cells by E1a and ras, the mechanism by which this occurs remains somewhat ambiguous. Investigations were therefore planned to determine whether p53-dependent suppression of transformation (by E1a and EJ-ras) involved an inhibitory effect on cell growth or an induction of cell death. For this purpose, various mutant p53 proteins (Figure 4.4) were used in an attempt to determine whether the genetic requirements for suppression of transformation are similar to those for either cellular function of p53. The abilities of the wild-type and mutant p53 protein to suppress transformation by E1a and EJ-ras was determined by transfecting REFs with the appropriate gene expression plasmids and counting the number of foci which arise from the transformed cells. This study showed that wild-type p53 (Nc9) and two mutant proteins, mp53 and 281EG can suppress transformation while the remaining mutant forms of p53 were defective for this activity (Figure 4.5C).

The studies described above and in Section 4.2.2 have demonstrated that dl163 can inhibit cell growth but cannot induce cell death nor suppress transformation, while mp53 and 281EG can induce cell death and suppress transformation but cannot inhibit cell growth (Figure 4.5). These results indicate that within the p53 protein, the regions which are important for suppression of transformation are different to those which are important for growth inhibition but similar to those which are important for cell death. Thus, p53-dependent suppression of transformation (by E1a and EJ-ras) is genetically separable from inhibition of cell growth, but not separable from induction of cell death.
4.2.6 Growth inhibition is not required for induction of cell death by p53

For each mutant form of p53, Table 4.1 provides a summary of its ability to suppress transformation by E1a and EJ-ras, induce cell death, and inhibit growth. Also summarised is the expression of the proteins in REFs and LFSFs and their conformational status.

The studies presented in this chapter have shown that dl163 can inhibit cell growth yet remains defective for inducing cell death, while mp53 and 281EG can induce cell death yet completely fail to inhibit cell growth (Figure 4.5A&B and Table 4.1). While it was already obvious from the nature of the activities that cell death is not required for growth inhibition, these results have importantly, demonstrated that growth inhibition is neither sufficient nor necessary for induction of cell death by p53. Figure 4.8A shows each mutant form of p53 plotted with respect to its ability to inhibit cell growth and its ability to induce cell death. The closeness of the relationship between the two variables is indicated by the co-efficient of correlation, R^2. (NB: The closer R^2 is to 1, the closer the relationship between the variables.) This graph clearly demonstrates the lack of correlation between the growth inhibitory and death inducing functions of p53 as the correlation co-efficient for these two activities is low: R^2 = 0.13. Thus, one can conclude that inhibition of cell growth is not necessary for p53 to cause cell death as these two activities are completely separable functions of p53.

4.2.7 p53-dependent suppression of transformation (by E1a and EJ-ras) correlates with induction of cell death and not inhibition of cell growth

As shown in Table 4.1, the studies in this chapter have demonstrated that dl163 can inhibit cell growth yet fails to suppress transformation by E1a and EJ-ras, while mp53 and 281EG can suppress transformation yet fail to inhibit cell growth (Figure 4.5A&C). Thus, one can conclude that inhibition of cell growth is neither sufficient nor necessary for the ability of p53 to suppress transformation by E1a and EJ-ras. Figure 4.8B shows each mutant form of p53 plotted with respect to its ability to inhibit cell growth and its ability to suppress transformation. Since the correlation co-efficient, R^2 for these two activities is zero, the lack of correlation between p53-dependent growth inhibition and suppression of transformation (by E1a and EJ-ras) is clearly demonstrated.

The studies presented in this chapter have also demonstrated that mp53 and 281EG can cause cell death and suppress cell transformation while the remaining mutant forms of p53 remain defective for both of these functions (Figure 4.5B&C and Table 4.1). These observations indicate that p53-induced cell death could be both necessary and sufficient for suppression of transformation by E1a and ras. Figure 4.8C shows each
Table 4.1. Comparisons of the cellular activities of mutant p53 proteins. ++ means: functional capacity is high; + means: functional capacity is moderate; and - means: no functional capacity. The conformation of each protein was determined by its interaction with pAb240 or pAb246 (refer to Figure 4.6B): + means: 'wild-type' conformation and - means: 'mutant' conformation. * Information provided by Antony Braithwaite - personal communication.
Figure 4.8. p53-dependent suppression of transformation (by E1a and EJ-ras) correlates with induction of cell death but not inhibition of cell growth. The data represented in Figure 4.5 were used to determine the ability of each p53 protein to perform each function (relative to the control whose ability to perform each function is 0%). Each protein was then plotted with respect to: (A) its ability to inhibit cell growth and its ability to induce cell death, (B) its ability to inhibit cell growth and its ability to suppress transformation (by E1a and EJ-ras), and (C) its ability to induce cell death and its ability to suppress transformation (by E1a and EJ-ras).

NB: For each graph, the equation used to fit the line of regression is $Y_i = aX_i + b + \text{error}$. The closeness in the relationship between the two functions of p53 is indicated by the correlation co-efficient, $R^2$ (as determined from a standard calculation by the Cricket Graph - version 1.3.2 computer package). The closer $R^2$ is to 1, the closer the relationship between the variables. Although the combination of data for induction of cell death and suppression of transformation (C) does not show a normal distribution (and appears to show a binomial distribution), the analyses of $R^2$ was the same as that used for examining the relationship between inhibition of cell growth and induction of cell death (A) or inhibition of cell growth and suppression of transformation (B). In both of these cases, the combination of data for the two variables shows a more normal distribution. The values of $R^2$ indicate that there is basically no correlation between inhibition of cell growth and induction of cell death (A) or inhibition of cell growth and suppression of transformation (B). However, there is a closeness in the relationship between induction of cell death and suppression of transformation (C).
A

ability to induce cell death

100%
50%
0%
-50%
-100%
-50%
-100%

R^2 = 0.130

ability to inhibit growth

-25% 0% 25% 50% 75% 100%

B

ability to induce cell death

100%
50%
0%
-50%
-100%
-50%
-100%

R^2 = 0.000

ability to inhibit growth

-25% 0% 25% 50% 75% 100%

C

ability to suppress transformation

100%
75%
50%
25%
0%

R^2 = 0.812

ability to induce cell death

-25% 0% 25% 50% 75% 100%

ability to suppress transformation
mutant form of p53 plotted with respect to its ability to induce cell death and its ability to suppress transformation. This graph clearly demonstrates a strong correlation between these two activities of p53 as the correlation co-efficient is high: $R^2 = 0.812$ (NB: since $R^2 = 0.812$, 65.9% of the data on the graph can be explained by a correlation between the two variables). The mechanism by which p53 suppresses cell transformation by E1a and EJ-ras therefore appears to involve the induction of cell death.

While comparisons in the activities of proteins in two different cell types are not always reasonable, it must be taken into account that REFs and LFSFs are both fibroblasts. Furthermore, the strong correlation between induction of cell death in LFSFs and suppression of transformation in REFs indicates that additional cellular factors are not affecting the functions of specific mutant p53 proteins.

Thus, the results of this chapter indicate that p53 suppresses transformation by E1a and EJ-ras through the induction of cell death rather than the inhibition of cell growth.
4.3 Discussion

4.3.1 The cytotoxic effects of p53 in LFSFs occur through apoptosis

The studies described in this chapter have shown that exogenous p53 causes the death of LFSFs (Figure 4.1). This observation is consistent with the finding that LFSFs are genetically deficient for expression of p53 (Rogan et al., 1995) since even low levels of endogenous p53 may be fatal to cells which have a susceptibility to the cytotoxic effects of this protein. In support of this suggestion, other cancer-derived cells which are subject to p53-induced cell death have previously undergone loss or inactivation of wild-type p53 (Yonish-Rouach et al., 1991; Shaw et al., 1992; Ramqvist et al., 1993; Ryan et al., 1993; Haupt et al., 1995a).

LFSFs are also genetically deficient for the expression of p16INK4 which (as discussed in Section 1.3.4) is known to inhibit the activity of cyclinD-cdk4 complexes (Serrano et al., 1993). Since cyclinD-cdk4 is important for the phosphorylation and consequent inactivation of pRb (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993) (refer also to Figure 1.4), one might expect that in LFSFs, the loss of p16INK4 would cause pRb to be readily inactivated. Thus, the death of LFSFs in the presence of p53 may be attributed to an apoptotic response as the functional disruption of pRb is associated with a high susceptibility p53-mediated apoptosis (Howes et al., 1994; Morgenbesser et al., 1994; Pan and Griend, 1994; Symonds et al., 1994; Williams et al., 1994a; Almasan et al., 1995). The functional disruption of pRb in LFSFs additionally implies that p53 would fail to inhibit cell growth since active pRb is required to mediate p53-dependent growth inhibition (refer to Section 3.3.2) (Slebos et al., 1994). It would therefore appear that the deficiency of p16INK4 causes LFSFs to undergo apoptotic cell death rather than growth inhibition in the presence of exogenous p53.

The prediction that pRb is functionally disrupted by the loss of p16INK4 in LFSFs is supported by the observed effects of E1a on these cells (Figure 4.3). Although E1a can induce apoptosis which is mediated by endogenous p53 in REFs (Debbas and White, 1993; Lowe and Ruley, 1993) (refer to Section 3.3.3), this effect is prohibited in LFSFs due to the deficiency of p53. Under conditions where the induction of apoptotic cell death is prevented, E1a is able to enhance cell proliferation. The growth stimulatory effects of E1a occur at least in part through the binding and inactivation of pRb (Egan et al., 1989; Whyte et al., 1989; Howe et al., 1990) as the consequent activities of E2F (Chellappan et al., 1992; Zamanian and La Thangue, 1992) stimulate the progression of cells from G1 into S phase of the cell cycle (reviewed in: Nevins, 1992) (refer to Section 3.3.3). However, studies presented in this chapter have shown that E1a is unable to stimulate the growth of LFSFs (Figure 4.3). This lack of effect with E1a could be attributed to pRb having been already inactivated due to the deficiency of p16INK4 in LFSFs (as predicted
above). Such functional disruptions to pRb activity can also explain the susceptibility of LFSFs to p53-mediated apoptosis in both the presence and absence of E1a.

An additional indication that the p53-dependent death of LFSFs occurs through apoptosis is presented in Figure 4.2. The cytotoxic effects of p53 in LFSFs can be (at least partially) inhibited by co-expression of either bcl-2 or E1b-19K both of which are known to inhibit apoptosis (Hockenbery et al., 1990; White et al., 1991; Rao et al., 1992) that is mediated by p53 (Debbas and White, 1993; Chiou et al., 1994). However, the effects of these proteins in LFSFs appears to be somewhat weaker than that observed when they completely inhibited apoptosis which is induced by E1a and mediated by endogenous p53 in REFs (Figure 3.9). This discrepancy is explained by the quantity of p53 which causes the apoptotic response as the levels of exogenous p53 in LFSFs would be considerably higher than the levels of endogenous p53 in REFs.

In light of the discussions presented above, one might reasonably conclude that the induction of cell death in LFSFs is caused by an apoptotic response of p53. For this reason, the cytotoxic effect of p53 in LFSFs will from here on be referred to as apoptosis.

4.3.2 Inhibition of cell growth requires certain regions of the p53 protein

In this chapter, the examination of various mutant forms of mouse p53 for their abilities to inhibit cell growth has revealed new information about regions of the p53 protein required for this activity. While this information is complementary to that reported by other such studies, it should be noted that comparisons between the results of this study and those of other studies (Crook et al., 1994; Pietenpol et al., 1994; Zhang et al., 1994b) (as discussed below) are based on the assumption that the p53-dependent growth inhibition observed in REFs is biochemically similar to that observed in cells derived from chronic myelogenous leukaemia, osteosarcoma, and lung adenocarcinoma.

The p53 protein may be divided into three structural domains: an N-terminal acidic region (approximately, amino acids 1-100), a central hydrophobic region (approximately, amino acids 100-300), and a C-terminal basic region (approximately, amino acids 300-390) (refer to Figure 1.3). Mutations in the central region often disrupt the conformation of p53 to such an extent that the resulting 'mutant' conformation can be distinguished from the 'wild-type' conformation by its association (or lack of association) with specific monoclonal antibodies (Gannon et al., 1990) (refer to Section 4.2.4 and Figure 4.6B). The importance of singular amino acids, protein regions, and the protein conformation in the growth inhibitory effects of p53 as revealed by this study is illustrated in Figure 4.9A.

The studies in this chapter have demonstrated that the presence of amino acids 14 to 66 in the N-terminus of mouse p53 is not required for inhibition of cell growth (Figure
4.5 and Table 4.1 - refer to dl163). However, the integrity of amino acids 76 and / or 78 is necessary for this function (Figure 4.5 and Table 4.1 - refer to mp53). While most of the early part of the N-terminus is not necessary for p53-dependent growth inhibition, a region which is important must occur towards the central region of this protein (Figure 4.9A). This finding is not incompatible with that of another study which showed that inhibition of cell growth requires a sequence which lies between amino acids 1 and 80 in the N-terminal region of human p53 (Pietenpol et al., 1994).

The results presented in this chapter have also shown that a mutant p53 protein (c5) which is conformationally altered (due to mutation of amino acids 168 and / or 234) (Figure 4.6B) remains capable of inhibiting cell growth (Figure 4.5 and Table 4.1). Integrity of protein conformation is therefore not critical for the p53-dependent growth inhibition (Figure 4.9A). This property may however contribute to the full growth inhibitory effects of p53 as the activity of this conformationally-disrupted protein (c5) is reduced in comparison to that of wild-type p53 (Figure 4.5). While the integrity of amino acids 168 and / or 234 may have some role, other amino acids in the central region of p53 are actually critical for growth inhibition (Figure 4.9A) as the deletion of amino acids 133 to 148 or the mutation (even a conservative mutation) of amino acid 281 completely abrogates this function (Figure 4.5 and Table 4.1 - refer to dl518, 281EG, and 281EK respectively). One or more properties of the central region of mouse p53 (other than protein conformation) must therefore be critical for inhibition of cell growth. This result is consistent with that of another study which showed that various sequences within the central region are required for the growth inhibitory effects of human p53 (Crook et al., 1994).

The studies in this chapter have demonstrated that the C-terminal region is also important for p53-dependent growth inhibition. The deletion of amino acids 304 to 322 and the deletion of amino acids 304 to 304 both abrogate the growth inhibitory activity of mouse p53 (Figure 4.5 and Table 4.1 - refer to dl1.1 and dl2.3 respectively). While some of the region between amino acids 323 and 361 may therefore be important, the critical sequence could be confined to that found between amino acids 304 and 322 (Figure 4.9A). This finding is compatible with those of other studies which showed that large deletions in the C-terminus of human p53 abrogate the growth inhibitory effects of this protein (Crook et al., 1994; Pietenpol et al., 1994; Zhang et al., 1994b).

In summary, the studies in this chapter have shown that p53-dependent growth inhibition requires sequences which occur in distal part of the N-terminus, within (and possibly throughout) the central region, and in the C-terminus of this protein. However, more information is required to precisely map the protein regions which are important for this function.
Figure 4.9. The growth inhibitory and apoptotic functions of p53 require different protein regions. The importance of an amino acid or a protein region for (A) growth inhibition or (B) apoptosis is indicated by its intensity. Information about the regions in the background is not provided by the studies presented in this chapter. The importance of the protein conformation for each function is indicated by the intensity of that part of the figure. Refer to Sections 4.3.2 and 4.3.3 for more details.
4.3.3 Induction of apoptosis requires most regions of the p53 protein

The studies presented in this chapter have examined various mutant forms of p53 for their abilities to induce apoptosis and have revealed new information about the regions of the p53 protein regions required for this function. The importance of singular amino acids, protein regions, and the protein conformation in the apoptotic effects of p53 as indicated by this study is illustrated in Figure 4.9B.

Because the deletion of amino acids 14 to 66 in the N-terminus abrogates the apoptotic activity of p53 (Figure 4.5 and Table 4.1 - refer to dl163), a sequence which is critical for this function must include amino acids which lie within this region (Figure 4.9B). While at least part of the N-terminus of mouse p53 is therefore critical for apoptosis, the studies have also demonstrated that conservative mutations of amino acids 45, 76 and / or 78 do not impede its apoptotic effects (Figure 4.5 and Table 4.1 - refer to mp53).

The central region of mouse p53 is also important for the induction of apoptosis as the deletion of amino acids 133 to 148, the mutation of amino acid 168 and / or 234, and a non-conservative mutation at amino acid 281 can each prevent the apoptotic activity of this protein (Figure 4.5 and Table 4.1 - refer to dl518, c5, and 281EK respectively). A conservative mutation at amino acid 281 does not however impede this function (Figure 4.5 and Table 4.1 - refer to 281EG). Since the deletion of amino acids 133 to 148 or mutation of amino acid 168 and / or 234 can also disrupt the conformation of p53 (Figure 4.6B - refer to dl518 and c5 respectively), one can suggest that integrity of protein conformation is at least one of the properties which make the central region of p53 necessary for apoptotic cell death (Figure 4.9B).

The studies presented in this chapter have also shown that the C-terminal region of mouse p53 is required for apoptosis. Since the deletion of amino acids 304 to 333 abrogates the apoptotic response, at least part of the necessary sequence must lie within this region. The additional deletion of amino acids 325 to 361 also prevents apoptotic activity and while some of this region may be important, the sequence which is critical for apoptosis could be confined to that found between amino acids 304 and 325 (Figures 4.5 and 4.9B and Table 4.1 - refer to dl1.1 and dl2.3 respectively). Interestingly, this finding is not consistent with those of another study which showed that the C-terminus (and a large part of the central region) is dispensable with regard to the apoptotic effects of mouse p53 (Haupt et al., 1995b).

From the results of this chapter, one can suggest that sequences which occur throughout the protein are necessary for the apoptotic function of p53. However, to precisely map the protein regions which are important for this function, much more information is required.
4.3.4 Inhibition of cell growth is not necessary for p53-mediated apoptosis

The analyses of mutant protein activity have demonstrated (as described above) that inhibition of cell growth and induction of apoptosis require the integrity of different regions within the p53 protein (Figure 4.9). One can therefore conclude that these are separable functions of p53. Figure 4.8A reiterates this finding by showing that the ability of p53 to inhibit cell growth does not correlate with its ability to induce apoptotic cell death. In particular, the studies in this chapter have found that dl163 inhibits cell growth but remains defective for inducing apoptosis, while mp53 and 281EG induce apoptosis but completely fail to inhibit cell growth (Figure 4.5A&B and Table 4.1). While it was already obvious from the nature of the activities that apoptosis is not required for growth inhibition, these results have importantly, demonstrated that growth inhibition is not sufficient and not even necessary for p53-dependent apoptosis (refer to Figure 4.10).

The concept of growth inhibition being insufficient for apoptosis has been suggested by other reports due to the observation that cells which have been arrested in a manner independent of p53 do not undergo apoptosis in the presence of p53. Since an arrest of cell growth does not automatically lead to an apoptotic response, an additional function of p53 must be required for the induction of this type of cell death (Ryan et al., 1993; Wagner et al., 1994). The concept of growth inhibition being unnecessary for apoptosis has also been suggested by another study which used a completely different approach to investigate the relationship between the cellular functions of p53. The findings of this chapter are very much in agreement with their observation that myeloid leukaemic cells undergo p53-mediated apoptosis while failing to arrest in the G1 phase of the cell cycle (Yonish-Rouach et al., 1993).

A popular model of p53 activity asserts that the accumulation of p53 in response to DNA damage arrests cell growth in G1 phase so that the DNA can be repaired before being replicated in S phase. If there is a failure to repair the damaged DNA, p53 eliminates the cell through apoptosis. These activities would ensure that the only cells which continue to proliferate are those which have maintained genomic integrity (Lane, 1992). This hypothesis is supported by various pieces of information regarding the activities of p53 (refer to Sections 1.3.2 and 4.1). However, because the model suggests that apoptosis is preceded by growth arrest, it is refuted by the observations (both of this chapter and of others (Yonish-Rouach et al., 1993)) that p53-dependent growth inhibition is not required for the apoptotic effects of this protein. An additional problem with this model relates to the nature of the p53-induced G1 arrest. While it assumes that this anti-proliferative effect is temporary and reversible, one report has shown that the growth arrest which is induced by p53 in response to ionising irradiation is actually prolonged and probably irreversible (Di Leonardo et al., 1994).
An alternative model may be that the accumulation of p53 in response to DNA damage arrests cell growth and if this response is somehow prevented, apoptosis is induced. Thus, p53 would use alternative rather than sequential mechanisms for preventing the continued proliferation of genetically damaged cells. While supported by the observations that growth arrest is not required for p53-mediated apoptosis, this model is also consistent with the results and arguments presented in Chapter 3 (refer to Sections 3.3.2 to 3.3.4). Since p53-dependent growth inhibition is mediated by active pRb (refer to Section 3.3.2) (Slebos et al., 1994), the loss or inactivation of this protein would prevent this anti-proliferative effect. However, under these conditions, cells become susceptible to p53-mediated apoptosis (Howes et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Symonds et al., 1994; Williams et al., 1994a; Almasan et al., 1995). Since the inactivation of pRb disrupts the regulation of cell cycle progression at the G1 / S phase boundary (refer to Section 1.3.3), the apoptotic function of p53 appears to be responsible for the elimination of cells whose growth is deregulated by a premature onset of DNA replication (refer to Section 1.3.5). This situation is additionally reflected by the effects of oncoproteins such as myc and adenovirus E1a both of which prevent p53-dependent growth inhibition (by binding and inactivating pRb, in the case of E1a) (Lowe et al., 1993a; Vousden et al., 1993; Wagner et al., 1994) yet stimulate the apoptotic effects this protein (Debbas and White, 1993; Lowe and Ruley, 1993; Hermeking and Eick, 1994). The ability of p53 to induce apoptosis when it fails to arrest cell growth may also explain the apoptotic response of LFSFs as the growth inhibitory effects of p53 may be prevented by the possible functional disruption of pRb in these cells (refer to Section 4.3.1).

Further evidence that growth inhibition and apoptosis are separate functions which occur under different cellular conditions has been provided by studies which showed that the supply of survival factors can determine the response of cells to p53. Upon the induction of p53, haematopoietic cells undergo growth arrest in the presence of certain interleukins but apoptosis in the absence of these survival factors (Levy et al., 1993; Canman et al., 1995). These and various other cellular factors are likely to be important for ensuring that cells respond to p53 in the most physiologically appropriate manner.

In summary, induction of apoptosis and inhibition of cell growth appear to be alternative functions which allow p53 to suppress tumourigenesis under different cellular conditions. Thus, the apoptotic function of p53 does not require (but occurs in place of) an inhibitory effect on cell growth.
4.3.5 The ability of p53 to suppress cell transformation (by E1a and EJ-ras) is due to apoptosis and not growth inhibition

By examining the activities of various mutant p53 proteins, the studies in this chapter have demonstrated an interesting relationship between the cellular functions of p53. While dl163 can inhibit cell growth but not suppress transformation, mp53 and 281EG can suppress transformation but not inhibit cell growth (Figure 4.5A&C and Table 4.1). Thus, an inhibitory effect on cell growth is neither sufficient nor necessary for the ability of p53 to suppress transformation of cells by E1a and EJ-ras. The lack of correlation between these functions is demonstrated in Figure 4.8B. The studies in this chapter have also demonstrated that mp53 and 281EG can induce apoptosis and suppress cell transformation while the remaining mutant forms of p53 remain defective for both of these activities (Figure 4.5B&C and Table 4.1). These observations indicate that p53-dependent apoptosis could be both necessary and sufficient for suppression of transformation by E1a and EJ-ras. Furthermore, the ability of a protein to induce apoptosis correlates very well with its ability to suppress transformation (Figure 4.8C). From the above results, one can conclude that the transformation of cells by E1a and EJ-ras is suppressed by an apoptotic rather than growth inhibitory function of p53 (refer to Figure 4.10).

With regard to the regions of p53 which are required for its ability to suppress transformation, the information gained from these studies is basically the same as that described for the induction of apoptosis (refer to Section 4.3.3). As illustrated in Figure 4.9B, p53-dependent apoptosis and therefore suppression of transformation (by E1a and EJ-ras) appears to require sequences which occur throughout the entire protein. Similarly, other studies of both mouse and human p53 have shown that most regions of the protein are important for suppression of transformation by E1a and activated ras (Reed et al., 1993) or by HPV-E7 and activated ras (Crook et al., 1994). Results obtained with the use of HPV-E7 can be compared to those obtained with E1a as there are several similarities in the biochemical and phenotypic effects of these proteins (refer to Sections 1.3.1 and 1.3.5). Interestingly, a different study of human p53 demonstrated that a large deletion in the early region of the N-terminus reduces, yet still permits suppression of transformation by E1a and activated ras (Unger et al., 1993). Also of interest is another study which showed a mouse p53 protein which has the C-terminus and a large part of central region deleted still retained some ability to suppress transformation by activated ras in combination with either myc, E7, or E1a (Haupt et al., 1995b). Since the activities of the mutant proteins in both of these studies were reduced in comparison to wild-type p53, the apparent inconsistencies in the literature may be attributed to differing sensitivities in the detection of the transformation suppressing effects of p53. In agreement with the findings presented in this chapter, the latter report
showed that there was some correlation between the abilities of a few mutant p53 proteins to suppress cell transformation and their abilities to induce apoptotic cell death (Haupt et al., 1995b).

Another study which compared the relationship between cellular functions of p53 showed that two mutant forms of human p53 (each containing a single point mutation) remained capable of inhibiting cell growth yet failed to suppress transformation by E7 and activated ras (Crook et al., 1994). Thus, growth inhibition is not sufficient for p53-dependent suppression of transformation. While consistent with this finding, the results of this chapter additionally demonstrate that the growth inhibitory effects of p53 are not necessary for suppression of cell transformation.

The results presented in Chapter 3 led to the conclusion that the mechanism by which p53 suppresses cell transformation is determined by the transforming oncoproteins (Sections 3.3.3 to 3.3.7). Transformation involving E1a was found to be suppressed by p53-mediated apoptosis (refer to Section 3.3.3), while transformation involving EJ-ras was found to be suppressed by p53-dependent growth inhibition (refer to Section 3.3.5). In addition, it was suggested that the cooperative effects of E1a and EJ-ras in cell transformation are due to each of these proteins overcoming the suppressive effects of endogenous p53 on the other protein (refer to Section 3.3.6). In support of this, E1a can overcome the growth inhibitory effects of p53 (Lowe et al., 1993a; Vousden et al., 1993; Lin et al., 1995). Since this effect of E1a appears to occur through the binding and inactivation of pRb (Whyte et al., 1988) which normally mediates p53-dependent growth inhibition (refer to Section 3.3.2) (Slebos et al., 1994), it would be expected to continue in the presence of high levels of p53. Additionally, EJ-ras was shown to inhibit an apoptotic response which is mediated by endogenous p53 in the presence of E1a (refer to Section 3.3.6) (Lin et al., 1995). There is however no evidence to suggest that EJ-ras can overcome apoptosis which is caused by high levels of exogenous p53. Thus, one might suggest that exogenous p53 suppresses transformation by E1a and EJ-ras through inducing apoptosis rather than inhibiting cell growth. This proposal has been verified by the results presented in this chapter.
Figure 4.10. Relationships between the different cellular activities of p53. The studies presented in this chapter have demonstrated that inhibition of cell growth and induction of apoptotic cell death are separable (and alternative) functions of p53 (refer to Section 4.3.4 for more details). Furthermore, the ability of p53 to suppress cell transformation by E1a and EJ-ras was shown to correlate with induction of apoptosis and not with inhibition of cell growth (refer to section 4.3.5 for more details).
Chapter 5

Biochemical activities of p53: involvement in growth inhibition and apoptosis
5.1 Introduction

The studies presented in Chapters 3 and 4 have demonstrated that inhibition of cell growth and induction of apoptosis are independent functions used by p53 to suppress cell transformation in response to particular oncoproteins and intracellular conditions. To complement these findings, it was shown that transformation by a commonly used combination of oncoproteins (E1a and EJ-ras) is suppressed by an apoptotic rather than growth inhibitory effect of p53. Having defined the relationships between the different cellular functions of p53, it was of interest to investigate the biochemical mechanisms which are involved in the inhibition of cell growth and the induction of apoptosis. This chapter therefore focusses on the main biochemical activities of p53: activation of gene transcription, repression of gene transcription, and inhibition of replication of DNA from the SV40 origin of replication.

The transactivation function of p53 was first demonstrated through the fusion of p53 with the DNA binding domain of yeast transcriptional activating protein, GAL4 as the anchoring of p53 to a promoter with a GAL4 binding site caused the activation of that promoter (Fields and Jang, 1990; O'Rourke et al., 1990; Raycroft et al., 1990). It was then found that p53 alone could bind to specific sequence of DNA (Kern et al., 1991; El-Deiry et al., 1992; Funk et al., 1992) and that the presence of these sequences within a promoter allowed it to be activated by p53 (Farmer et al., 1992; Kern et al., 1992; Zambetti et al., 1992). While artificial templates consisting of multiple copies of a p53-binding site fused upstream of a basic promoter have been commonly used to demonstrate the transactivation function of p53 (Farmer et al., 1992; Kern et al., 1992), a number of natural promoters (as listed below) which contain p53-binding sites are also known to be activated by this protein.

Transcriptional activation by p53 appears to involve the binding of this protein to both the promoter and the transcriptional machinery. The machinery required for mRNA transcription includes RNA polymerase II, several basal transcription factors (TFIIA, -B, -D, -E, -F, -G, -H, -J), and various sequence-specific regulatory transcription factors (such as p53). In promoters containing a TATA-box sequence, the assembly of the basal transcription factors is initiated by TFIID which is composed of the TATA-box binding protein (TBP) and several TBP-associated factors (TAFs) (reviewed in: (Goodrich and Tijan, 1994)). p53 is known to bind TBP (Seto et al., 1992) and in the presence of a p53-binding site, this interaction stabilises the binding of both p53 and TFIID to DNA (even in the absence of a TATA-box sequence) (Chen et al., 1993b). This observation suggests that p53 activates transcription by causing the formation of a stable complex between the promoter and the basal transcriptional machinery. The role of p53 in transcriptional activation may however be more complicated as this protein is also capable
of binding certain TAFs (TAFn40 and TAFn60) (Thut et al., 1995) and another basal transcription factor, TFIIH (Xiao et al., 1994).

Genes which are transcriptionally activated by p53 include those which encode: mouse muscle-specific creatinine kinase (MCK) (Weintraub et al., 1991; Zambetti et al., 1992); GADD45 (which is associated with growth arrest in response to DNA damage) (Kastan et al., 1992); p21WAF1 (El-Deiry et al., 1993); the murine double-minute (mdm-2) gene product (Barak et al., 1993; Wu et al., 1993); epidermal growth factor receptor (EGFR) (Deb et al., 1994); cyclin G (Okamoto and Beach, 1994); thrombospondin (which inhibits angiogenesis) (Dameron et al., 1994); and bax (Miyashita and Reed, 1995). While the contributions of some of these proteins remain uncertain, others are potentially important for the cellular activities of p53. The ability of p53 to arrest cell growth is thought to occur through the induction of p21WAF1 as this protein inhibits the activities of the cyclin dependent kinases which are necessary for the progression of cells from G1 phase into S phase of the cell cycle (Harper et al., 1993; Xiong et al., 1993) (refer to Section 3.3.2). The transactivation function may also contribute to the induction of apoptosis by p53 as the expression of bax is known to promote the apoptotic response (Oltvai et al., 1993).

In addition to activating gene transcription, p53 is known to repress transcriptional activity from various gene promoters (Ginsberg et al., 1991; Subler et al., 1992). At least in some cases, this function appears to involve the binding of TBP (Seto et al., 1992) as promoters whose activity depends upon the binding of TBP to a TATA-box sequence are specifically repressed by p53 (Mack et al., 1993). The repressive effects on gene transcription may be explained by the ability of p53 to prevent the binding of TBP to the TATA-box (Chen et al., 1993b; Ragimov et al., 1993) as this event is normally necessary for the assembly of the transcriptional machinery. However, since p53 has been shown to repress transcription in the absence a TATA-box sequence (Perrem et al., 1995), inhibitory effects on other transcription factors also must also be important for this function. In support of this, p53 is known to bind both the CCAAT-binding protein (CBP) (Agoff et al., 1993) and Sp-1 (Borellini and Glazer, 1993) and to inhibit the activities of both Sp1 (Perrem et al., 1995; Shaulian et al., 1995) and VP16 (Shaulian et al., 1995).

Viral and cellular promoters from which transcription is repressed by p53 include those of: simian virus 40 (SV40); cytomegalovirus (CMV); Rous sarcoma virus (RSV) (Subler et al., 1992; Jackson et al., 1993); herpes simplex virus (thymidine kinase and UL9 promoters); human T-cell lymphotropic virus; human immunodeficiency virus; proliferating cell nuclear antigen (PCNA) (Subler et al., 1992); c-fos; c-jun; p53; β-actin; (Ginsberg et al., 1991); heat shock protein, hsp70 (Ginsberg et al., 1991; Agoff et al., 1993); interleukin 6 (Santhanam et al., 1991); multidrug resistance (MDR) protein (Chin et al., 1992); pRb (Shiio et al., 1992); c-myc; mouse β-major globin (Ragimov et al.,
1993); basic fibroblast growth factor (basic FGF) (Ueba et al., 1994); and possibly, bcl-2 (Miyashita et al., 1994a). While the repression of cellular genes may represent a contribution of p53 to a generalised shut down of intracellular activity (during cell growth arrest or cell death), some specific cases of transcriptional repression may be important for the cellular functions of p53. One such example might be that down-regulation of bcl-2 expression is necessary at least in part for p53-dependent apoptosis as the presence of bcl-2 inhibits an apoptotic response (Hockenbery et al., 1990). The additional suggestion that both bcl-2 and adenovirus Elb-19K may inhibit apoptosis by overcoming the transrepression function of p53 (Shen and Shenk, 1994; Sabbatini et al., 1995) has provided further support for a role of transcriptional repression in p53-mediated apoptosis.

Another biochemical activity of p53 is the inhibition of viral DNA replication (Braithwaite et al., 1987). The initiation of DNA replication from SV40 origins of replication requires the combined activities of the SV40 large T antigen (LTag) and the cellular DNA replication machinery. In preparation for the synthesis of new DNA, LTag normally binds to the origin and with the assistance of replication protein A (RPA) unwinds the DNA template (Borowiec et al., 1990). These activities are however prevented in the presence of p53 (Sturzbecher et al., 1988; Wang et al., 1989) which may exert this effect by binding to LTag (Lane and Crawford, 1979; Linzer and Levine, 1979) and/or by binding to and inhibiting the functions of RPA (Dutta et al., 1993; Li and Botchan, 1993). The binding of p53 to LTag may also interfere with the actual synthesis of new DNA as the interaction of these proteins is known to prevent the binding of LTag to DNA polymerase α (Gannon and Lane, 1987). p21WAF1 and GADD45 both of which are transcriptionally activated by p53 also appear to inhibit the replication of DNA by binding to the proliferating cell nuclear antigen (PCNA) (Smith et al., 1994; Waga et al., 1994) which is part of the replication machinery. However, the induction of these proteins is probably not important for the effects of p53 on SV40 DNA replication as the observations that these effects occur in vitro (Sturzbecher et al., 1988; Wang et al., 1989) suggest that p53 has a direct role in inhibiting the replication of DNA.

The ability of p53 to inhibit the activities of the cellular protein, RPA indicates that the inhibitory effects of p53 on DNA replication may not be confined to that which occurs from viral origins of replication. In support of this idea, a form of p53 which is activated for DNA binding has been shown to inhibit the replication of cellular DNA (Cox et al., 1995). However, the contribution of such a function to the known cellular effects of p53 remains ambiguous.

The studies presented in this chapter have investigated the abilities of p53 to activate gene transcription, repress gene transcription, and inhibit SV40 DNA replication, with an aim to determine the relationships of these functions to the growth inhibitory and apoptotic effects of this protein. For these purposes, various mutant p53 proteins were
used in order to compare the genetic requirements of each biochemical activity to each cellular function of p53. These types of studies are useful for determining the biochemical mechanisms which are important for the phenotypic effects of a protein.
5.2 Results

5.2.1 Mutant forms of p53 vary in their abilities to activate transcription

Although p53 is known to activate transcription from various gene promoters, the importance of this biochemical activity in the cellular functions of p53 remains ambiguous. Investigations were therefore planned to determine the relationships between transcriptional activation and the growth inhibitory and apoptotic effects of p53. For this reason, the abilities of various mutant forms of p53 to inhibit growth or induce apoptosis were compared with their abilities to activate transcription from the muscle-specific creatinine kinase (MCK) promoter. This promoter was used as it is known to be strongly activated by wild-type p53 (Weintraub et al., 1991; Zambetti et al., 1992). The various mutant forms of mouse p53 used in this study are diagrammatically represented in Figure 5.1. The ability of each of these proteins to activate transcription was determined by transfecting REFs with the appropriate gene expression plasmids and using CAT assays to determine the levels of transcription.

This study showed that wild-type p53 (Nc9), mp53, dl1.1 (and possibly 281EG and dl2.3) can activate transcription of the MCK promoter, while the remaining mutant forms of p53 are defective for this activity (Figure 5.2A). Because the ability of 281EG to activate transcription remained uncertain, increasing doses of this protein were used to determine whether they had an increasing effect on gene transcription. This experiment demonstrated that 281EG does activate transcription in a dose-dependent manner, as does wild-type p53 (Figure 5.2B). The same experiment also showed that dl163 completely failed to activate transcription even when three times the normal dose of this protein was used (Figure 5.2B).

The studies described above and in the previous chapter (Section 4.2.2) have demonstrated that mp53, 281EG, and dl1.1 can activate gene transcription but cannot inhibit cell growth, while dl163 can inhibit cell growth (as effectively as wild-type p53) but fails to activate gene transcription (Figures 4.5 and 5.2 and Table 5.1). Thus, one can conclude that transactivation by p53 is neither sufficient nor necessary for the growth inhibitory effects of this protein. Furthermore, the studies have demonstrated that dl1.1 can activate gene transcription but cannot induce apoptosis, while there were no mutant p53 proteins which can induce apoptosis without activating gene transcription (Figures 4.5 and 5.2 and Table 5.1). These observations indicate that transcriptional activation may be necessary but is not sufficient for p53-mediated apoptosis.

While the assays for transactivation and growth inhibition were both performed in REFs, the assays for apoptosis were performed in LFSFs. Although comparisons in the activities of proteins between two different cell types are not always practical, it must be taken into account that REFs and LFSFs are both fibroblasts. Furthermore, the strong
Figure 5.1. Wild-type and mutant forms of the mouse p53 protein. Amino acids which are mutated are indicated by a single line, while amino acids which are deleted are indicated by a shaded region. Refer to Figure 1.3 for more details on the p53 protein. The conformational status of each protein was defined by its interactions with certain antibodies (refer to Figure 4.6B). Proteins whose conformations remain similar to wild-type p53 bind pAb246, but not pAb240, while proteins whose conformations are significantly disrupted bind pAb240, but not pAb246. * Information provided by Antony Braithwaite - personal communication.
Figure 5.2. Mutant p53 proteins exhibit varying capacities for transcriptional activation. (A) REFs were transfected (through the use of CaPO₄) with: 10μg pETCAT (encodes CAT under the control of the MCK promoter) along with either 10μg CMVneo (control) or 10μg of a plasmid encoding wild-type (Nc9) or mutant p53 protein. Three days post-transfection, cell lysates were prepared and CAT activities were measured to indicate the levels of transcription from the MCK promoter. The represented data were derived from at least three independent experiments. (B) REFs were transfected with 5μg pETCAT along with either 15μg CMVneo (control) or 5μg (1x), 10μg (2x), or 15μg (3x) of a plasmid encoding either Nc9, dl163, or 281EG. Appropriate amounts of CMVneo were also used so that the total amount of plasmid was 20μg in each case. CAT activities were then determined from three independent experiments (as described above).
correlation between induction of apoptosis in LFSFs and suppression of transformation in REFs (refer to Section 4.2.7) indicates that additional cellular factors are not affecting the functions of specific mutant p53 proteins.

5.2.2 Mutant forms of p53 vary in their abilities to repress transcription

Although p53 is known to repress transcription from various gene promoters, the importance of this biochemical activity in the cellular functions of p53 remains ambiguous. Investigations were therefore planned to determine the relationships between transcriptional repression and the growth inhibitory and apoptotic effects of p53. For this reason, the abilities of various mutant forms of p53 to inhibit growth or induce apoptosis were compared with their abilities to repress transcription from the simian virus 40 (SV40) promoter which is known to be strongly repressed by wild-type p53 (Subler et al., 1992; Jackson et al., 1993). The various mutant forms of mouse p53 used in this study are diagrammatically represented in Figure 5.1. The ability of each of these proteins to repress transcription was determined by transfecting REFs with the appropriate gene expression plasmids and using CAT assays to determine the levels of transcription.

This study showed that wild-type p53 (Nc9), mp53, c5, and 281EG can repress transcription from the SV40 promoter, while the remaining mutant forms of p53 are defective for this function (Figure 5.3A). d11.1 and d12.3 not only failed to repress transcription, but actually activated transcription from the SV40 promoter (between 3 and 5 fold) (Figure 5.3). Although the repressive effect of mp53 was minimal, it is most probably significant as this protein has been shown to repress transcription from the SV40 promoter in a dose-dependent manner in other cell types (Paul Jackson - personal communication).

The studies described above and in Section 4.2.2 have demonstrated that mp53 and 281EG can repress gene transcription but cannot inhibit cell growth, while d1163 can inhibit cell growth (as effectively as wild-type p53) but appears not to repress gene transcription (Figures 4.5 and 5.3 and Table 5.1). Thus, it would appear that transrepression by p53 is neither sufficient nor necessary for the growth inhibitory effects of this protein. Furthermore, the studies have demonstrated that c5 can repress gene transcription but cannot induce apoptosis, while there were no mutant p53 proteins which can induce apoptosis without repressing gene transcription (Figures 4.5 and 5.3 and Table 5.1). These observations indicate that transcriptional repression may be necessary but is not sufficient for p53-mediated apoptosis.

While the assays for transrepression and growth inhibition were both performed in REFs, the assays for apoptosis were performed in LFSFs. However, for the reasons explained in the preceding section, comparisons in the activities of proteins between these two cell types appear to be reasonable.
Figure 5.3. Mutant p53 proteins exhibit varying capacities for transcriptional repression. (A) REFs were transfected (through the use of CaPO₄) with: 10μg pSV₂CAT (encodes CAT under the control of the SV40 promoter) along with either 10μg CMVneo (control) or 10μg of a plasmid encoding wild-type (Nc9) or mutant p53 protein. Three days post-transfection, cell lysates were prepared and CAT activities were measured to indicate the levels of transcription from the SV40 promoter. The represented data were derived from at least three independent experiments. (B) The full graph is shown to indicate the levels of transactivation by dl1.1 and dl2.3.
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Table 5.1. Comparisons of the cellular and biochemical activities of mutant p53 proteins. ++ means: functional capacity is similar to that of wild-type p53; + means: functional capacity is less than that of wild-type p53; and - means: no functional capacity. The conformation of each protein was determined by its interaction with pAb240 or pAb246 (refer to Figure 4.6B): + means: 'wild-type' conformation and - means: 'mutant' conformation. * Information provided by Antony Braithwaite - personal communication.
5.2.3 Mutant forms of p53 vary in their abilities to inhibit SV40 origin-dependent DNA replication

Another biochemical activity of p53 is the inhibition of DNA replication from the SV40 origin of replication. This activity was demonstrated by transfecting COS cells (which have been transformed by the SV40 large T antigen (LTag)) with a control plasmid or a plasmid encoding wild-type mouse p53 (both of which contain an SV40 origin of replication). Three days post-transfection, DNA was extracted from the cells and treated with a methylation-dependent restriction enzyme, DpnI. While the original transfected plasmids (prepared in bacterial cells) were methylated and sensitive to DpnI digestion, the replicated plasmids (synthesised in COS cells) were unmethylated and resistant to this enzyme. The quantity of undigested (replicated) plasmid DNA was then determined by transformation of E.coli. as the number of colonies of transformed cells is proportional to the number of intact plasmids. The same procedure was used for HeLa cells except that a plasmid encoding LTag was co-transfected with the other plasmids as HeLa (unlike COS cells) do not have endogenous LTag (which is needed for SV40 DNA replication).

While thousands of bacterial colonies (representing the quantity of replicated plasmid DNA) were formed after transforming E.coli. with the samples of DNA from the control cells, very few colonies were produced with the samples of DNA from the cells which expressed wild-type mouse p53 (Figure 5.4A&B). Thus, p53 was shown to inhibit the replication of plasmids from SV40 origins of replication in both COS and HeLa cells.

Although the ability of p53 to inhibit SV40 DNA replication is well known, the importance of this type of activity in the cellular functions of p53 has not been studied. Investigations were therefore planned to determine the function(s) which enable p53 to inhibit viral DNA replication are involved in the growth inhibitory and / or apoptotic effects of p53. For this reason, the abilities of various mutant forms of p53 to inhibit growth or induce apoptosis were compared with their abilities to inhibit SV40 DNA replication. The mutant forms of mouse p53 used in this study are diagrammatically represented in Figure 5.1. Because the accurate measurement of replicated plasmid DNA depends upon the presence of a reliable number of plasmids within the cells, those plasmids which might transfect cells poorly (ie. CMV281EK, CMVd1.1, and CMVd12.3 - as indicated by the amount of protein normally expressed from these plasmids (refer to Section 4.2.4 and Figure 4.6)) were excluded from this assay. The ability of various mutant p53 proteins (as represented in Figure 5.1) to inhibit SV40 DNA replication was determined by the procedure described above.

This study showed that wild-type p53 (Nc9), mp53, dl163, c5, and 281EG can inhibit SV40 origin dependent DNA replication, while dl518 remains defective for this function. While mp53, dl163, and 281EG had moderate inhibitory effects, Nc9 and c5
Figure 5.4. Mouse p53 inhibits SV40 DNA replication in different cell types. (A) COS cells were transfected (through the use of DEAE-dextran) with plasmids which each contain an SV40 origin of replication: either 10μg CMVneo (control) or 10μg CMVNe9 (encodes wild-type mouse p53). Three days post-transfection, DNA was extracted from the cells and treated with a methylation-dependent restriction enzyme, DpnI. While the original transfected plasmids were methylated and therefore sensitive to DpnI digestion, the replicated plasmids were unmethylated and therefore resistant to this enzyme. The quantity of undigested (replicated) plasmid DNA was then determined by transformation of E.coli. as the number of colonies of transformed cells is proportional to the number of intact plasmids. (B) COS cells were transfected as described above, and HeLa cells were transfected (through the use of CaPO₄) with 10μg RSV-T (encodes LTag) along with either 10μg CMVneo or 10μg CMVNe9. The DNA was then extracted, treated with DpnI and used for transforming E.coli. as described above. The average numbers of bacterial colonies were determined from at least three independent experiments.
could almost completely inhibit the replication of DNA from SV40 origins (Figure 5.5A&B).

Figure 5.6A shows the expression of the p53 proteins in COS cells, as determined from immunoprecipitations of labelled cellular proteins with a p53-specific antibody. While dl518 is expressed very well, there is moderate expression of mp53, dl163, and 281EG, and very poor expression of Nc9 and c5. These results provide further support for the inhibitory effects of the p53 proteins on SV40 DNA replication as the quantity of each protein reflects the quantity of replicated plasmid DNA from which the protein is expressed.

Because replication of DNA from SV40 origins requires LTag, the ability of p53 to bind this protein may be important for the inhibition of SV40 DNA replication. This possibility was investigated by determining which of the mutant p53 proteins were able to bind LTag. Figure 5.6A&B shows the complexes of certain p53 proteins with LTag as determined by immunoprecipitations of these complexes with a p53-specific antibody. While Nc9, mp53, dl163, and 281EG bound LTag well, c5 bound LTag quite weakly and dl518 showed no affinity for this protein at all. Thus, these studies have demonstrated that c5 binds LTag weakly yet almost completely inhibits SV40 DNA replication, while mp53, dl163, and 281EG bind LTag well yet exhibit a moderate inhibition of SV40 DNA replication (Figures 5.5 and 5.6). While the binding of p53 to LTag may be involved, these observations suggest that it is certainly not the only factor which contributes to the ability of p53 to inhibit replication of DNA from SV40 origins.

The studies described above and in the previous chapter (Section 4.2.2) have demonstrated that mp53 and 281EG can inhibit the replication of DNA from SV40 origins but cannot inhibit cell growth, while each of the proteins which can inhibit cell growth can also inhibit SV40 DNA replication (Figures 4.5 and 5.5 and Table 5.2). Thus, one can conclude that the function(s) which enable p53 to inhibit SV40 DNA replication might be necessary but are not sufficient for the ability of p53 to inhibit the growth of REFs. These studies have also demonstrated that dl163 and c5 can inhibit SV40 DNA replication but cannot induce apoptosis, while each of the proteins which can induce apoptosis can also inhibit the replication of SV40 DNA (Figures 4.5 and 5.5 and Table 5.2). Thus, one can additionally conclude that the function(s) which enable p53 to inhibit SV40 DNA replication might be necessary but are not sufficient for the ability of p53 to induce apoptosis.

While the assays for growth inhibition and apoptosis were performed in REFs and LFSFs (respectively), the assays for inhibition of SV40 DNA replication were performed in COS cells. This must be taken into account when comparing the activities of proteins in these assays as additional cellular factors may affect the functions of specific mutant p53 proteins.
Figure 5.5. Mutant p53 proteins exhibit varying capacities for inhibition of SV40 DNA replication. COS cells were transfected with 10μg CMVneo (control) or 10μg of a plasmid encoding wild-type (Ne9) or mutant p53 protein. Three days post-transfection, the DNA was then extracted, treated with DpnI and used for transforming E.coli. (refer to Figure 5.4 for more details). The average numbers of bacterial colonies (representing the amounts of replicated plasmid DNA) were determined from at least three independent experiments. The data is represented on both linear (A) and logarithmic (B) scales.
Figure 5.6. Wild-type and mutant p53 proteins: expression in COS cells and binding of LTag. (A) To determine the expression levels of proteins: COS cells were transfected (through the use of DEAE-dextran) with 10μg CMVneo (control) or 10μg of a plasmid encoding wild-type (Nc9) or mutant p53 protein. Three days post-transfection, the cellular proteins were radiolabelled, cell lysates were prepared, and immunoprecipitations were performed with normal sheep serum (NSS) or pAb200-47 (recognises p53). (B) While complexes of p53 and LTag are observed in COS cells, variations in expression levels of p53 proteins obscures their relative abilities to bind LTag. HeLa cells were therefore transfected (through the use of CaPO₄) with 10μg RSV-T (encodes LTag) along with either 10μg CMVneo (control) or 10μg of a plasmid encoding wild-type (Nc9) or mutant p53 protein. Three days post-transfection, cells were treated and immunoprecipitations were performed as described above (except that normal rabbit serum (NRS) was used instead of NSS).
### Table 5.2. Comparisons of the cellular and biochemical activities of mutant p53 proteins, including inhibition of SV40 DNA replication.

++ means: functional capacity is similar to that of wild-type p53; + means: functional capacity is less than that of wild-type p53; and - means: no functional capacity. The conformation of each protein was determined by its interaction with pAb240 or pAb246 (refer to Figure 4.6B): + means: 'wild-type' conformation and - means: 'mutant' conformation.

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<tr>
<th>p53 protein</th>
<th>Suppression of Transformation (E1a+ras)</th>
<th>Apoptosis</th>
<th>Growth Inhibition</th>
<th>Activation of Transcription</th>
<th>Repression of Transcription</th>
<th>Inhibition of SV40 DNA Replication</th>
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5.3 Discussion

5.3.1 Activation of gene transcription requires the N-terminal and central regions of p53

In this chapter, the examination of various mutant forms of p53 for their abilities to activate gene transcription (in REFs) has revealed new information about the regions of the p53 protein required for this activity. The importance of certain amino acids and protein regions as well as the protein conformation in the transactivation function of mouse p53 (as indicated by this study) is illustrated in Figure 5.7A.

While at least part of the region between amino acids 14 and 66 in the N-terminus of mouse p53 was found to be necessary for transcriptional activation, the integrity of amino acids 45, 76, and 78 was not important for this function (Figures 5.2 and 5.7A and Table 5.1 - refer to dll163 and mp53 respectively). These findings are consistent with those of other studies which have shown that regions within the N-terminus of human p53 are critical for transactivation (Unger et al., 1993; Pietenpol et al., 1994; Sang et al., 1994; Subler et al., 1994; Zhang et al., 1994b). In human p53, the region of importance must lie between amino acids 1 and 73 as the fusion of this sequence to a GAL4 DNA binding domain is sufficient for the activation of promoters which contain a GAL4 binding site (Fields and Jang, 1990). Regions which are necessary for transactivation within the N-terminus of p53 are also known to bind to TBP (Liu et al., 1993; Truant et al., 1993) and TAFII40 and TAFII60 (Thut et al., 1995) (all of which are sub-units of the transcription factor, TFIID) as well as the transcription factor, TFIIH (Xiao et al., 1994). Thus, interactions of p53 with basal transcription factors appear to be important for transcriptional activation. This model is supported by the observation that the abilities of regions within p53 to bind TBP correlated with their abilities to activate gene transcription (Liu et al., 1993).

The studies in this chapter have demonstrated that the central region of mouse p53 is also important for transcriptional activation as the deletion of amino acids 133 to 148, the mutation of amino acids 168 and / or 234, and a non-conservative mutation of amino acid 281 each abrogate the transactivation function of this protein (Figures 5.2 and 5.7A and Table 5.1 - refer to dl518, c5, and 281EK). Additionally, a conservative mutation of amino acid 281 does not abrogate, but does reduce the effects of p53 on gene transcription (Figures 5.2 and 5.7A and Table 5.1 - refer to 281EG). These observations are in agreement with studies which have used various mutations and deletions in the human p53 protein to show that most of the central region is necessary for transcriptional activation (Kern et al., 1992; Crook et al., 1994). Since the deletion of amino acids 133 to 148 or the mutation of amino acids 168 and / or 234 disrupt the conformation of p53 (Figure 4.6 - refer to dl518 and c5 respectively), one can suggest that p53-dependent
transactivation requires the integrity of protein conformation (Figure 5.7A). However, this is not the only property of the central region which is of importance as some mutant forms of p53 retain the 'wild-type' conformation yet fail to activate gene transcription (Ory et al., 1994). As discussed in Section 5.1, the presence of a p53-binding site within a promoter allows that promoter to be activated by p53 (Farmer et al., 1992; Kern et al., 1992; Zambetti et al., 1992). Therefore, since studies of both human and mouse p53 have shown that the sequence-specific DNA binding function is contained within the central hydrophobic domain (Bargonetti et al., 1993; Pavletich et al., 1993; Wang et al., 1993), the requirement to bind specific sequences of DNA means that the central region of p53 is critical for activation of gene transcription. Within this region, the importance of particular amino acids for p53-dependent transactivation appears to depend upon the promoter and the actual p53-binding site contained therein (Chen et al., 1993a).

With regard to the C-terminus of mouse p53, the studies presented in this chapter have indicated that this region contributes to but is not necessarily essential for activation of gene transcription. While the deletion of amino acids 304 to 333 reduces but does not eliminate transcriptional activation, the larger deletion of amino acids 304 to 364 further reduces but may or may not eliminate this function of p53 (Figures 5.2 and 5.7A and Table 5.1 - refer to dl1.1 and dl2.3). Interestingly, some studies of human and mouse p53 have shown that most of the C-terminus is not necessary for transactivation (Wang et al., 1993; Sang et al., 1994; Subler et al., 1994; Zhang et al., 1994a), while other studies have shown that this region is necessary for transcriptional activation by p53 (Crook et al., 1994; Pietenpol et al., 1994; Zhang et al., 1994a). These inconsistencies in the literature may be explained by the finding that the requirement of the C-terminus in p53-dependent transcriptional activation is dependent upon the promoter and in particular, the p53-binding site contained therein (Zhang et al., 1994a). Other studies have demonstrated that while regions within the C-terminus are often not critical for transactivation, they can contribute to ability of p53 to tightly bind specific sequences of DNA (Hupp et al., 1992; Shaulian et al., 1993; Tarunina and Jenkins, 1993; Stenger et al., 1994). Therefore, if the C-terminus contributes to the activation of transcription by stabilising the interaction of p53 with the promoter, one might expect that the importance of this region would vary with the different p53-binding sites. The reason for the ability of C-terminal sequences to increase the stability of DNA binding may be because these sequences are also required for the oligomerization of p53 proteins (Milner et al., 1991; Shaulian et al., 1992; Sturzbecher et al., 1992) as this property is known to correlate with enhanced sequence-specific DNA binding (Shaulian et al., 1993; Tarunina and Jenkins, 1993; Hainaut et al., 1994; Stenger et al., 1994). However, it should also be noted that a region toward the end of the C-terminus which allows p53 to bind DNA in a non-specific manner (Foord et al., 1991; Wang et al., 1993; Wu et al., 1995) appears to negatively regulate the sequence-specific DNA binding by p53 (Hupp et al., 1992; Bayle et al., 1995). Thus, the
Figure 5.7. Regions of p53 required for (A) transcriptional activation and (B) transcriptional repression. The importance of an amino acid or a protein region for (A) activation or (B) repression of gene transcription is indicated by its intensity. Information about the regions in the background is not provided by the studies presented in this chapter. The importance of the protein conformation for each function is indicated by the intensity of that part of the figure. Refer to Sections 5.3.1 and 5.3.2 for more details.
influences of the C-terminus of p53 on transcriptional activation may be complex and appears to vary between promoters.

In summary, the results of this chapter have shown that transactivation by p53 requires both the N-terminal and central regions of this protein. While the N-terminus is important for its interactions with basal transcription factors (such as TBP), the central region is important for its interaction with specific sequences of DNA. As mentioned in Section 5.1, the binding of p53 to both DNA and basal transcription factors may activate transcription by promoting the formation of a stable complex between the promoter and the transcriptional machinery (Chen et al., 1993b). While not necessarily essential for transcriptional activation, the C-terminal region of p53 may contribute to this function in a promoter-dependent manner by regulating the sequence-specific DNA binding activity of this protein.

5.3.2 Repression of gene transcription requires the N-terminal and C-terminal regions of p53

The studies presented in this chapter have examined various mutant forms of p53 for their abilities to repress gene transcription (in REFs) and have revealed new information about the regions of the p53 protein required for this function. The importance of certain amino acids and protein regions in the transrepression function of p53 (as indicated by the results of this chapter) is illustrated in Figure 5.7B.

In the N-terminus of mouse p53, at least part of the region between amino acids 14 and 66 is necessary for transcriptional repression, while the integrity of amino acids 45, 76 and / or 78 appears to contribute to this function. These findings are similar to those of a previous study which used the same p53 proteins in a different cell type (Jackson et al., 1993) and consistent with those of other studies which have demonstrated that transrepression by human p53 requires the N-terminal region of the protein (Sang et al., 1994; Subler et al., 1994). Since this region is also known to bind TBP (Liu et al., 1993; Truant et al., 1993), the repression of gene transcription may be explained at least in part by an inhibitory effect on TBP due to its interaction with the N-terminus of p53. In agreement with this suggestion, p53 is known to prevent the binding of TBP to the TATA-box sequence (Chen et al., 1993b; Ragimov et al., 1993) and repress transcription from promoters which rely on this activity (Mack et al., 1993) (refer to Section 5.1). Thus, interactions which inhibit the binding of TBP (and probably other transcription factors) to DNA may be important for the contribution of the N-terminus of p53 to the transrepression effects of this protein.

With regard to the central region of mouse p53, the studies in this chapter have shown that the deletion of amino acids 133 to 148 or a non-conservative mutation of amino acid 281 (Figures 5.2 and 5.7B and Table 5.1 - refer to d1518 and 281EK
respectively) abrogate p53-dependent transcriptional repression, while the mutation of amino acids 168 and / or 234 or a conservative mutation of amino acid 281 does not affect this function (Figures 5.2 and 5.7B and Table 5.1 - refer to c5 and 281EG respectively). Thus, the integrity of central region appears to be dispensable for the transrepression activity of p53. The above findings only partly agree with a previous study which showed that the deletion of amino acids 133 to 148 or the mutation of amino acids 168 and / or 234 abrogated the transcriptional repression function of p53 in HeLa cells (Jackson et al., 1993). The discrepancy observed with c5 (which has mutations of amino acids 168 and 234) may be due to different levels of protein expression in the different cell types. With regard to the results presented in this chapter, this mutant form of p53 has a disrupted conformation (Figure 4.6 - refer to c5) yet retains its ability to repress gene transcription. Thus, one can conclude that at least in REFs, integrity of protein conformation is not necessary for transrepression by p53. The other observation (as presented above) that some changes within the central region of p53 abrogate transcriptional repression is consistent with those of another study which showed that various point mutations within the this region of human p53 have the same effect (Deb et al., 1992; Ory et al., 1994). These results imply that a property of the central region is required for p53-dependent transrepression. However, since dl518 and 281EK fail in each of the functions examined in this and the previous chapter (refer to Table 5.1), the changes in these mutant p53 proteins might be so severe that each of their activities are abrogated even if a property of the central region of p53 is not specifically required. Such may also be the case with the mutant forms of human p53 (that were used in the studies mentioned above) which failed to repress transcriptional due to mutations within the central region of the protein.

The results presented in this chapter have also demonstrated the C-terminus of mouse p53 is necessary for transcriptional repression as the removal of part of this region prevents transrepression and even activates transcriptional activity from this promoter (Figures 5.2 and 5.7B and Table 5.1 - refer to dl1.1 and dl2.3). This type of effect has also been observed with mutations in the central region of p53 (Deb et al., 1992). The activation rather than repression of gene transcription might be explained by the mutant form of p53 inadvertently binding to part of the promoter so that the interactions of the protein with basal transcription factors does not inhibit but actually stabilises the binding of the transcriptional machinery to the promoter (refer to Section 5.1 for information on mechanisms of transactivation and transrepression). Regardless of this effect, the failure of the C-terminal deletion proteins to repress transcription indicates that at least part of the region between amino acids 304 and 322 is critical for this activity of p53. This finding is in agreement with other studies which have shown that removal of most of the C-terminus of human p53 abrogates transcriptional repression by this protein (Crook et al., 1994; Sang et al., 1994; Subler et al., 1994). As with the N-terminus of p53, the role of
the C-terminus in repression of gene transcription may also be an inhibitory effect on TBP activity as the region of the C-terminus which is required for transrepression is also known to bind TBP (Horikoshi et al., 1995). Further support for the idea that the C-terminal region of p53 causes transcriptional repression through interactions with basal transcription factors comes from the observation that this region alone can repress the effects of several transcriptional activators (Shaulian et al., 1995).

In summary, the studies in this chapter have demonstrated that while the central region may have some role in transcriptional repression, the N-terminal and C-terminal regions of p53 are critical for this function. These regions appear to contribute to the repression of gene transcription by binding to and inhibiting the activities of transcription factors such as TBP.

5.3.3 p53-dependent inhibition of SV40 DNA replication requires more than the binding of LTag

In this chapter, the examination of various mutant forms of mouse p53 for their abilities to inhibit SV40-origin dependent DNA replication has revealed new information about the particular amino acids and protein regions required for this function. The results of this study have also provided an indication of the basic activities which contribute to the inhibitory effect of p53 on the replication of SV40 DNA.

The ability of p53 to inhibit SV40 DNA replication is reduced but not eliminated by the deletion of amino acids 14 to 66 or the mutation of amino acids 45, 76 and / or 78 in the N-terminal region of the protein (Figure 5.5 and Table 5.2 - refer to dl163 and mp53 respectively). These findings are consistent with the those of other studies which showed that the integrity of the N-terminus is not critical for p53-dependent inhibition of DNA replication (Braithwaite et al., 1987; Sturzbecher et al., 1988; Ridgway et al., 1994). However, the results presented above indicate that sequences within the N-terminus do contribute to the full inhibitory effects of p53 on the replication of viral DNA. Since this region is also known to bind RPA (Dutta et al., 1993; Li and Botchan, 1993), the N-terminus of p53 may contribute to the complete inhibition of SV40 DNA replication by interfering with the functions of RPA and consequently obstructing the unwinding of the DNA template (refer to Section 5.1).

With regard to the central region of mouse p53, the integrity of some parts of this region appear to be important for inhibition of SV40 DNA replication while other parts are not. In agreement with previous studies (Braithwaite et al., 1987; Sturzbecher et al., 1988), the deletion of amino acids 133 to 148 was found to abrogate p53-dependent inhibition of DNA replication, while a conservative mutation at amino acid 281 does not eliminate but does impair this function (Figure 5.5 and Table 5.2 - refer to dl518 and 281EG respectively). The mutation of amino acids 168 and / or 234 however has no
effect on the ability of p53 to inhibit the replication of SV40 DNA (Figure 5.5 and Table 5.2 - refer to c5). Since the conformation of p53 is disrupted by the mutation of amino acids 168 and / or 234 (Figure 4.6 and Table 5.2 - refer to c5), the integrity of protein conformation must not be necessary for the inhibition of SV40 DNA replication. The binding of specific sequences of DNA also appears to be dispensable for this function as the mutations of amino acids 168 and / or 234 would also be expected to prevent sequence-specific DNA binding (as evidenced by the inability of c5 to activate gene transcription - refer to Section 5.3.1). Thus, while the inhibition of SV40 origin-dependent DNA replication requires at least part of the central region of p53, it does not require the integrity of protein conformation nor the ability to bind specific sequences of DNA.

Another property which involves the central region of p53 and which may be important for the inhibition of SV40 DNA replication is the binding of LTag. In agreement with a previous study (Jenkins et al., 1988), the results presented in this chapter showed that the binding of LTag by p53 is abrogated by the deletion of amino acids 133 to 148 (Figure 5.6 - refer to d1518). The ability of p53 to bind LTag was also shown to be impaired by the mutation of amino acids 168 and / or 234 (Figure 5.6 - refer to c5). These findings are consistent with other studies which demonstrated that regions within the central region of p53 are necessary for this function (Tan et al., 1986; Jenkins et al., 1988). Interestingly, it was observed that a mutant p53 protein (c5) which binds LTag weakly almost completely inhibits SV40 DNA replication, while other proteins (mp53, dl163, and 281EG) which bind LTag well can only moderately inhibit the replication of SV40 DNA (Figures 5.5 and 5.6 and Table 5.2). Thus, the binding of LTag may contribute but is not sufficient for the full inhibitory effects of p53 on SV40 DNA replication. These findings are consistent with those of other studies which have shown that the binding of LTag is neither sufficient nor necessary for this function (Jenkins et al., 1989; Sturzbecher et al., 1992). In further support of these conclusions, it has been demonstrated that p53 which is already bound to LTag inhibits DNA replication as efficiently as unbound p53 (Wang et al., 1989). Other activities of p53 must therefore be important for the ability of this protein to inhibit the replication of DNA from SV40 origins.

While the studies presented in this chapter do not address the importance of the C-terminus in the ability of p53 to inhibit SV40 DNA replication, this region may be necessary as it is capable of binding RPA (Dutta et al., 1993). On the other hand, the inhibitory effects of p53 on RPA activity may be fully achieved through the interaction of the N-terminus of p53 with this protein (as mentioned above). If the C-terminal region of p53 does contribute to the inhibition of SV40 DNA replication, it is unlikely to involve the oligomerization function of this region as certain mutant forms of p53 fail to oligomerize yet retain the ability to inhibit the replication of DNA (Sturzbecher et al.,
1992). Thus, monomeric forms of p53 appear to be sufficient for inhibition of SV40 origin-dependent DNA replication.

In summary, the ability of p53 to inhibit SV40 DNA replication involves considerably more than the binding of LTag. From the results presented in this chapter, it would appear that a function of the N-terminus and a function of the central region (and possibly the C-terminus) are both necessary for the full inhibitory effect of p53 on the replication of DNA. The binding and inhibition of RPA may account for at least one of these functions. Whatever mechanisms are necessary for inhibition of SV40 DNA replication, they must involve a direct effect of the p53 protein on the DNA replication process as this function of p53 is known to occur in vitro (Sturzbecher et al., 1988; Wang et al., 1989).

5.3.4 Transcriptional activation is not necessary for p53-dependent growth inhibition

The studies presented in this and the previous chapter have demonstrated that some mutant forms of p53 (mp53, 281EG, and dl1.1) retain the ability to activate gene transcription in REFs (Figure 5.2) yet fail to inhibit the growth of these cells (Figure 4.5 and Table 5.1). It would therefore appear that transcriptional activation is not sufficient for p53-dependent growth inhibition. However, in comparing the effects of a protein in two different assays, one must consider the differences in the levels of protein required for each activity. Because the assays for gene transcription involve the protein acting on promoters which are exogenous and therefore present in high quantities, while the assays for cell growth involve the protein acting on endogenous target molecules, more protein would be required for an effect on transcription than for an effect on cell growth. Therefore, since the failure of the above proteins to inhibit cell growth cannot be attributed to insufficient protein quantities, one can conclude that transactivation is not sufficient for the growth inhibitory effects of p53.

The results of this and the previous chapter have also demonstrated that a mutant form of p53 (dl163) fails to activate gene transcription in REFs (Figure 5.2) yet retains the ability to inhibit the growth of these cells as efficiently as wild-type p53 (Figure 4.5 and Table 5.1). Thus, one can suggest that transcriptional activation is not necessary for p53-dependent growth inhibition. However, since more protein would be required for an effect on gene transcription than for an effect on cell growth, the failure of dl163 to transactivate may be due to insufficient protein quantities. However, this is unlikely as the expression of dl163 in REFs is not significantly different to that of wild-type p53 (No9) (Figure 4.6B) which transactivates very efficiently. Even so, this possibility was addressed by examining increasing amounts of this protein for dose-dependent effects on gene transcription. Since three times the normal amount of dl163 still failed to activate
transcription, it would appear that the deletion of amino acids 14 to 66 in the N-terminus completely abolishes the transactivation function of mouse p53. This conclusion is supported by the observations that a similar region in the N-terminus of human p53 is necessary for transcriptional activation (Unger et al., 1993; Pietenpol et al., 1994; Sang et al., 1994; Subler et al., 1994; Zhang et al., 1994b) as the critical region occurs between amino acids 1 and 73 (Fields and Jang, 1990) and relies on the integrity of amino acids 22 and 23 (Lin et al., 1994). In light of the above results, one can reasonably conclude that the ability of p53 to activate gene transcription is not necessary for the inhibition of cell growth.

One explanation for the growth inhibitory effects in the presence of dl163 could be suggested from a report which showed that this mutant form of p53 increases the levels of endogenous wild-type p53 by enhancing the stability of the protein (May et al., 1991). However, this effect was not observed with the levels of endogenous p53 in REFs which express dl163 (Figure 4.6A, NB: antibody 200-47 detects both mouse and rat p53). Increases in the levels of endogenous p53 with dl163 were also not observed when this experiment was repeated in the same cells (ie. HeLa cells) as those used in the original study (Jackson et al., 1994). Further evidence that growth inhibition in the presence of dl163 cannot be attributed to the activity of endogenous wild-type p53 is that the presence of dl518 which was also shown to enhance the levels of endogenous p53 (May et al., 1991), does not inhibit the growth of REFs (Figure 4.5A). Since dl518 fails to exhibit any of the activities observed with wild-type p53 (Table 5.1), the possibility of enhanced levels of endogenous p53 is not relevant to the studies in this thesis. Thus, one can still conclude that a transactivation-deficient p53 protein (dl163) is capable of inhibiting cell growth.

Interestingly, other studies which have examined the activities of mutant p53 proteins have found a correlation between the transactivation and growth inhibitory functions of p53 (Crook et al., 1994; Ory et al., 1994; Pietenpol et al., 1994). While these findings contrast with the finding that p53-dependent transcriptional activation is neither sufficient nor necessary for inhibition of cell growth, the two results may not be incompatible as a correlation between two functions does not necessarily mean that one function is sufficient or even necessary for the other. However, it should also be noted that the type of cells used to observe p53-dependent growth inhibition may influence the results. Since the studies mentioned above (Crook et al., 1994; Ory et al., 1994; Pietenpol et al., 1994) used cells which had been derived from different cancers, a growth inhibitory effect which occurs in normal fibroblasts and which is independent of transactivation (ie. that which is caused by dl163 in REFs) may not have been observed as the cancer-derived cells may have been resistant to this effect. Thus, p53 may have more than one mechanism for inhibiting cell growth.
In agreement with the results presented in this and the previous chapter, another study of mutant p53 activity has indicated that transcriptional activation by human p53 is not sufficient for growth inhibition (Zhang et al., 1994b). However, in this study, the C-terminal deletion protein which fails to inhibit growth yet activates transcription is only capable of activating a certain type of p53-responsive promoter (Zhang et al., 1994a). The possibility therefore remains that some cases of p53-dependent transactivation are sufficient for inhibition of cell growth. The ability of mutant forms of p53 to activate transcription in a promoter-dependent manner might also affect the conclusions made from the studies in this chapter. However, since the effect of a mutated p53 protein on a particular promoter appears to be determined by the stability of its interaction with the p53-binding site contained therein (Zhang et al., 1994a), only those proteins which have an altered ability to bind specific sequences of DNA (ie. those with mutations in the central region and C-terminus) would be expected to exhibit variations in activity between different p53-responsive promoters (refer to Section 5.3.1). The transcriptional activity of proteins which have changes within the N-terminus should therefore remain relatively constant from one promoter to the next. Thus, the results obtained with dl163 and mp53 (as mentioned above) still allow one to conclude that transactivation (of p53-responsive promoters in general) is neither sufficient nor necessary for the growth inhibitory effects of p53.

One of the main reasons for the association between the transactivation and growth inhibitory functions of p53 is that p21\textsuperscript{WAF1} is induced in cells which are subject to p53-dependent growth inhibition (Dulic et al., 1994; El-Deiry et al., 1994) (refer to Section 3.3.2). While the gene for p21\textsuperscript{WAF1} is transcriptionally activated by p53 (El-Deiry et al., 1993), the encoded protein can inhibit the functions of the cyclins and cyclin-dependent kinases (cdks) which are necessary for progression of cells from G1 phase into S phase of the cell cycle (Harper et al., 1993; Xiong et al., 1993) (refer to Section 1.3.4). Since one of the mechanisms by which these cyclin-cdk\textsuperscript{s} act is the hyperphosphorylation and consequent inactivation of pRb (Hinds et al., 1992; Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993), the induction of p21\textsuperscript{WAF1} would cause pRb to remain in an active state (Harper et al., 1993; Slebos et al., 1994) so that the cells are prevented from entering S phase (refer to Section 1.3.3). Thus, p53 is thought to arrest cell growth (in G1 phase) through the transactivation of p21\textsuperscript{WAF1}.

These findings appear to be contradicted by the observation that transcriptional activation is not necessary for p53-dependent growth inhibition. However, as suggested above, p53 may have more than one biochemical mechanism for inhibiting cell growth. While one pathway would involve the transactivation of p21\textsuperscript{WAF1} and possibly other proteins, an alternative pathway would make use of one or more other functions of p53. The existence of this latter pathway has also been suggested by a study which showed that human p53 can arrest cell growth without inducing the expression of p21\textsuperscript{WAF1}.
The observation that transcriptional activation is not sufficient for p53-dependent growth inhibition also has implications for the above model of p53 activity. While one of the biochemical pathways leading to cell growth inhibition may certainly involve the transactivation of p21WAF1, this activity is not sufficient for a cellular effect and must therefore be accompanied by at least one other function of p53. While this type of function might use a separate mechanism for increasing the levels of p21WAF1, it might alternatively influence the activity of one or more completely separate intermediary proteins. The biochemical mechanisms which appear to be important for the ability of p53 to inhibit cell growth are illustrated in Figure 5.8. Whatever these mechanisms may be, the findings in chapter 3 indicate that each of the pathways leading to growth inhibition must be somehow mediated by pRb as the binding and inactivation of this protein by LTag can completely overcome the growth inhibitory effects of p53 (refer to Section 3.3.2).

5.3.5 Multiple biochemical activities may be involved in p53-dependent growth inhibition

Since transcriptional activation fails to account for much of the growth inhibitory effect of p53 (as discussed in the previous section), it is possible that transcriptional repression is involved. In relation to this, the results of this and the previous chapter have demonstrated that some mutant forms of p53 (mp53 and 281EG) retain the ability to repress gene transcription in REFs (Figure 5.3) yet fail to inhibit the growth of these cells (Figure 4.5 and Table 5.1). As explained in the preceding section, the quantities of protein required for an effect on transcription would be greater than that required for an effect on cell growth. Therefore, since the failure of the above proteins to inhibit cell growth cannot be attributed to insufficient protein quantities, one can conclude that transcriptional repression is not sufficient for p53-dependent growth inhibition. The studies presented in this and the previous chapter have also shown that a mutant form of p53 (dl163) fails to repress gene transcription in REFs (Figure 5.3) yet retains the ability to inhibit the growth of these cells as efficiently as wild-type p53 (Figure 4.5 and Table 5.1). Thus, one can suggest that transcriptional repression is not necessary for p53-dependent growth inhibition. However, since more protein would be required for an effect on gene transcription than for an effect on cell growth, the failure of dl163 (which has a deletion of amino acids 14 to 66) to transrepress could be due to insufficient protein quantities. As mentioned in the previous section, this is unlikely as the expression of dl163 in REFs is not significantly different to that of wild-type p53 (Nc9) (Figure 4.6B) which represses transcription very efficiently. An inability of dl163 to transrepress is consistent with studies which demonstrate that the N-terminal region of p53 is necessary for transcriptional repression (Sang et al., 1994; Subler et al., 1994) (refer to
Section 5.3.2). However, another study which showed that the C-terminus alone can repress the activity of several transcriptional activators (Shaulian et al., 1995) suggests that dll63 may retain some ability repress gene transcription. Thus, the transrepression function of p53 may or may not be necessary but is definitely not sufficient for the ability of this protein to inhibit cell growth.

Another known function of p53 is the inhibition of SV40 origin-dependent DNA replication. This activity may reflect a role for p53 in the regulation of cellular DNA replication as an activated form of p53 is known to inhibit the replication of DNA within the nucleus (Cox et al., 1995). The possibility that an inhibitory effect on DNA replication could be involved in the growth inhibitory effects of p53 is addressed by the studies presented in this and the previous chapter. The results demonstrated that some mutant forms of p53 (mp53 and 281EG) retained the ability to inhibit SV40 DNA replication (Figure 5.5) yet failed to inhibit the growth of cells (Figure 4.5 and Table 5.2). The assay for SV40 DNA replication involves the protein acting against the activities of high levels of LTag (in COS cells) on SV40 origins which are exogenous and therefore present in high quantities, while the assays for cell growth involve the protein acting entirely on endogenous target molecules. Therefore, more protein would be required for an effect on viral DNA replication than for an effect on cell growth. Thus, the failure of the above proteins to inhibit cell growth cannot be attributed to insufficient protein quantities. One can therefore conclude that the function(s) which allow p53 to inhibit SV40 DNA replication are not sufficient for growth inhibition. However, since in this study, each of the proteins (Nc9, dll63, and c5) which retained an ability to inhibit cell growth (Figure 4.5) could also inhibit SV40 DNA replication (Figure 5.5 and Table 5.2), the function(s) which allow p53 to inhibit the replication of SV40 DNA may be necessary for the growth inhibitory effects of this protein. It is therefore possible that the ability of p53 to inhibit cell growth involves a direct impediment to the replication of DNA during S phase.

The arguments presented in this and the preceding section have indicated that the biochemical mechanisms involved in p53-dependent growth inhibition are somewhat complicated. As independent functions, transcriptional activation, transcriptional repression, and inhibition of DNA replication are each insufficient for the inhibition of cell growth, while transcriptional activation and possibly transcriptional repression are not even necessary for this cellular activity of p53. However, since there is considerable evidence that the transactivation of p21WAF1 is involved in p53-dependent growth inhibition (refer to Section 5.3.4), it would appear that p53 has more than one biochemical mechanism for inhibiting cell growth. Because transcriptional activation of p21WAF1 would only contribute to one pathway, other biochemical activities must be involved in the growth inhibitory effects of p53 (refer to Figure 5.8).
5.3.6 Multiple biochemical activities may be involved in p53-dependent apoptosis

The studies presented in this and the previous chapter have shown that each of the mutant forms of p53 (Neo, mp53, and 281EG) which retained an ability to induce apoptosis (Figure 4.5) were capable of activating and repressing gene transcription (Figures 5.2 and 5.3 and Table 5.1). Either (or both) of these functions may therefore be necessary for the apoptotic effects of p53. These studies also demonstrated that a mutant form of p53 (dl1.1) fails to induce apoptosis (Figure 4.5) yet retains the ability to activate gene transcription (Figure 5.2 and Table 5.1), while a different mutant p53 protein (c5) fails to induce apoptosis (Figure 4.5) yet retains the ability to repress gene transcription (Figure 5.3 and Table 5.1). It would therefore appear that transcriptional activation or transcriptional repression alone is not sufficient for p53-dependent apoptosis. Because the quantities of protein required in an assay for gene transcription would be greater than that required in an assay for a cellular effect (as explained in Section 5.3.4), the failure of the above proteins to induce apoptosis cannot be attributed to insufficient protein quantities. One can therefore conclude that as independent functions, transcriptional activation and transcriptional repression are not sufficient for the apoptotic effects of p53. However, since each of the proteins which remained capable of both activating and repressing gene transcription in this study could also induce apoptosis, one can suggest that the combined effects of transactivation and transrepression may be sufficient for the induction of apoptotic cell death.

In Chapter 4, it was demonstrated that the ability of p53 to suppress the transformation of cells by E1a and EJ-ras was due to the induction of apoptosis rather than the inhibition of cell growth. Therefore, since the biochemical mechanisms involved in these two functions would be the same, the information regarding p53-dependent apoptosis (as discussed in this section) also applies to the suppression of transformation by E1a and EJ-ras.

The finding that transcriptional activation may be necessary but is not sufficient for p53-dependent apoptosis (and suppression of transformation by E1a and EJ-ras) is compatible with those of other studies of both mouse and human p53. With regard to the effects of p53 on transformation, results obtained with the use of HPV-E7 can be compared to those obtained with the use of E1a as there are several similarities in the biochemical and phenotypic effects of these proteins (refer to Sections 1.3.1 and 1.3.5). While one study showed that the transactivation function of p53 is not sufficient for suppression of transformation by E7 and activated ras (Crook et al., 1994), other studies have shown that this function is not even necessary for the induction of apoptosis (Haupt et al., 1995b) or suppression of transformation by E1a and activated ras or by E7 and activated ras (Unger et al., 1993; Haupt et al., 1995b). It was noted however that the
ability to activate gene transcription did contribute to the complete apoptotic and transformation suppressing effects of p53 (Unger et al., 1993; Haupt et al., 1995b).

While no other studies have used mutant forms of p53 to investigate the relationship between repression of gene transcription and induction of apoptosis, a correlation between these functions has been observed through the use of bcl-2 and E1b-19K. Since these two proteins have been shown to overcome both the transcriptional repression and apoptotic activities of p53 (Shen and Shenk, 1994; Sabbatini et al., 1995), one can suggest transrepression is a necessary part of p53-dependent apoptosis. This conclusion is consistent with the findings presented in this chapter.

As mentioned above, transcriptional activation and transcriptional repression may both contribute to the apoptotic effects of p53. While neither of these functions alone is sufficient for the induction of apoptosis, the combined effects of transactivation and transrepression may be enough to cause the apoptotic response. A possible explanation for these observations is that p53 is able to activate transcription of the gene for bax (Miyashita and Reed, 1995) and possibly repress transcription of the gene for bcl-2 (Miyashita et al., 1994a). The consequent changes in the levels of these proteins (Haldar et al., 1994; Miyashita et al., 1994b; Selvakumaran et al., 1994; Zhan et al., 1994) may be sufficient for the induction of apoptosis as bax (which promotes apoptosis) forms heterodimers with bcl-2 (which prevents apoptosis) and the ratio between the levels of these two proteins appears to determine whether or not there is an apoptotic response (Oltvai et al., 1993). However, another study has shown that the regulation of gene transcription is not necessary for p53-dependent apoptosis since this type of cell death can occur in the absence of new RNA or protein synthesis (Caelles et al., 1994). While this observation suggests that p53 has a direct role in the induction of apoptosis, it does not exclude the possibility that changes in gene transcription may contribute to the full apoptotic effect.

The possibility that an inhibitory effect on DNA replication could somehow be involved in the apoptotic effects of p53 is also addressed by the studies presented in this and the previous chapter. The results demonstrated that some mutant forms of p53 (dl163 and c5) retained the ability to inhibit SV40 DNA replication (Figure 5.5) yet failed to induce apoptosis (Figure 4.5 and Table 5.2). As discussed in Section 5.3.5, the levels of protein required for an effect on SV40 origin-dependent DNA replication would be greater than that required for a cellular effect. Thus, the failure of the above proteins to cause apoptosis cannot be attributed to insufficient protein quantities. One can therefore conclude that the function(s) which allow p53 to inhibit SV40 DNA replication are not sufficient for the induction of apoptosis. However, since in this study, each of the proteins (Nc9, mp53, and 281EG) which retained an ability to induce apoptosis (Figure 4.5) could also inhibit SV40 DNA replication (Figure 5.5 and Table 5.2), the function(s) which allow p53 to inhibit the replication of SV40 DNA may be necessary for the
apoptotic effects of this protein. The ability of p53 to inhibit SV40 origin-dependent DNA replication could reflect a role in the regulation of cellular DNA replication as an activated form of p53 is known to inhibit the replication of DNA within the nucleus (Cox et al., 1995). While the relationship between this activity and apoptosis is ambiguous, it is possible that obstructions to the replication of cellular DNA may serve as a signal for an apoptotic response.

In summary, p53-dependent apoptosis appears to involve multiple biochemical mechanisms. As independent functions, transcriptional activation, transcriptional repression, and inhibition of DNA replication are each insufficient for the apoptotic effects of p53. However, the combined effects of transcriptional activation and transcriptional repression may be sufficient for p53-dependent apoptosis (refer to Figure 5.8). The mechanism by which p53 induces an apoptotic response (and suppresses transformation of cells by E1a and EJ-ras) may therefore depend upon a shift in the balance between quantities of proteins which are activated and proteins which are repressed by p53.
Figure 5.8. Relationships between the different cellular and biochemical functions of p53. Despite evidence which suggests that p53 inhibits cell growth by transactivation of p21\textsuperscript{WAF1} (refer to Section 3.3.2), the studies in this chapter have demonstrated that transcriptional activation is neither sufficient nor necessary for p53-dependent growth inhibition. Thus, p53 appears to have more than one biochemical mechanism for inhibiting cell growth. While one pathway would involve the transactivation of p21\textsuperscript{WAF1}, this activity alone would not be sufficient for a cellular effect. Other functions of p53 must therefore contribute to each of the pathways leading to growth inhibition (refer to Sections 5.3.4 and 5.3.5 for more details). Additionally, each pathway must involve pRb as the growth inhibitory effects of p53 rely on the activity of this protein (refer to Section 3.3.2). The studies in this chapter have also shown that transcriptional activation and repression may each be necessary for p53-dependent apoptosis and the combination of these functions may even be sufficient for this cellular effect (refer to Section 5.3.6 for more details). While the mechanisms by which p53 inhibits cell growth or induces apoptosis remain unclear, each function appears to involve multiple biochemical activities.
Chapter 6

Cellular and biochemical mechanisms by which p53 suppresses oncogenic transformation
6.1 Growth inhibition and apoptosis are separate and alternative functions of p53

Because cells which have undergone genetic damage are highly susceptible to oncogenic transformation, the p53 protein is induced in order to prevent the continued proliferation of these cells. The failure of p53 to respond appropriately in this situation has been implicated in development of a large number of human cancers. Through the manipulation of cells in culture, the role of p53 in suppressing tumourigenesis has been demonstrated by its ability to suppress the transformation of cells by a number of viral and activated cellular oncoproteins (Eliyahu et al., 1989; Finlay et al., 1989). The mechanism by which transformation is suppressed probably involves a reduction in cell growth and / or an increase in cell death as p53 is known to arrest growth predominantly in the G1 phase of the cell cycle (Baker et al., 1990; Diller et al., 1990; Mercer et al., 1990; Michalovitz et al., 1990; Kastan et al., 1991) and to induce an apoptotic response (Yonish-Rouach et al., 1991; Shaw et al., 1992; Clarke et al., 1993; Lowe et al., 1993b) (refer to Section 4.1 for more details).

The studies presented in this thesis have demonstrated that inhibition of cell growth and induction of apoptosis are genetically separable functions of p53. It would therefore appear that the growth inhibitory function of p53 is not followed by and not even required for the induction of apoptotic cell death (refer to Section 4.3.4). These findings are very much in agreement with the observation that haematopoietic cells can undergo p53-mediated apoptosis while failing to arrest in the G1 phase of the cell cycle (Yonish-Rouach et al., 1993). A popular model of p53 activity is that the accumulation of this protein in response to genetic damage arrests the growth of cells until the DNA is repaired, but if the DNA is beyond repair, p53 eliminates the cell through an apoptotic response (Lane, 1992). However, because this model suggests that growth arrest is a necessary prerequisite for p53-dependent apoptosis, it is not consistent with the observations mentioned above. An alternative model may be that the accumulation of p53 in the presence of DNA damage causes an arrest of cell growth, but if this response is somehow prevented, apoptosis is induced (refer to Section 4.3.4). Thus, p53 would use alternative rather than sequential mechanisms for preventing the continued proliferation of genetically damaged cells. This model is supported by the observations that depending upon the supply of certain survival factors, haematopoietic cells can undergo either growth arrest or apoptosis in response to high levels of p53 (Levy et al., 1993; Canman et al., 1995). The concept that certain cellular conditions can determine whether p53 causes growth inhibition or apoptosis is further supported by the observed effects of p53 in the presence of different oncoproteins, as discussed in the following section.
6.2 The cellular effect of p53 is determined by oncoprotein activity and the status of pRb

Although p53 is known to suppress the transformation of cells by various combinations of oncoproteins, it is unable to suppress transformation by LTag. While the ability of LTag to bind (and presumably inactivate) p53 is thought to be important for transformation by this oncoprotein (Peden et al., 1989; Srinivasan et al., 1989; Lin and Simmons, 1991a; Zhu et al., 1992), the studies in this thesis have shown that this is not the case. Interestingly, the binding of p53 is also unnecessary for the ability of LTag to overcome the suppressive effects of p53 on cell growth and cell transformation (refer to Section 3.3.1). These findings which complement the observations of other studies (Michael-Michalovitz et al., 1991; Quartin et al., 1994), suggest that the binding of p53 is somewhat redundant in the normal functioning of LTag. The ability of LTag to bind and inactivate pRb (DeCaprio et al., 1988; Ewen et al., 1989) is however critical not only for cell transformation but also for overcoming the growth and transformation suppressing effects of p53 (refer to Section 3.3.2). Since growth inhibition (and transformation suppression) by p53 cannot occur in the absence of active pRb, it would appear that pRb actually mediates the inhibitory effect of p53 on cell growth. This finding is consistent with another report which showed that when cells are subject to a p53-dependent growth arrest, pRb is maintained in an active state (Slebos et al., 1994).

The adenovirus E1a proteins are also known to bind and inactivate pRb (Whyte et al., 1988; 1989; Egan et al., 1989) and (consequently) overcome the inhibitory effects of p53 on cell growth (Lowe et al., 1993a; Vousden et al., 1993). However, the studies presented in this thesis have demonstrated that transformation involving E1a remains susceptible to suppression by p53. This observation cannot be explained by p53-dependent growth inhibition, but may be attributed to p53-dependent apoptosis. In agreement with previous reports (Rao et al., 1992; Debbas and White, 1993; Lowe and Ruley, 1993), E1a was found to cause the death of cells through an apoptotic effect of endogenous p53 (refer to Section 3.3.3). Increased susceptibility to apoptosis in the presence of E1a is consistent with the observations that an apoptotic response is induced by the functional inactivation of pRb (Howes et al., 1994; Morgenbesser et al., 1994; Pan and Griep, 1994; Symonds et al., 1994; Williams et al., 1994a; Almasan et al., 1995) (refer to Sections 1.3.3 and 3.3.4). Thus, it would appear that when p53 fails to cause a pRb-mediated growth arrest, apoptosis is induced as a reserve mechanism for suppressing oncogenic transformation.

The studies in this thesis have shown that transformation involving EJ-ras is also susceptible to suppression by p53. In contrast to E1a, EJ-ras does not cause a susceptibility to p53-dependent apoptosis, but actually inhibits this type of cell death
(refer to Section 3.3.6). The ability of activated ras to protect cells from apoptosis has also been indicated by another study which examined the effects this protein in relation to those of E1a (Lin et al., 1995). Although EJ-ras can also stimulate the growth of cells, high levels of p53 were found to inhibit this growth stimulatory effect (refer to Section 3.3.5). It would therefore appear that the transformation-related activities of EJ-ras are suppressed by a growth inhibitory rather than apoptotic effect of p53.

The observations described above (and in the preceding section) demonstrate that p53-dependent growth inhibition and p53-dependent apoptosis are separate and alternative functions which occur in response to different cellular conditions. Certain oncoproteins can influence the cellular activity of p53 by altering the status of pRb. Because p53-dependent growth inhibition is mediated by active pRb, the loss or inactivation of this protein would prevent this growth inhibitory effect. Under these conditions however cells become susceptible to p53-dependent apoptosis. Since the inactivation of pRb disrupts the regulation of cell cycle progression at the G1 / S phase boundary (refer to Section 1.3.3), the apoptotic function of p53 may be responsible for the elimination of cells whose growth is deregulated by a premature onset of S phase (refer to Section 4.3.4).

6.3 Oncoprotein cooperation is necessary for overcoming the effects of endogenous p53

As discussed in the preceding section, the growth inhibitory and apoptotic effects of p53 are alternative functions which occur in response to different types of oncogenic activity. Thus, the mechanism by which p53 suppresses cell transformation appears to be determined by the transforming oncoproteins. Generalisations with regard to particular classes of oncoproteins (refer to Section 1.3.5) may be suggested from the observed effects of p53 in the presence of E1a or EJ-ras. While the transformation-related activities of class I oncoproteins (including activated ras) may be suppressed by p53-dependent growth inhibition, the transformation-related activities of class II oncoproteins (including E1a) may be suppressed by p53-dependent apoptosis. Since class III oncoproteins (including bcl-2 and E1b-19K) are those which are known to prevent apoptotic cell death, it may be suggested that their transformation-related activities are suppressed (if at all) by p53-growth inhibition. Because EJ-ras was also shown to inhibit apoptosis, there may be some overlap in the activities of class I and class III oncoproteins.

LTag is unusual in that it does not belong to any one class of oncoproteins. Another unusual feature of LTag is that it can transform cells by itself and this transformation is not suppressed by high levels of p53. In part, this can be attributed to the ability of LTag to overcome the growth inhibitory effects of p53 by binding and inactivating pRb (refer to Section 3.3.2). However, this activity would cause a
susceptibility to p53-dependent apoptosis. Therefore, in order to transform cells and overcome the transformation suppressing effects of p53, LTag must prevent the apoptotic response. Although this could be achieved by the binding (and inactivation) of p53, the studies in this thesis have indicated that there must be an additional anti-apoptotic function associated with LTag (refer to Section 3.3.4). Thus, transformation by LTag would remain refractory to the suppressive effects of p53 due to the abolition of both the growth inhibitory and apoptotic effects of this protein.

Since LTag transforms alone and overcomes both cellular effects of p53, one may suggest that the process of cell transformation normally requires cooperation between oncoproteins of different classes in order to overcome the activities of endogenous p53. As examples, the studies in this thesis demonstrated that neither E1a nor EJ-ras can transform alone, but each will transform in cooperation with proteins which abrogate the functions of p53 within the cell (refer to Sections 3.3.3 and 3.3.5). Thus, the cooperative effects of E1a and EJ-ras with each other may be explained by each of these proteins overcoming the suppressive effects of endogenous p53 on the other protein. While transformation by EJ-ras is normally prevented by p53-dependent growth inhibition, E1a is able to overcome this effect (Lowe et al., 1993a; Vousden et al., 1993). Additionally, EJ-ras was shown to inhibit p53-dependent apoptosis which would normally prevent transformation by E1a (refer to Section 3.3.6) (Lin et al., 1995).

The ability of E1a to overcome p53-dependent growth inhibition would occur through the binding and inactivation of pRb which normally mediates this activity of p53 (refer to Section 3.3.2). Thus, even high levels of p53 would fail to inhibit cell growth in the presence of E1a. While EJ-ras can inhibit apoptosis which is caused by endogenous p53, there is no evidence to suggest that it can prevent an apoptotic response in the presence of high levels of p53. Thus, one might suggest that exogenous p53 suppresses transformation by E1a and EJ-ras by inducing apoptosis rather than inhibiting cell growth. Studies of the genetic requirements for different functions of p53 have demonstrated that this proposal is correct. While growth inhibition was found to be dispensable for the transformation suppressing effects of p53, the induction of apoptosis could be both necessary and sufficient for the ability of p53 to suppress the transformation of cells by E1a and EJ-ras (refer to Section 4.3.5).

6.4 The cellular activities of p53 may involve multiple biochemical mechanisms

The studies in this thesis have also examined the abilities of p53 to activate and repress gene transcription for possible contributions to the cellular effects of this protein. The ability of p53 to inhibit SV40 origin-dependent DNA replication was also examined as this activity may reflect a role for p53 in regulating the replication of cellular DNA.
The transcriptional activation function of p53 has been associated with the inhibition of cell growth since the observation that p21\textsuperscript{WAF1} is a transcriptional target of p53 (El-Deiry et al., 1993). The induction of this protein in response to p53 is thought to cause an arrest of growth by inhibiting the activity of cyclin-cdkks which normally inactivate pRb (Harper et al., 1993; Xiong et al., 1993). Thus, the maintenance of pRb in an active state would prevent the progression of cells from G1 phase into S phase of the cell cycle (refer to Sections 1.3.3 and 3.3.2). However, the studies presented in this thesis have demonstrated that transcriptional activation is not necessary for the growth inhibitory effects of p53 (refer to Section 5.3.4). It can therefore be suggested that p53 has more than one biochemical mechanism for inhibiting cell growth. While one pathway would involve the transactivation of p21\textsuperscript{WAF1}, one or more other functions of p53 would initiate an alternative pathway for the same cellular effect. Since transcriptional activation was also shown to be insufficient for p53-dependent growth inhibition, the pathway which involves the transactivation of p21\textsuperscript{WAF1} must also include at least one other activity of p53.

The studies in this thesis have also shown that transcriptional repression by p53 may or may not be necessary, but is definitely not sufficient for growth inhibition (refer to Section 5.3.5). While the function(s) which allow p53 to inhibit SV40 DNA replication are also not sufficient, they too may be necessary for the growth inhibitory effects of this protein (refer to Section 5.3.5). It is therefore possible that the ability of p53 to inhibit cell growth involves a direct impediment to the replication of DNA during S phase. In light of these findings, the biochemical mechanisms involved in p53-dependent growth inhibition appear to be somewhat complicated. However, it must be noted that pRb is a necessary part of each of the pathways which lead to the inhibition of cell growth as the inactivation of this protein prevents this cellular effect of p53 (refer to Section 3.3.2).

With regard to the induction of apoptosis by p53, the studies in this thesis have demonstrated that as independent functions, transcriptional activation and transcriptional repression are not sufficient for this cellular effect. However, the activation and / or repression of gene transcription may be necessary and the combined effects of these activities may even be sufficient for the induction of apoptotic cell death (refer to Section 5.3.6). The initiation of an apoptotic response may therefore depend upon the ratio between the quantities of proteins whose expression is activated or repressed by p53. Additionally, the studies have shown that the function(s) which allow p53 to inhibit SV40 DNA replication may be necessary but are not sufficient for the induction of apoptosis (refer to Section 5.3.6). It is therefore possible that obstructions to the replication of cellular DNA may serve as a signal for an apoptotic response. While the mechanisms involved in p53-dependent apoptosis remain ambiguous, it may be concluded that more than one biochemical activity is involved.
6.5 Future research directions

As summarised in Figure 6.1, the studies presented in this thesis have been useful in defining the relationships between the cellular functions of p53 and the activities of certain oncoproteins. The effects of p53 in the presence of LTag, E1a, and EJ-ras have indicated that the mechanism by which p53 suppresses cell transformation is determined by the transforming oncoproteins, and in particular the status of pRb. In order to test the proposal that the response of p53 varies with regard to the oncogenic activity, a greater number of oncoproteins would need to be analysed.

While the studies in this thesis have shown that inhibition of cell growth and induction of cell death are independent and alternative functions of p53, the actual changes which cause these effects require further definition. Although various factors indicate that the cytotoxic effects of p53 can be attributed to apoptosis, additional experiments would be required to demonstrate the classic signs of apoptosis (such as DNA fragmentation, chromatin condensation, and cell shrinkage). If these apoptotic indicators were not detected, one could suggest that p53 has alternative means by which it can induce cell death. While the growth inhibitory effects of p53 are likely to occur in the G1 phase, additional experiments may also be performed to determine whether growth is arrested entirely in G1 phase or in both G1 and G2 phases of the cell cycle.

These analyses of the cell cycle could be important in defining the different mechanisms involved in p53-dependent growth inhibition. Since the transactivation function of p53 is not required, it would appear that transcriptional activation of p21WAF1 (and the consequent inhibitory effects on cell cycle progression) contributes to only one of the pathways through which p53 inhibits cell growth. Further studies are required to clearly define the biochemical mechanisms which are important for both the growth inhibitory and apoptotic effects of p53.

6.6 Conclusion

Over the course of these studies, one of the things which has impressed me most is the versatility of p53 in preventing tumourigenesis. Inhibition of cell growth and induction of apoptotic cell death are independent functions used by p53 for suppressing transformation under different oncogenic conditions. p53 also appears to have multiple biochemical mechanisms through which these cellular functions can be achieved. With regard to the life of an individual cell, the activities of p53 are most unwelcome. However, with regard to our own lives, the ultimate purpose of this protein is much appreciated.
Figure 6.1. Relationships between the different cellular and biochemical functions of p53, and the activities of certain oncoproteins. Inhibition of cell growth and induction of apoptosis are separate (and alternative) functions used by p53 to suppress the transformation of cells in response to different oncoproteins. In the presence of active pRb, p53-dependent growth inhibition can suppress transformation involving EJ-ras. However, this effect is overcome by E1a and LTag both of which bind and inactivate pRb. In the absence of active pRb, transformation involving E1a can be suppressed by p53-dependent apoptosis. However, bcl-2, E1b-19K, and a function of LTag (other than the binding of p53) can overcome this apoptotic response. EJ-ras can also inhibit this effect, but only with low levels of p53 as transformation by E1a and EJ-ras is suppressed through apoptosis which is induced by high levels of p53. While transactivation of p21\textsuperscript{WAF1} is thought to mediate p53-dependent growth inhibition, transcriptional activation was found to be neither sufficient nor necessary for this effect. It would therefore appear that p53 has more than one biochemical mechanism for inhibiting cell growth and that the transactivation of p21\textsuperscript{WAF1} contributes to only one of these pathways. Other biochemical functions must therefore be involved in the growth inhibitory effects of p53. With regard to p53-dependent apoptosis, transcriptional activation and repression may each be necessary, and the combination of these functions may even be sufficient for this type of cell death. Although the mechanisms by which p53 inhibits cell growth or induces apoptosis remain unclear, each function appears to involve multiple biochemical activities.
p53

E1a LTag - binding of pRb

p21_{WAF1} \rightarrow pRb + ??

?\rightarrow pRb

transactivation + transrepression ??

bcl-2

E1b-19K (EJ-ras)

LTag (not binding of p53)

cell growth inhibition

suppression of transformation involving E1a - EJ-ras

apoptotic cell death

suppression of transformation involving E1a - EJ-ras
References


