

Cloning of cDNA for murine interleukin-3
and its expression in eukaryotic and
prokaryotic systems

by

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(i)

STATEMENT

All the experimental work reported in this thesis was performed by the author, unless specifically stated otherwise in the text.

A handwritten signature in blue ink, appearing to read "MC Fung". The letters are cursive and somewhat stylized.

Ming-Chiu Fung

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Abbreviations

Abbreviations used in this thesis without definition include:

- PBS: Phosphate buffered Saline, pH7.4.
- BFU-E: Blast forming unit erythroid.
- GM-CFC: Granulocyte macrophage colony forming cell.
- SDS-PAGE: Sodium dodecyl Sulfate polyacrylamide gel electrophoresis.
- Kb: Kilo-base.
- dNTPs: deoxyribonucleotides (dATP, dTTP, dCTP, dGTP).
- DMEM: Dulbecco's minimal essential medium.

Abstract

The aim of this thesis was to isolate the cDNA for the murine IL-3 and to express it in eukaryotic and prokaryotic systems.

The WEHI-3B cell line was used as the main source of IL-3 mRNA. A sensitive assay for IL-3 mRNA was developed using microinjection of Xenopus laevis oocytes. The IL-3 secreted from the oocytes was assayed using the IL-3 dependent cell line 32Dcl-23.

IL-3 mRNA was partially purified by 10-20 fold on sucrose density gradients. The enriched IL-3 mRNA was then used for the construction of the cDNA library which was screened using the hybridization-translation method. A short cDNA clone, pILM1, was isolated. An oligonucleotide corresponding to the pILM1 cDNA insert was synthesized and used as a probe to screen a second cDNA library which was enriched for longer cDNA clones. A longer clone, pILM3, carrying the entire coding sequence for murine IL-3 was isolated. The cDNA insert of pILM3 codes for a polypeptide of 166 amino acids including a signal peptide of possibly 27 amino acids. The predicted protein sequence of mature processed IL-3 indicates a MW of 15,102 with four potential N-glycosylation sites. The N-terminal sequence of the mature IL-3 (Ihle et al., 1983a) is in

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complete agreement with the reading frame but starts at residue 33 suggesting an additional proteolytic cleavage has occurred apart from the cleavage of the signal peptide.

The pILM3 cDNA insert carrying the coding sequence for IL-3 was subcloned into an SV40 expression vector (pSV₂-neo). The recombinant expression plasmid, pILM4, was microinjected into the nucleus of the Xenopus oocytes and also used for the transformation of monkey COS cells. In both cases, biologically active IL-3 was produced. The recombinant IL-3 produced from the pILM4 transformed monkey COS cells was used to study the biological activities of recombinant IL-3. The recombinant IL-3 supported growth of the IL-3 dependent cell lines 32Dcl-23 and FDC-P1, induced 20 α SDH in splenic lymphocytes from nu-nu mice and showed multilineage hemopoietic growth factor activity.

The IL-3 cDNA was also expressed in E. coli as a mature protein starting with residue 28 (Serine) or as a fused polypeptide with an extra 9 amino acids and starting with the residue 27 (Alanine). In both cases, the expressed IL-3 was biologically active. This indicated that glycosylation is not essential for biological activity and that the expression in bacteria is feasible. The IL-3 expressed as a mature

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protein was less stable than the IL-3 fusion polypeptide.

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CHAPTER 1

General Introduction

This chapter briefly reviews relevant literature prior to the commencement of the work described in this thesis. More recent work pertaining to this area of research will be reviewed in the Discussion (Chapter 5) together with the results of the thesis.

A. A general review of interleukin-3.

1.1 Introduction.

The immune response to antigen depends on a network of interactions between the various leukocytes of the blood and lymphatic systems. Regulation of these interactions has been shown to be mediated, at least in part, by chemical 'messengers' secreted by the cells involved. The generic name of 'lymphokines' was initially proposed by Dumonde et al. (1969) for these chemical messengers, because the first to be described were produced by antigen-stimulated T-lymphocytes. Similar factors produced by cells of the macrophage/monocyte series have likewise become known as 'monokines'.

Lymphokines and monokines have traditionally been defined on the basis of their biological activity in a particular assay, rather than on the basis of

definitive biochemical and biological properties. The practice of naming such factors, like lymphocyte-activating-factor (LAF) (Gery and Handschumacher, 1974) and T-cell growth factor (TCGF) (Koopman et al., 1978), with acronyms after activities peculiar to various bioassay systems has generated an unsystematic nomenclature. To try and remedy this, a new term 'interleukin' was proposed in the Second International Lymphokine Workshop (Ermatingen, Switzerland, May 27-31, 1979) to name those factors that act as communication signals between different populations of leukocytes (Aarden et al., 1979).

Interleukin-1 (IL-1) and interleukin-2 (IL-2) were distinguished by their biochemical properties and biological activities. IL-1 was defined as a macrophage derived factor with a molecular weight of 12,000-18,000 which plays an essential role by facilitating the production of other lymphokines by helper T cells (Larsson et al., 1980; Smith et al., 1980a, b). IL-2 was defined as a factor with a molecular weight of 30,000-35,000 which appears to mediate the differentiation and amplification of cytotoxic T cells (Morgan et al., 1976; Gillis and Smith, 1977; Watson et al., 1979; Nabholz et al., 1980).

Another group of factors which is relevant to this research is the family of haemopoietic regulators

called colony stimulating factors (CSF's). This nomenclature derives from the ability of these factors to stimulate the development of colonies of differentiated cells from committed progenitor cells in vitro. Four different CSF's have been distinguished by Metcalf (1981) viz. M-CSF, GM-CSF, G-CSF and multi-CSF. The CSF of most relevance to the present work is multi-CSF (interleukin-3).

1.2 Interleukin-3.

Ihle et al. (1981) described what they believed to be a unique lymphokine named interleukin-3 (IL-3), which was isolated and purified from ConA-stimulated T lymphocytes and which could induce the expression of 20- α -hydroxysteroid dehydrogenase (20 α SDH) in splenic lymphocytes from athymic mice in vitro. A convenient source of IL-3 was found to be the WEHI-3B cell line (Warner et al., 1969), a myelomonocytic leukemia line which produces relatively high levels of IL-3 constitutively (Hapel et al., 1981; Lee et al., 1982). IL-3 can also be obtained from lectin- or antigen-stimulated T lymphocytes and a number of T cell lymphoma and hybridoma lines (Ihle et al., 1981; Iscove et al., 1982, Yung et al., 1981; Clark-Lewis and Schrader, 1981).

WEHI-3B conditioned medium also possesses a variety of other CSF-type activities, viz. burst

promoting activity (BPA) (Iscove et al., 1982); P-cell stimulating factor (PSF) (Clark-Lewis and Schrader, 1981); mast cell growth factor (MCGF) (Yung et al., 1981); hemopoietic cell growth factor (HCGF) (Bazill et al., 1983); histamine producing cell stimulating factor (HCSF) (Dy et al., 1981); and multicolony-stimulating factor (Multi-CSF) (Metcalf, 1981). At the commencement of the work described in this thesis there was already general agreement that many, if not all, of these activities could be attributable to a single factor and that IL-3 was a CSF. In terms of biochemical characterization of this factor, the most extensive characterization was of IL-3 from WEHI-3B cells by Ihle et al. (1982a, 1983a). Since we used the WEHI-3B cell line as the source of mRNA for cloning experiments and because IL-3 is a more systematic name it will be used in preference to the alternatives throughout this thesis.

1.3 Biochemical properties of interleukin-3.

IL-3 was recently purified to apparent homogeneity (Ihle et al., 1982a; Ihle et al., 1983a). The purification procedure described results in a 1,800,000-fold purification, with yields of 5-10% of the initial activity and gives 2-10 μ g of IL-3 from 150 litres of WEHI-3B conditioned medium. The molecular weight of IL-3 was reported to be approximately 28,000

when analyzed using SDS-PAGE. The specific activity of the purified IL-3 was from 0.05ng/unit (Ihle et al., 1982a) to 0.2ng/unit (Ihle et al., 1983a). The NH₂-terminal sequence reported for the homogeneous IL-3 was: Asp-Thr-His-Arg-Leu-Thr-Arg-Thr-Leu (Ihle et al., 1983a). Comparison with known protein sequences indicated that the NH₂-terminal sequence of IL-3 was unique.

1.4 Biological activities of IL-3.

IL-3 was initially defined as the factor in conditioned media from ConA-stimulated splenic lymphocytes that induces 20 α SDH in culture of nu/nu splenic lymphocytes (Ihle et al., 1981). Although it was originally proposed that 20 α SDH was a specific T cell marker, it is now clear that this enzyme is also found in myeloid and stromal cell lines (Schaffar-Deshayes et al., 1983; Garland and Dexter, 1982; see Discussion). Thus the induction of 20 α SDH is not a satisfactory definition of IL-3. Recently the biological activities of IL-3 were studied using highly purified IL-3 from WEHI-3B conditioned medium (Ihle et al., 1983a). It was demonstrated that purified IL-3 showed a typical dose-response curve in all the assays for WEHI-3B growth factor, mast cell growth factor (MCGF), P-cell stimulating factor (PSF), histamine-producing cell-stimulating factor (HCSF) and colony-stimulating factor activities (CSF) (Ihle et

al., 1983). These results taken together with the data obtained with cDNA clones encoding IL-3 (see below) indicate that IL-3, burst promoting activity (Iscove et al., 1982), histamine-producing cell-stimulating factor (Dy et al., 1981), P-cell stimulating factor (Clark-Lewis and Schrader, 1981), multi-CSF (Metcalf, 1981), hemopoietic cell growth factor (Bazill et al., 1983) are alternative names for the same polypeptide.

The spectrum of biological activities observed for IL-3 indicate that it supports the growth of cells in all hemopoietic lineages in vitro including early precursors which are still pluripotent. It is thus the most broadly acting member of the family of hemopoietic growth factors identified to date and could potentially regulate hemopoiesis in vivo.

1.5 IL-3 dependent cell lines.

Greenberger and his coworkers first reported that continuous cell lines could be established from Moloney retrovirus-infected long term bone marrow cultures using WEHI-3B conditioned media (Greenberger et al., 1979). Because of the dependency on the WEHI-3 conditioned medium, the cells were termed 'factor-dependent'. Subsequently, both Greenberger (1980) and Dexter et al. (1980) found that viral infection was not necessary to obtain factor-dependent cells.

Several of these factor-dependent cell lines were examined for the ability of IL-3 to support their growth. Partially purified IL-3 did support the growth of C3Hffv, 32Dcl-23 and FDC-P1 lines (Ihle et al., 1982b; Hapel et al., 1982) and these cell lines responded in a dose dependent way to IL-3.

1.6 Hemopoietic growth regulators related to IL-3.

A number of glycoprotein growth factors active on cultured mouse hemopoietic cells have been described which are biochemically distinct from IL-3. Most of these act individually on cells in only one or two of the various hemopoietic lineages (Nicola and Vadas, 1984). For example erythropoietin (Miyake et al., 1977), M-CSF, CSF-1 (Tertian et al., 1981) and GM-CSF (Burgess et al., 1981). GM-CSF is the most closely related to IL-3 in terms of its biological activity. Both GM-CSF and IL-3 are produced by antigen- or mitogen-stimulated T lymphocytes and also by T-cell hybridomas and T-cell lymphomas (Burgess et al., 1981). However, no GM-CSF is produced by the WEHI-3B cell line. Although GM-CSF can also support the growth of the macrophage-granulocyte precursors, it differs from IL-3 in not supporting mast cell growth (Clark-Lewis and Schrader, 1981) or the formation of eosinophil (Clark-Lewis and Schrader, 1981), or megakaryocyte colonies (Iscove et al., 1982). GM-CSF

is readily separated from IL-3 by column chromatography because it is more strongly retarded than IL-3 on DEAE at pH7.2 in low salt (Ihle et al., 1983a; Burgess et al., 1977).

B. Recombinant DNA approaches to lymphokine biology.

As can be seen from the discussion of the biochemical properties of IL-3, purification of lymphokines by conventional biochemical methods is quite difficult due to the minute amounts of material available and their very high biological activities (50% activity at approx 10^{-12} M). Even after extensive purification there is still the problem that part of the biological activities observed might be attributable to other contaminating factors.

A complementary approach is to isolate cDNA clones encoding the lymphokine. This enables definition of the lymphokine from its predicted protein sequence and the biological properties of 'recombinant' lymphokine which can be determined after expression of the cDNA clones in eukaryotic or prokaryotic systems. Production of recombinant lymphokines on a large scale enables wide ranging biological studies to be carried out including clinical trials in the case of human lymphokines. The availability of cDNA clones opens up the approach of site directed mutagenesis for structure/function

studies and enables research on lymphokine gene expression and rearrangements to be done.

At the commencement of the work described in this thesis, recombinant DNA techniques had been applied to two of the lymphokines produced by antigen-activated T lymphocytes, viz. γ -interferon and interleukin-2.

1.1 Isolation of cDNA clones encoding γ -interferon.

Human immune interferon (IFN- γ) cDNA clones have been isolated by two groups (Gray et al., 1982; Devos et al., 1982). In both cases, enriched human IFN- γ mRNA was used to construct the cDNA library. Total mRNA was fractionated by electrophoresis on denaturing agarose gels (Gray et al., 1982) or by sucrose gradient centrifugation (Devos et al., 1982). The IFN- γ mRNA activity was determined by injecting aliquots of each fraction into Xenopus laevis oocytes (Gray et al., 1982; Devos et al., 1982). The IFN- γ mRNA was enriched about 20 fold (Gray et al., 1982) or 10-30 fold (Devos et al., 1982). Two different methods, differential hybridization and hybridization-translation were used by Gray et al. (1982) and Devos et al. (1982) respectively to isolate the human IFN- γ cDNA clones. The frequency of the IFN- γ mRNA in total mRNA was estimated to be about 1:2,400 (Gray et al., 1982). The DNA sequence of the cDNA clone codes for a polypeptide of 166 amino acids, the first 20 of which

comprise a signal peptide (Gray et al., 1982; Devos et al., 1982). The encoded amino acid sequence has two potential glycosylation sites and is quite basic in character.

Identification of the sequence as IFN- γ was confirmed by expression of the cDNA clone in bacterial (Gray et al., 1982) and mammalian cells (Gray et al., 1982; Devos et al., 1982). The expression of IFN- γ in bacterial cells was performed by joining the cDNA sequence starting from amino acid 21 in phase with the trp promoter (Gray et al., 1982). For the expression of IFN- γ in mammalian cells, the whole cDNA sequence was cloned after the SV40 late promoter in the same orientation (Gray et al., 1982; Devos et al., 1982). In both systems the expression product was shown to have antiviral activity which was immunologically indistinguishable from that of the IFN- γ produced by stimulated human lymphocytes.

1.2 Isolation of cDNA clones encoding interleukin-2.

cDNA clones coding for human interleukin-2 (IL-2) were isolated from a cDNA library using similar methods to those used to isolate the human IFN- γ cDNA clones (Taniguchi et al., 1983; Devos et al., 1983). The human IL-2 mRNA from Jurkat III cell line (Taniguchi et al., 1983) or from normal splenocytes (Devos et al., 1983) was enriched about 10 fold

(Taniguchi et al., 1983) by sucrose gradient centrifugation. IL-2 mRNA activity was assayed by translation in the Xenopus laevis oocytes. A cDNA library was constructed from the enriched IL-2 mRNA and the plasmid containing the cDNA copy of human IL-2 was identified by the hybridization-translation method. The frequency of the human IL-2 mRNA in total mRNA was estimated to be 1:20,000 (Taniguchi et al., 1983). The DNA sequence of the cDNA codes for a polypeptide of 153 amino acids. The first 20 amino acids probably comprise the signal peptide. The deduced amino acid sequence contains no potential glycosylation sites. Although the amino acid sequences deduced from the DNA sequences of the cDNA clones obtained by Taniguchi et al. (1983) and by Devos et al. (1983) are the same, there is one base different between their DNA sequences.

The cDNA was cloned after the SV40 early promoter in the same orientation and was expressed in monkey COS cells. The expression product had biological activities characteristic of human IL-2 (Taniguchi et al., 1983). Biologically active human IL-2 was also directly expressed in E. coli under the control of trp & PL promoter (Devos et al., 1983)

1.3 Gene organisation for γ -interferon and interleukin-2.

The two lymphokines, human interleukin-2 and human immune interferon, appear to have similar regulation. Both lymphokines are produced by mitogen- or antigen-activated spleen cells (Morgan et al., 1976; Gillis et al., 1978; Yip et al., 1981). Their expression appears to be regulated at the level of transcription since spleen cells do not have detectable levels of mRNA prior to antigen activation (Wallace et al., 1981a; Efrat et al., 1982). It is therefore interesting to compare the gene organization of these two lymphokines.

Both the human IL-2 and IFN- γ genes exist as a single copy per haploid human genome (Fujita et al., 1983; Gray et al., 1982). The human IL-2 chromosomal gene organization is similar to that of human IFN- γ . Both contain three introns interrupting the coding sequence (Degrave et al., 1983; Fujita et al., 1983; Gray and Goeddel, 1982; Taya et al., 1982). There is no significant homology between the cDNA sequence of human IL-2 and human IFN- γ (Degrave et al., 1983) and the human IL-2 and human IFN- γ genes only show weak homology in the 5' flanking region (Fujita et al., 1983).

C. Aims of the thesis

At the commencement of this work there was sufficient information to suggest that IL-3 was an important hemopoietic growth factor. Its biological activities were not fully defined, however, and its relationship to other factors required clarification. No work had been reported on the molecular biology of IL-3. The aim of this project was to complete the critical first steps in the application of recombinant DNA techniques to the study of IL-3 by isolating a cDNA clone for murine IL-3 and expressing it in eukaryotic and prokaryotic systems.

Chapter 2

Translation of mRNA encoding murine interleukin-3 in Xenopus laevis oocytes.

I. Introduction.

Xenopus laevis oocytes provide a very sensitive and convenient translation system for the identification of mRNA and for the study of translational control in living cells. The translation of injected mRNA in the Xenopus laevis oocytes was first demonstrated by Gurdon et al. (1971). The Xenopus laevis oocytes are enormous cells with a diameter of 1 mm or more and are resilient enough to withstand the injection of up to 100 nanoliters of liquid, a volume which represents 10-20% of the volume of the oocyte (Lane and Knowland, 1975).

The characteristics of the Xenopus oocyte translation system are discussed below:-

(i) Variety of mRNA's translated.

It has been demonstrated that the oocyte translation machinery can translate a wide variety of eukaryotic mRNAs including mRNAs from vertebrates, invertebrates, plants, fungi and viruses (Table 2.1).

The translation of normal prokaryotic mRNA such

Table 2.1 Examples of mRNAs translated by Xenopus oocytes.

<u>Nature and source of RNA</u>	<u>New proteins synthesized</u>	<u>References</u>
<u>Xenopus</u>		
Vitellogenin and albumin mRNA from <u>Xenopus laevis</u> liver	<u>Xenopus</u> vitellogenin and albumin	Berridge and Lane (1976)
<u>Other vertebrates</u>		
Human		
13S mRNA from conA induced human leukaemic T cells (Jurkat-111)	Human interleukin-2	Taniguchi et al. (1983)
Poly(A)-RNA from poly(rI). poly(rC) induced cultured human foreskin fibroblasts	Human interferon	Reynolds et al., (1975)
Gibbon		
14-16S RNA from gibbon lymphosarcoma cell line, MLA 144	Gibbon interleukin-2	Lin et al., (1982)
Calf		
14S RNA from calf lens epithelial cells	α A2 crystallin	Berns et al., (1972)
Rabbit		
9S RNA from rabbit reticulocytes	α - and β -rabbit globin chains	Lane et al., (1971)
Mouse		
11-12S mRNA from cultured murine T lymphoma line, EL4	mouse interleukin-2	Bleackley et al., (1981)
Duck		
9S RNA from duck reticulocytes	α^A , α^D , and β -duck globin chains	Lane et al., (1973)
Carp		
poly(A)-RNA from carp islet	carp proinsulin	Rapoport (1981)

Table 2.1 (continued)

Invertebrate:

Honey bee

Crude RNA from queen bees venom glands	Promellitin-like substance	Kindas-Mugge et al., (1974)
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Plants

Crude RNA from self- pollinated styles of <u>Petunia hybrida</u>	Undefined plant protein	Van der Donk (1975)
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Poly(A)-RNA from parsley cell suspension cultures (<u>Petroselinum hortense</u> Hoffm).	Flavanone synthase (FS) and phenylalanine ammonia-lyase (PAL)	Schroder et al., (1977)
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Poly(A)-RNA from pea and French bean cotyledons	Vicilin and legumin related proteins	Bassuner et al., (1983)
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Mould

Poly(A)-RNA from <u>Dictyostelium discoideum</u> axenic strain AX2 cell culture	Secretory proteins	Dicou et al., (1979)
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Viruses

60-70S RNA and 30-40S RNA from avian myeloblastosis virus	Viral structural proteins p27, p19, p15 and p12	Ghysdael et al., (1977)
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38S poly(A)-RNA from bovine leukemia virus	viron proteins p24, p15, p10	Ghysdael et al., (1979)
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Bacteria

5'-end capped β -Lactamase mRNA from <u>E. coli</u>	β -Lactamase	Wiedmann et al., (1984)
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as those from f2, MS2 or Q β phage in Xenopus oocytes has not been detected (Gurdon et al., 1971; Marbaix and Huez, 1979). However, it was recently demonstrated that 5'-end capped β -lactamase mRNA of E. coli could be successfully translated in oocytes (Wiedmann et al., 1984).

(ii) The stability of the introduced mRNA in the Xenopus oocytes.

It has been demonstrated that the rabbit and mouse α and β globin mRNA is very stable in Xenopus oocytes and is efficiently translated for four to fifteen days (Gurdon et al., 1973). It is also known that human globin mRNA (Maniatis et al., 1976), human placental lactogen mRNA (Mous et al., 1979), and Xenopus liver vitellogenin and albumin mRNAs (Berridge and Lane, 1976) are translated for long periods of time after their injection into Xenopus oocytes.

Although the 3'-OH poly(A) segment is not required for the translation of mRNAs (Bard et al., 1974, Williamson et al., 1974, and Soreq et al., 1974), the 3'-OH poly(A) segment of the eukaryotic mRNA plays a very important role in the stability of the mRNA in the cytoplasm during translation (Gurdon et al., 1973; Marbaix et al., 1979). The deadenylated globin mRNA was degraded rapidly after injection into the Xenopus oocyte (Marbaix et al., 1977). When the

poly(A) was readded at the 3'-OH end of poly(A)-free globin mRNA using an ATP:RNA adenylyltransferase from E. coli (Sippel, 1973), this "reconstituted" mRNA was indeed shown to be as stable as native poly(A)-containing mRNA (Huez et al., 1975, 1978). The minimal length of the poly(A) tail required for ensuring the stability of the mRNA was demonstrated to be at least 20 adenylate residues (Nudel et al., 1976).

Besides of the 3'-OH poly(A) segment of the eukaryotic mRNA, the 5'-end "cap" also plays an important role in stabilizing the mRNA in the cytoplasm (Furuichi et al., 1977) as well as its role in the initiation of translation (Paterson and Rosenberg, 1979; Revel and Groner, 1978; Shatkin, 1976). The 5'-end "cap" was formed by adding post-transcriptionally a 7-methylguanosine to the mRNA nuclear precursor and linked by a 5' to 5' bond to the first 5' nucleotide of the transcribed RNA molecule. The work of Furuichi et al. (1977) showed that the presence of a "cap" or even 5'-blocking of mRNA with unmethylated guanosine protects reovirus against degradation after injection into Xenopus laevis oocytes. However, for the initiation of translation a complete 5'-end "cap" is required.

(iii) The fidelity of translation of injected mRNA.

In a detailed study concerned with the translation of rabbit globin mRNA in Xenopus oocytes (Lane et al., 1971; Marbaix and Lane, 1972), it was demonstrated that the oocyte-derived globin chains were very similar to rabbit reticulocyte globin chains as judged by carboxymethyl cellulose chromatography of the whole chains and by cation-exchange chromatography, paper chromatography and paper electrophoresis of tryptic peptides derived from the separated α - and β -chains. It therefore seems likely that mRNAs from different species are faithfully translated in Xenopus oocytes.

The fidelity of the oocyte system is also confirmed by the fact that injected mRNAs are translated to biologically active products, for example mouse β -glucuronidase (Labarca and Paigen 1977); human interferons (Cavalieri et al., 1977); human interleukin-2 (Taniguchi et al., 1983).

(iv) Translation efficiency of the oocyte system.

It is now generally believed that the translational machinery in Xenopus oocytes is fully saturated and that any injected mRNA has to compete against endogenous mRNA for translation.

At low concentrations of injected mRNA (less than

10ng per oocyte for globin mRNA), a linear relationship is observed between the mRNA concentration and the translation rate; at higher concentrations, a sort of "saturation" is reached (Moar et al., 1971; Marbaix and Gurdon, 1972). Independent of the concentration of mRNA, the maximal rate of translation of the injected mRNA is reached after 7 to 20 hours (Asselbergs et al., 1979).

The rate of translation of rabbit β -globin mRNA at 19° C in Xenopus oocytes was estimated to be about 30 β -globin molecules synthesized per molecule of mRNA per hour (Gurdon et al., 1973). Although the translation rate of injected oocytes is approximately one quarter the rate observed in reticulocytes at the same temperature, 110 molecules of β -globin per molecule of mRNA per hour (Hunt et al., 1969), the injected mRNA can be translated in the Xenopus oocytes for 4 to 15 days (Gurdon et al., 1973) as compared with one or two hours in the reticulocyte lysate system. Thus the mRNA is translated about 100 times more efficiently in Xenopus oocytes than in a reticulocyte lysate. It is possible to detect picogram quantities of mRNA using oocytes (Gurdon et al., 1971) while the cell-free system requires much larger amounts (1 to 10ng) of mRNA (Pelham and Jackson, 1976). Even though most of the eukaryotic mRNAs are efficiently translated in the Xenopus oocytes, several other mRNA are only translated with a

relatively low yield in Xenopus oocytes, such as thyroglobulin mRNA (Vassart et al., 1975), and the α -globin mRNA in the absence of haemin (Gigliani et al., 1973).

(v) Post-translational modifications of the translation products.

Another advantage of the Xenopus oocyte system for the translation of heterologous mRNAs is that a wide range of secondary modifications that take place in terminally differentiated cells can also occur in oocytes (reviewed by Asselbergs, 1979). Some examples of the secondary modifications of the translation products of mRNAs injected into Xenopus oocytes are:

- (a) N-Acetylation of terminal methionine in calf lens epithelium α_2 crystallin (Berns et al., 1972),
- (b) proline hydroxylation of mouse collagen (Lane and Knowland, 1975),
- (c) phosphorylation of testis protamines (Gedamu et al., 1978),
- (d) glycosylation of Phaseolus vulgaris L. phaseolin (Matthews et al., 1981),
- (e) glycosylation of human interferon, a secreted polypeptide (Reynolds et al., 1975),
- (f) proteolytic cleavage of rabbit pre-uteroglobin to uteroglobin, a secreted polypeptide (Beato and Rungger, 1975),

- (g) assembly of multimeric proteins bound by S-S bonds, like rabbit uteroglobin (Beato and Rungger, 1975),
- (h) assembly of multimeric proteins with non-covalent bonds between subunits, like bovine α -crystallin (Asselbergs et al., 1978), and also
- (i) proteolytic processing of viral polyproteins like the processing of the precursor polypeptides of Avian Myeloblastosis Virus to virion structural proteins (Ghysdael et al., 1977).

It thus appears that the post-translational modifications which occur in Xenopus oocytes are not cell-type specific and are probably determined by the availability of amino acid sequences of the translational product (Gurdon, 1974). However some post-translational modifications are not performed by Xenopus oocytes:

- (a) the carp proinsulin is not converted to insulin (Rapoport, 1981),
- (b) the C-terminal amide of the honey bee promitellin is not synthesized (Kindas-Mugge et al., 1974; Kreil et al., 1977),
- (c) bovine thyroglobulin is not iodinated (Vassart et al., 1975),
- and (d) the mouse β -glucuronidase probably does not undergo post-synthetic cleavage (Labarca and Paigen, 1977).

Apart from post-translational modification,

another great advantage of Xenopus oocyte translation system is that the foreign protein translated in the oocyte is directed to its own original destination. For example the membrane proteins like murine immunoglobulin E receptor (Liu and Orida, 1984), human serotonin receptor and sodium channel (Gundersen et al., 1984) and Semliki Forest virus envelope proteins (Huth et al., 1984) are also transported to the oocyte membrane after translation in the Xenopus oocyte. Secreted proteins such as murine interleukin-2 and bacterial β -lactamase are secreted outside the oocyte after translation (Bleackley et al., 1981; Wiedmann et al., 1984) and appear to have their signal peptides processed normally.

Thus the Xenopus laevis oocyte translation system is an efficient and complete system for the translation of foreign mRNA. Since murine interleukin-3 is a secretable glycoprotein (Ihle et al., 1981), this system appeared to be the system of choice for the assay of the murine interleukin-3 mRNA and for the screening of the cDNA library in the molecular cloning of the murine interleukin-3 using the hybridization-translation method.

II. Materials

Reagents

1. Guanidine-HCl, an ultra pure reagent, was obtained from Bethesda Research Laboratories, Inc. (No. 5502).
2. Guanidine thiocyanate, purum, was obtained from Fluka (No. 50990).
3. Sarcosyl, NL 97; was a gift from CIBA-GEIGY Pty. Ltd.
4. Phenol, Anala R, was obtained from BDH Chemicals Australia Pty. Ltd. (No. 10188) and stored at -20°C.
5. Sucrose, ultra pure density gradient grade, was obtained from Schwarz/Mann Pty. Ltd. (No. 821721).
6. Oligo (dT)-cellulose type 7 was obtained from P-L Biochemicals, Inc. (No. 5543).
7. Calcium chloride, Anala R, was obtained from BDH Chemicals Australia Pty. Ltd. (No. 10067).
8. 2-Mercaptoethanol, 90%, was obtained from Eastman Kodak Co. (No. 4196).
9. Urea, ultra pure reagent, was obtained from Bethesda Research Laboratories Inc. (No. 5505-UA).
10. SDS (Sodium dodecyl sulfate), specially pure, was obtained from BDH Chemical Australia Pty. Ltd. (No. 4244).

11. Penicillin G, potassium, was obtained from Sigma (No. PEN-K).
12. Streptomycin, was obtained from Glaxo (No. 325794).
13. [methyl-³H] thymidine, was obtained from Amersham (No. TRA120).
14. Trypan blue, was obtained from Sigma (No. T6146).
15. DTT (DL-Dithiotheritol), was obtained from Sigma (No. D-0632).

All other chemicals used were of the highest purity commercially available.

Cell lines

WEHL-3B and EL-4 cells were provided by Dr. Andrew J. Hapel.

Xenopus laevis

Xenopus laevis was obtained from Xenopus Ltd., 151 Frenches Road, Red Hill, Surrey RM1 2HZ, England.

III. Solutions.

1. Guanidine thiocyanate buffer: 4M guanidine thiocyanate, 0.1M Tris-HCl pH7.5 in sterile water. The solution was millipore filtered and stored at room temperature in a sealed sterile bottle. 0.143ml 2-mercaptoethanol (14M stock solution, Eastman Kodak Co., No. 4196) was added to 20ml of the guanidine thiocyanate buffer before use.
2. Guanidine-HCl buffer: 6M guanidine-HCl, 10mM EDTA pH7.0 in sterile water. It was stored at room temperature. 0.1ml 1MDTT was added to 10ml of this buffer before use.
3. Urea buffer: 7M urea, 0.1M Tris-HCl pH8.5, 0.1mM EDTA, 0.1% SDS. (This solution was made up immediately before use.
4. Oocyte incubation medium: (Cavalier et al., 1977). 88mM NaCl, 1mM KCl, 2.4mM NaHCO₃, 0.82mM MgSO₄·7H₂O, 0.33mM Ca(NO₃)₂·4H₂O, 0.41mM CaCl₂·6H₂O, 7.5mM Tris-base, Penicillin G, potassium, 11µg/ml, Streptomycin, 18µg/ml. The final pH was adjusted to pH7.6 with HCl and the solution was sterilized by filtration.

5. Phenol: Solid phenol from the freezer was melted in equal volume of 200mM Tris-HCl pH9, 100mM NaCl, 1mM EDTA. It was vortexed well and two phases allowed to separate. The aqueous phase was removed by aspiration. The neutralized phenol was then used for phenol extraction of nucleic acids. Neutralized phenol was prepared immediately before use.

IV. Methods

2.1 Isolation of RNA by centrifugation through CsCl. This method is modified from Chirgwin's method (Chirgwin et al., 1979).

WEHI-3B cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum to a density of 2×10^6 /ml. 10^9 cells were then collected by centrifugation in an MSE centrifuge at 300xg for 10 min and resuspended in 2ml of serum-free RPMI-1640 medium. The suspension was added dropwise to 40ml guanidine thiocyanate buffer (4M guanidine thiocyanate, 0.1M Tris-HCl pH7.5, 0.1M 2-mercaptoethanol) with continuous stirring. The cell lysate was stored at -70°C until it was processed further.

1/20th volume of 10% sarcosyl was added to the thawed cell lysate. After adding 0.5g CsCl/ml, the light-brown solution was heated at 65°C for 5 min, quick-cooled in ice, layered on top of a 2.5ml cushion of 5.7M CsCl, 25mM NaOAc pH5 in a sterilized SW41 centrifuge tube (Beckman No. 331372, polyallomer) and centrifuged in an SW41 rotor at 28,000rpm for 18 hr at 18°C . The overlaying solutions were removed by aspiration, and the tubes were inverted quickly and drained for 15 min. The tubes were aspirated dry whilst still inverted and the bottom 0.5cm of the

tubes containing the clear RNA pellet were cut off with a sterile scalpel blade. The RNA pellet was rinsed out very carefully from the bottom of the tube 4 times with 200 μ l 10mM Tris-HCl pH7.5, 1mM EDTA, 5% sarcosyl, 5% phenol. The RNA suspension was vortexed for about 5 min to ensure it was completely dissolved. It was then extracted twice with 10ml phenol, chloroform and isoamylalcohol mixture (50:48:2). The aqueous phase was brought to 0.3M NaOAc pH5.5 and the RNA precipitated with 2.5 volumes of absolute ethanol in a dry ice-ethanol bath for 1 hr. The tube was centrifuged in a Sorvall SS34 rotor at 20,000 rpm for 30 min at -15°C. The RNA pellet was washed with cold absolute ethanol and dried under vacuum. It was resuspended in 10mM Tris-HCl pH7.5, 0.1mM EDTA.

2.2 Isolation of RNA by ethanol precipitation (Brooker et al., 1980).

The guanidine thiocyanate cell lysate was prepared as in Method 2.1. The cell lysate was centrifuged in an SS34 rotor at 15,000rpm for 10 min at 0°C. The supernatant containing the RNA and other cellular components was acidified to pH5 by the addition of 0.04 volume of 1N acetic acid, and the RNA was precipitated by the addition of 0.5 volume of absolute ethanol. After mixing, the solution was kept in dry-ice ethanol bath for 1 hr and centrifuged in an

SS34 rotor at 20,000rpm for 30 min at -15°C . The pellet was dissolved in approximately 0.5 volume (relative to the original volume of the lysate) of guanidine-HCl buffer (6M guanidine-HCl, 10mM EDTA pH7.0, 10mM DTT). The solution was again acidified to pH5 by the addition of 0.04 volume of 1N acetic acid and precipitated with 0.5 volume of ethanol. The mixture was kept in a dry-ice ethanol bath for 30 min and centrifuged in an SS34 rotor at 20,000rpm for 30 min at -15°C . The precipitation of the RNA in guanidine-HCl buffer was repeated once, reducing the volume to one third relative to that of the first precipitation. The final pellet was resuspended in 0.5 volume of 0.24M sodium acetate pH5.5. 2.5 volumes of absolute ethanol were added, mixed well and the mixture held in a dry-ice ethanol bath for 1 hr. It was centrifuged in an SS34 rotor at 20,000rpm for 30 min at -15°C . The RNA pellet was resuspended in the same volume of urea buffer (7M urea, 0.1M Tris-HCl pH8.5, 0.1mM EDTA, 0.1% SDS). It was extracted twice with equal volume of phenol, chloroform and isoamylalcohol mixture (50:48:2). The SDS was raised to 1% in the second extraction. The nucleic acid was precipitated with 2.5 volumes of absolute ethanol in a dry-ice ethanol bath for 1 hr and centrifuged in a SS34 rotor at 20,000rpm for 30 min at -15°C . The pellet was suspended again in 10ml 30mM potassium acetate, 70% ethanol, held in a dry-ice ethanol bath

for 1 hr and centrifuged in a SS34 rotor at 20,000rpm for 30 min at -15°C . The final pellet was dried under vacuum and resuspended in 10mM Tris-HCl pH7.5, 0.1mM EDTA.

2.3 Isolation of poly(A)⁺-RNA from total RNA.

Poly(A)⁺-RNA was isolated from the total RNA by chromatography on oligo(dT)-cellulose (Bantle, J.A. et al., 1976). About 4 to 5mg total RNA was adjusted to 0.5M NaCl and applied to a column containing 1g of oligo(dT)-cellulose in buffer (0.5M NaCl, 10mM Tris-HCl pH7.5, 1mM EDTA) at a flow rate of about 20ml/hr. The column was washed with the same buffer until the optical density was $< 0.08 A_{260}$ unit/ml. The column was washed again with 10ml 0.1M NaCl, 10mM Tris-HCl pH7.5, 1mM EDTA. Poly(A)⁺-RNA was then eluted with 10mM Tris-HCl pH7.5, 1mM EDTA. 500 μ l fractions were collected. Fractions containing RNA were pooled together. 0.1 volume of 3M KOAC pH5.5 and 2.5 volumes of absolute ethanol were added and mixed well. The mixture was held in a dry-ice ethanol bath for 1 hr and centrifuged in an SS34 rotor at 20,000rpm for 30 min at -15°C . The RNA pellet was washed with cold absolute ethanol and dried under vacuum. It was resuspended in 10mM Tris-HCl pH7.5, 0.1mM EDTA.

The oligo(dT)-cellulose column was regenerated by washing with 10ml 0.1M KOH. The oligo(dT)-cellulose

was then neutralized with 0.5M NaCl, 10mM Tris-HCl, pH7.5, 1mM EDTA and stored at 4°C in the same buffer containing 0.2g/l NaN₃.

2.4 Preparation of injection needles.

Five-microliter microdispenser tubes (DADE Diagnostics, Inc. P4518-5X) were pulled out using a needle puller (Leitz, Typ 027-035.002). The needles were cut with fine scissors under a dissecting microscope to form a needle with a tip of 15-20nm in diameter. The microdispenser capillaries consist of uniform bore tubing (15mm equivalent to 1µl). The needles were sterilized at 180°C overnight before use.

2.5 Preparation of oocytes.

A Xenopus laevis female was cooled in ice water for 30 min, sacrificed by quickly removing the head with a scalpel and paralysed by pithing. The abdominal area was swabbed with 0.5% w/v hibitane in 70% v/v ethanol and the skin of the abdomen removed. The abdomen was cut open and the oocytes were removed and immediately placed in 150ml of oocyte incubation medium in a sterile beaker. Individual oocytes were obtained by gently teasing apart the oocyte sacs with 2 pairs of watchmakers forceps. Individual oocytes were transferred with a sterile pasteur pipette (1mm internal diameter) to another petri dish and washed with the oocyte incubation medium. The oocytes could

be kept in the oocyte incubation medium in a sterilized petri dish in cold room at 4°C for at least two weeks with a change of medium every 2 days.

2.6 Injection of mRNA.

mRNA (1µg/µl in 10mM Tris-HCl pH7.5, 0.1mM EDTA) was heated at 80°C for 2 min and rapidly cooled in ice before injection. The injection needle was clamped to the handle of the micromanipulator (Leitz) and attached to tubing connected to a suction-pressure system (Figure 2.1). The injection needle was rinsed with sterile water 1.5µl of the treated mRNA was placed in the bottom of a sterile petri dish. The injection needle was filled from the drop using suction. A single oocyte of about 1mm in diameter was picked up using sterile glass spoon and placed facing the injection needle. The injection needle was punched into the oocyte at an angle of about 45°. Positive pressure, 20lbs/in² was applied to the system using compressed N₂ and about 50nl mRNA, equivalent to approximately 0.75mm displacement of the meniscus, was injected per oocyte. The needle was removed from the oocyte. The injected oocytes were kept in a drop of oocyte incubation medium. After injection, the injected oocytes were observed under the dissecting microscope. Any leaking oocytes were discarded. The injected oocytes were incubated in oocyte incubation

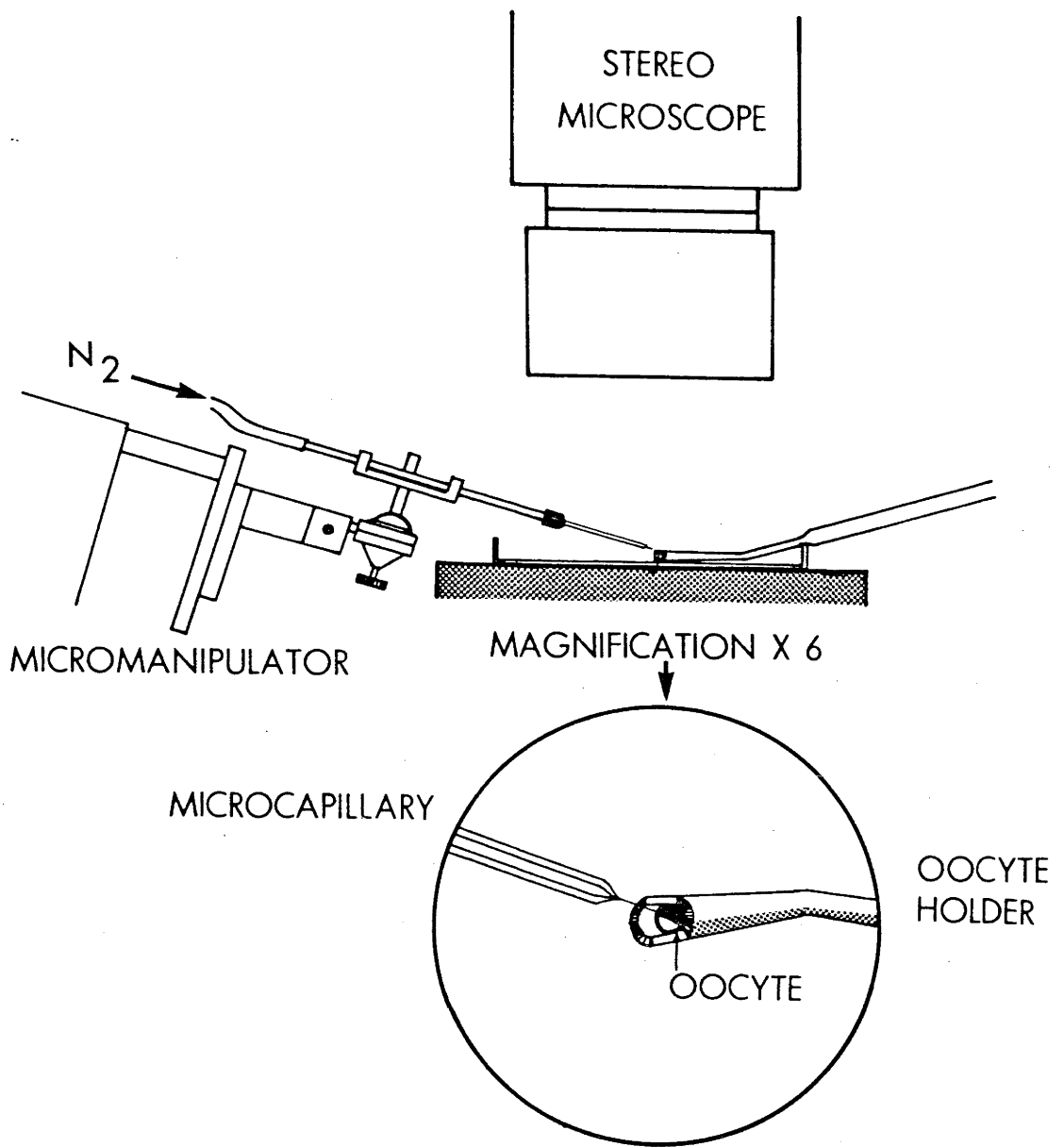


Figure 2.1 Schematic diagram summarizing the equipment used for microinjection into Xenopus laevis oocytes. Full details of the procedures used are given in Method 2.6.

medium (10/100 μ l) in a loosely capped sterilized Eppendorf tube at 20°C for 48 hrs. After incubation, the incubation medium was transferred to another sterile Eppendorf tube and centrifuged in an Eppendorf centrifuge for 15 min. The supernatant was stored at -70°C until assayed.

2.7 Assay for Interleukin-3 activity.

All the assays for IL-3 activity were done by Dr. Andrew Hapel's laboratory. Samples to be examined were diluted in RPMI-1640 containing 10% FCS. Two fold dilutions were prepared in 96-well microtiter plates containing 0.05ml/well. Log phase, washed 32Dcl-23 or FDC-P1 IL-3-dependent cells (10^5) were subsequently added in 0.05ml and were incubated for 24 hrs at 37°C. After the initial incubation, 1 μ Ci of 3 H-thymidine was added and the cells were further incubated for approximately 6 hr at 37°C. The cells were then harvested with an automated cell harvester unit onto filter paper. The level of thymidine incorporation was determined by liquid scintillation counting.

2.8 Sucrose gradient centrifugation of IL-3 mRNA.

Linear gradients were made by mixing equal volumes of 5% and 20% (w/v) sucrose in 0.02M sodium acetate, pH5, in the chamber of a Buchler gradient

maker which had been treated with 0.1% pyrocarbonate in 70% ethanol. Gradients were made in sterilized Beckman SW41 centrifuge tubes by running the heavy sucrose solution into the tube first and lowering the tube very slowly. IL-3 mRNA in 10mM Tris-HCl, pH7.5, 0.1mM EDTA was heated at 80°C for 2 min and quick-cooled in an ice bath to reduce aggregation. The sample was layered over one gradient (500µg/gradient). The gradients were centrifuged at 30,000rpm, 4°C for 20 hr. After centrifugation, gradients were carefully pierced at the bottom with a gauge 25 needle. Fractions (20 drops per fraction) were collected. 20µl of samples from each fraction were taken to measure the absorbance at 260nm. 0.1 volume of 3M KCl and 2.5 volumes of absolute ethanol were added and mixed well. The mixture was kept in dry-ice ethanol bath overnight and centrifuged in an Eppendorf centrifuge for 30 min at 4°C. The mRNA pellet was washed with cold absolute ethanol and dried under vacuum. It was finally resuspended in 10mM Tris-HCl, pH7.5, 0.1mM EDTA.

V. Results

1. Isolation of total RNA and poly(A)⁺ RNA from WEHI-3B cells.

It was previously shown that the WEHI-3B cell line constitutively produces IL-3 at high titers (Lee et al., 1982). Therefore the WEHI-3B cell line was used as the main source of IL-3 mRNA. WEHI-3B cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 20mM glutamine, and antibiotics to a titer of 2×10^6 /ml. 10^9 cells were then collected by centrifugation and lysed in guanidine thiocyanate buffer (Method 2.1). The RNA was purified by pelleting through a CsCl step gradient (Method 2.1). In this method, since DNA and protein do not pellet with the RNA and care was taken to completely remove these contaminants from the RNA pellet. The pellet was then dissolved in buffer, phenol extracted and the RNA precipitated (Method 2.1). Four preparations of the total RNA from 10^9 WEHI-3B cells were made and yields ranged from 2.5mg to 5.4mg (Table 2.2).

In addition to the WEHI-3B cell line, the EL-4 cell line is also another source of IL-3 mRNA since it can be induced to produce IL-3 by stimulation with phorbol esters (Lee et al., 1982). One batch of RNA was prepared from induced EL-4 cells. The cells were grown to a cell density of about 10^6 cells per ml in

Table 2.2 Preparation of IL-3 mRNA

preparation		yield of total RNA/ 10 ⁹ cells	yield of poly(A) ⁺ RNA/ 10 ⁹ cells		
			<u>OD_{260/280}</u>	<u>OD_{260/280}</u>	
WEHI-3	1	2.70mg	1.90	370μg	1.77
	2	5.40mg	1.72	950μg	1.64
	3	2.80mg	1.70	353μg	1.84
	4	2.47mg	1.71	384μg	1.75
induced EL-4	1	4.61mg	1.71	480μg	1.75

The yield of total RNA and poly(A)⁺ RNA from each preparation was measured by optical density at 260nm. Poly(A)⁺RNA was isolated from total RNA by chromatography on oligo(dT)-cellulose (Method 2.3).

spinner culture in DMEM medium supplemented with 10% heat-inactivated fetal calf serum. 2×10^9 cells were collected by centrifugation, resuspended in serum-free medium containing 100ng per ml phorbol myristic acetate and subcultured into 150cm^2 culture flasks at 5×10^8 cells per flask. The cells were incubated for 10 hr before collection. The viability of the cells after induction was checked by staining with 0.0625% of Trypan blue. 90% of the induced cells were still viable.

2×10^9 cells were collected by centrifugation and resuspended in 4ml PBS. The resuspended cells were lysed in 4M guanidine thiocyanate buffer (Method 2.2). The RNA was purified by the guanidine HCl precipitation technique (Method 2.2). 9.2 mg of total RNA was obtained from 2×10^9 induced EL-4 cells.

Poly(A)⁺-RNA was isolated from the total RNA by chromatography on oligo (dT)-cellulose (Method 2.3). In a typical preparation, 3.5mg of total RNA was chromatographed on a column containing 1g of oligo(dT)-cellulose. The RNA was applied in 0.5M NaCl buffer and washed with the same buffer and then 0.1M NaCl buffer. The RNA was eluted in buffer containing no salt. The optical densities of the column fractions are shown in Table 2.3. Fractions 4-9 containing the eluted RNA were pooled and the RNA recovered by ethanol precipitation.

Table 2.3 Oligo (dT)-cellulose chromatography of the WEHI-3 total RNA.

Fraction no.	Absorbance of fractions (260nm)		
	0.5M NaCl	0.1M NaCl	Elution
1	0.002	0.003	0.001
2	0.585	0.003	0.001
3	1.240	0.014	0.003
4	0.180	0.009	0.114
5	0.024	0.006	0.223
6	0.010	0.004	0.089
7	0.007	0.004	0.031
8	0.006	0.003	0.010
9	0.005	0.003	0.005
10	0.005	0.003	0.004
11	0.005		0.003
12	0.004		0.003
13	0.003		
14	0.004		
15	0.004		
16	0.003		
17	0.003		
18	0.003		
19	0.003		
20	0.003		

3.7mg total RNA was adjusted to 0.5M NaCl and loaded onto a lg oligo(dT)-cellulose column. It was firstly washed with 0.5M NaCl, 10mM Tris-HCl pH7.5 and then washed with 0.1M NaCl, 10mM Tris-HCl pH7.5. In the first and second wash, 1ml fractions were collected and 50 μ l samples were taken out from each fraction to measure the absorbance at 260nm. After washing, the poly(A)⁺ RNA was eluted using 10mM Tris-HCl, pH7.5. Fractions of 500 μ l were collected and 20 μ l samples were taken out from each fraction to measure the absorbance at 260nm.

A summary of the RNA preparations from WEHI-3B and EL-4 cells is given in Table 2.2.

2. Translation of IL-3 mRNA in oocytes.

Attempts were made to translate the poly(A)⁺ mRNAs from WEHI-3B and EL-4 by microinjection into Xenopus laevis oocytes. mRNAs from EL-4 or WEHI-3B were diluted to 1µg/µl and heated at 80°C for 2 min then cooled in ice before injection. The microinjection system is shown schematically in Figure 2.1 and the technique is described in detail in Method 2.6. After injection, the oocytes were observed under the microscope and any leaking oocytes were discarded. 10 injected oocytes were incubated in 100µl of oocyte incubation medium in a loosely capped sterilized Eppendorf tube at 20°C for 48 hr. After incubation, the incubation medium was removed to another sterilized Eppendorf tube and was centrifuged in an Eppendorf centrifuge for 15 min. The supernatant was stored at -70°C. For the oocyte extract, 20 incubated oocytes were homogenized manually in 100µl fresh incubation medium using a sterile glass rod. The extract was then centrifuged for 15 min in an Eppendorf centrifuge. The supernatant was carefully removed to avoid contamination by the lipid layer formed at the top. The oocyte extract was also stored at -70°C. For the total extract the incubated oocytes

were homogenized in the same tube without changing the incubation medium. The incubation medium, oocyte extract and total extract were assayed for IL-3 activity using the IL-3 dependent cell line 32Dcl-23 (Ihle et al., 1982, Method 2.7). It was found that mRNA from EL-4 and WEHI-3B cells could be translated in the oocytes to give readily detectable levels of IL-3 after 48 hr incubation (Table 2.4). Most of the detectable activity was present in the incubation medium indicating that the oocytes were secreting mature IL-3. Comparison of the activities of the total extracts and the activities in the incubation media showed that the oocyte extracts were inhibitory. mRNA from WEHI-3B cells regularly gave higher levels of IL-3 in the translation assays than preparations from EL-4 (Table 2.4), in keeping with the higher levels of IL-3 found in culture supernatants of the WEHI-3B cell line.

The effect of incubation time on the levels of IL-3 activity in the incubation medium was examined (Table 2.5). It was found that the level of IL-3 activity only marginally increased over the period of 48 hr to 96 hr of incubation. Thus 48 hr incubation time was routinely used.

The effect of the age of the oocytes after harvesting from the toad on their ability to translate IL-3 mRNA was also examined. It was found that the

Table 2.4 Translation of IL-3 mRNA in Xenopus laevis oocytes

mRNA	Incubation time	IL-3 activity (c.p.m.)		
		oocyte	medium	total extract
EL-4	24h	0	3,100	3,700
	48h	-	20,000	5,600
WEHI-3	48h	6,000	47,000	28,700
<hr/>				
WEHI-3B	conditioned medium		70,000	

mRNA from EL-4 or WEHI-3B at 1µg/µl was heated at 80°C for 2 min and cooled on ice. 50nl of the treated mRNA was injected per oocyte. 10 injected oocytes were incubated in 100µl oocyte incubation medium at 20°C for 24 hr or 48 hr. After incubation, the medium was withdrawn and an extract of the oocytes prepared as described in the text. The incubation medium, oocyte extract and total extract were assayed for IL-3 activity using the IL-3 dependent cell line 32Dcl-23 (Method 2.7) measuring incorporation of ³H-thymidine as an index of cell growth.

Table 2.5 Translation of WEHI-3B mRNA in Xenopus laevis oocytes

<u>Incubation time</u>	<u>IL-3 activity (c.p.m.)</u>
24 hr	7,800
48 hr	15,000
72 hr	16,000
96 hr	17,000

WEHI-3B conditioned medium	21,000
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WEHI-3B mRNA was translated in Xenopus oocytes and the incubation medium assayed for IL-3 activity (see Table 2.4) after different lengths of incubation.

isolated oocytes could be stored in a sterile petri-dish in the oocyte incubation medium at 4° for 2 weeks with a change of fresh medium every two days. The oocytes still looked healthy after 2 weeks storage. In order to study the effect of oocyte age on the translation efficiency of IL-3 mRNA. WEHI-3B mRNA was injected into the oocytes after different times of storage. The results indicated that there was no significant drop in the ability to translate IL-3 mRNA with up to 14 days storage (Table 2.6).

There was a linear relationship between the amount of IL-3 mRNA injected into oocytes and the level of IL-3 activity secreted into the incubation medium between 0 to 12.5ng per oocyte. As more IL-3 mRNA was injected per oocyte the relationship between the amount of IL-3 mRNA injected into the oocyte and the level of IL-3 activity secreted into the incubation medium was no longer linear. However, up to 50ng of IL-3 mRNA injected per oocyte, the translation rate had still not reached the "saturation" state (Figure 2.2).

3. Enrichment of IL-3 mRNA by sucrose gradient centrifugation.

In order to enrich the IL-3 mRNA, WEHI-3B mRNA was fractionated on a sucrose gradient. A linear sucrose gradient was prepared in a Beckman SW41 tube

Table 2.6 Effect of oocyte age on their ability to translate IL-3 mRNA

<u>age of the oocytes</u>	<u>IL-3 activity (c.p.m.)</u>
day 0	16,000
day 4	13,000
day 8	14,000
day 14	14,000
WEHI-3B conditioned medium	21,000

The IL-3 mRNA was microinjected into Xenopus oocytes and the incubation medium assay for IL-3 activity as described in Table 2.4.

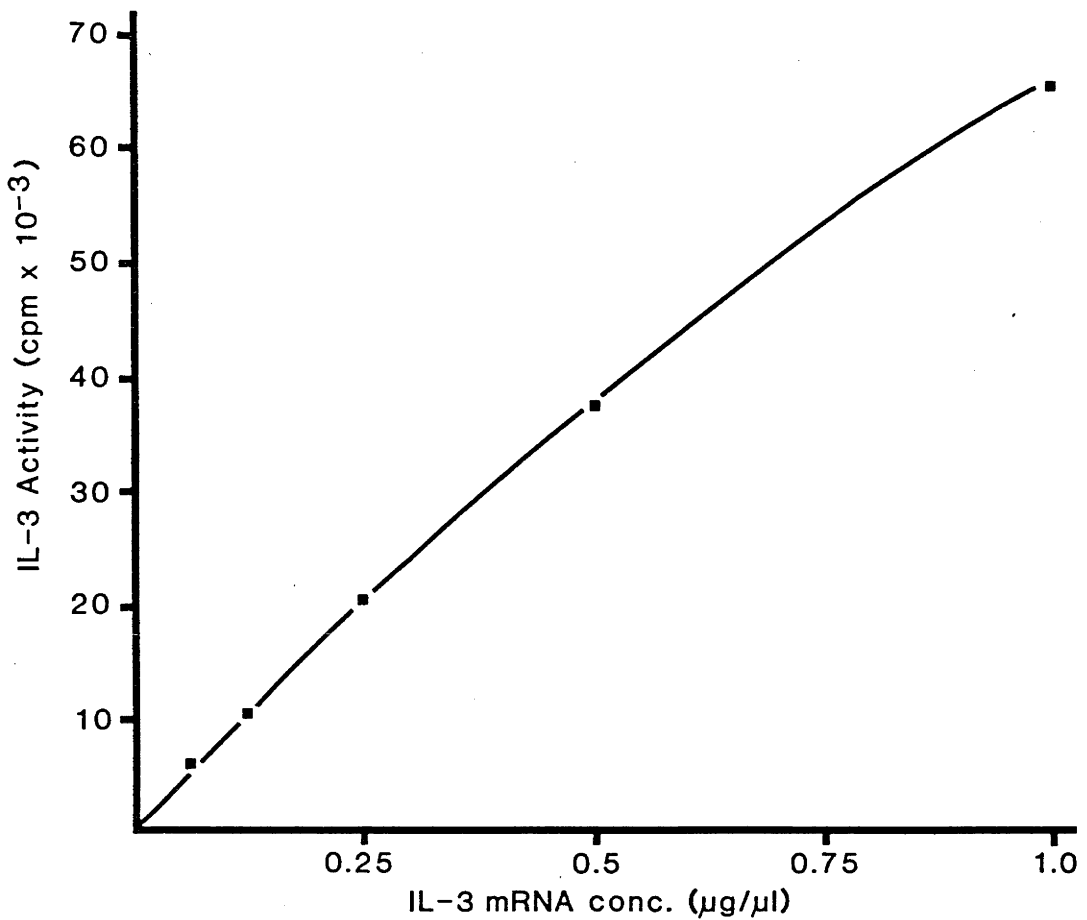


Figure 2.2 Relationship between the amount of IL-3 mRNA injected and the IL-3 activity produced in oocytes. Twofold dilutions of WEHI-3B mRNA were prepared in 10mM Tris-HCl pH7.5, 0.1mM EDTA. 50nl of each diluted sample, which had been heated at 80°C and chilled in ice was microinjected into each of 10 oocytes and the secreted IL-3 activity determined as described in Table 2.4.

(Method 2.8). 500 μ g WEHI-3B poly(A⁺) RNA in 150 μ l of 10mM Tris-HCl pH7.5, 0.1mM EDTA, was heated at 80° for 2 min and cooled rapidly in an ice bath to reduce aggregation. The sample was layered over the gradient. The gradient was centrifuged for 20 hr at 30,000rpm at 4°C using the Beckman SW41 rotor. After centrifugation, the gradient was fractionated into 15 fractions of about 600 μ l. 20 μ l samples from each fraction were taken to measure the absorbance at 260nm. Two distinct peaks at 28S and 18S were observed (Figure 2.3). Each fraction was divided into two parts and to each tube 33 μ l of 3MKCl and 825 μ l absolute ethanol were added and mixed well. The tubes were kept in dry-ice ethanol bath overnight and were centrifuged in an Eppendorf centrifuge at 4°C for 30 min. The RNA pellets were washed with cold absolute ethanol and dried under vacuum. The pellets were suspended in 20 μ l of 10mM Tris-HCl pH7.5, 0.1mM EDTA and samples of each fraction were injected into Xenopus laevis oocytes as described in Method 2.6. The major peak of IL-3 activity sedimented at -12S, that is fraction 8 and 9 (Figure 2.3). This procedure resulted in a 10-20 fold purification of the IL-3 mRNA.

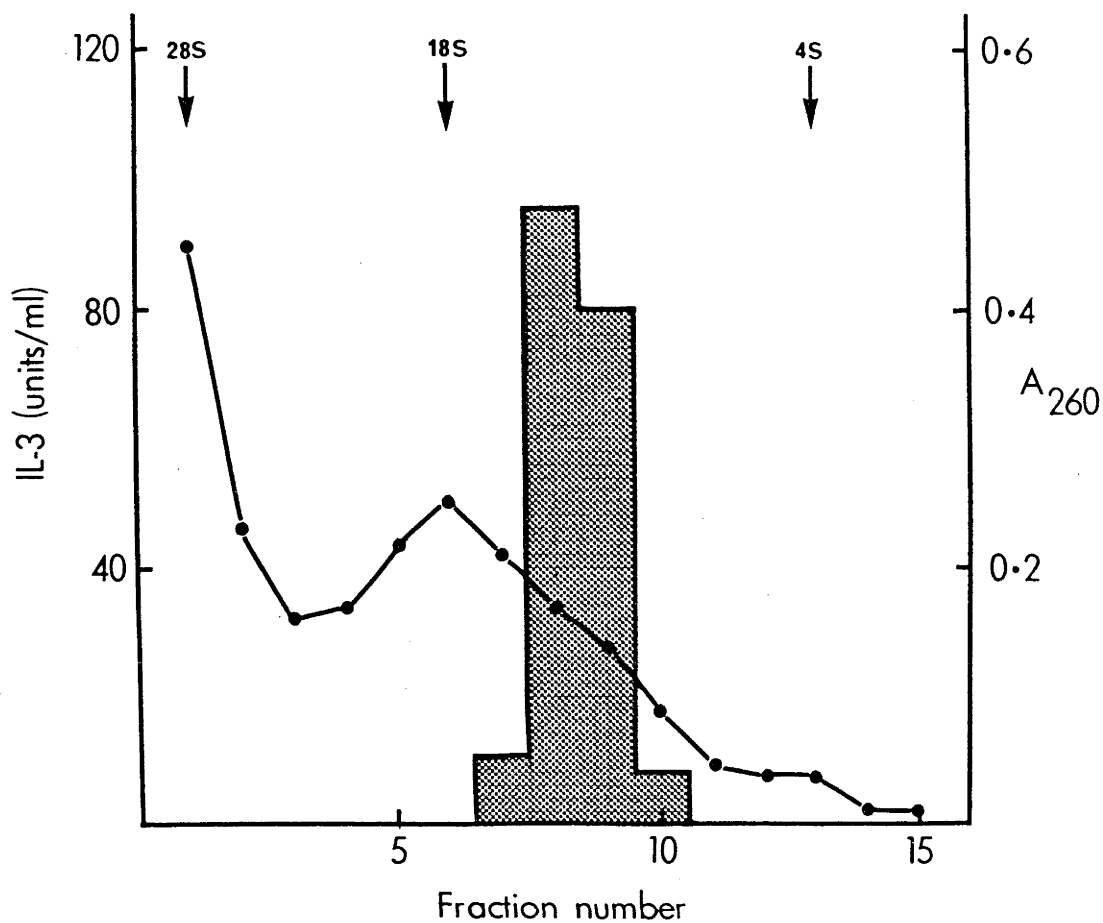


Figure 2.3

Sedimentation of IL-3 mRNA through a sucrose density gradient. A_{260} ; hatched areas, IL-3 activity. Poly(A)⁺-RNA (500 μ g) from WEHI-3 was heated at 80°C for 2 min, chilled in ice and applied to a 5-20% sucrose gradient prepared in a Beckman SW41 polyallomer tube. The gradient was centrifuged at 30,000rpm for 20 hr at 4°C and divided into 15 fractions by the collection of drops. The RNA was recovered by ethanol precipitation and samples injected into oocytes as described in Table 2.3. One unit of IL-3 activity is that required to give 50% of the maximum incorporation of ³H-thymidine in the assay.

VI. Discussion

In the work described in this chapter a sensitive assay for IL-3 mRNA was developed using microinjection of Xenopus oocytes. The WEHI-3B cell line was used as the main source of IL-3 mRNA. On average, 3.34mg of total RNA and 514µg poly(A)⁺RNA were isolated from 10⁹ WEHI-3B cells.

Although the ribosomal RNAs cannot be completely eliminated from the poly(A)⁺RNA after one oligo(dT)-cellulose chromatography (data not shown), the poly(A)⁺ IL-3 mRNA was translated efficiently and the IL-3 secreted into the oocyte incubation medium. 48 hr of incubation for the injected oocytes gave a high enough level of IL-3 activity. There was only a marginal increase in the IL-3 activity when the incubation time was increased to 96 hr and so 48 hr incubation time was routinely used. At low concentrations of injected mRNA (smaller than 12.5ng per oocyte for IL-3 mRNA (Figure 2.2); 10ng per oocyte for globin mRNA (Moar et al., 1971)) a linear relationship was observed between the amount of mRNA injected and the IL-3 appearing in the incubation medium. It was previously reported that injection of about 30ng of globin in RNA gave "saturation" of translation per oocytes (Moar et al., 1971). In contrast, the "saturation" state was not reached even up to 50ng per oocyte for IL-3 mRNA (Figure 2.2),

although this could be due to a lower degree of purity for the IL-3 mRNA preparations. Since the translation efficiency remained high with up to 14 days of storage of the oocytes (Table 2.6), a single batch of oocytes could be used for a number of successive assays.

Using the oocyte translation assay the IL-3 mRNA was partially purified 10-20 fold on sucrose density gradients. The purified IL-3 mRNA was then used for the construction of the cDNA library. By this means the degree of enrichment of IL-3 cDNA clones in the library was significantly increased. This strategy was believed to be essential since it was expected that the IL-3 mRNA would be present in low abundance in the poly(A)⁺RNA preparations.

Chapter 3 Molecular cloning of cDNA for murine interleukin-3.

I. Introduction.

Although their isolation can sometimes be difficult and time consuming, cDNA clones provide an extremely powerful tool for analyzing the structure, organization and expression of eukaryotic genes (Breathnach et al., 1977; Catterall et al., 1979; Fujita et al., 1983). The cDNA can be used as a hybridization probe to enable the isolation of the corresponding genes from chromosomal DNA of the same species (Breathnach et al., 1977) or of different species (Maki et al., 1984). By comparing the DNA sequence of cDNA clone and the corresponding genomic clone, one can identify the intervening sequences (Tilghman et al., 1978a), study the splicing of the mRNA (Tilghman et al., 1978b) and discover whether genomic rearrangements have occurred (Brack et al., 1978). The cloned cDNA can also be expressed in bacterial cells (Itoh et al., 1984; Rosenberg et al., 1984). cDNA cloning of RNA viruses such as vesicular stomatitis virus (Rose, 1980), polio virus (Kitamura et al., 1981) and influenza viruses (Sleigh et al., 1979) has provided an important route for studying their structure, replication, and expression and for the development of antiviral vaccines (Heiland and

Gething, 1981; Panicali and Paoletti, 1982; Smith et al., 1983; Moss et al., 1984).

The method used for the synthesis and cloning of cDNAs have been progressively refined since the first demonstration that avian myeloblastosis virus reverse transcriptase can be used to synthesis a DNA copy of an RNA molecule (Temin and Mizutani, 1970; Baltimore, 1970). The strategies for cloning double strand cDNA can be divided into five steps: (i) first strand cDNA synthesis, (ii) second strand cDNA synthesis, (iii) insertion of the double strand cDNA into a cloning vector, (iv) transformation of the host cell, and (v) screening of the cDNA library.

(i) First strand cDNA synthesis.

The first strand cDNA synthesis is a primer requiring step. Oligo(dT) polynucleotide is generally used as a primer for synthesis of cDNA from a poly(A)⁺RNA (Rougeon and Mach, 1976). This ensures that the cDNA synthesis is initiated near the 3' terminus of the RNA. For the nonpolyadenylated RNAs a poly(A) tail can be added to their 3' terminus using poly(A) polymerase from Escherichia coli (Hell et al., 1976; Emtage et al., 1980). Alternatively, a specific primer can be used to synthesise a particular pure cDNA from a heterogeneous population of mRNA sequences (Chan et al., 1979). The optimal conditions for

producing full-length first strand have been studied by several laboratories (Efstratiadis et al., 1976; Buell et al., 1978; Retzel et al., 1980). It was found that the optimal conditions are to some extent dependent on the particular mRNA. However, the general recommendations are (1) the use of highly purified reverse transcriptase at pH8.3 and 42°C; (2) about 4 units/ μ g of mRNA; (3) 140-150mM KCl, 6-10mM MgCl₂, and 0.1mM-1mM dNTPs.

(ii) Second strand synthesis.

The first method used for the synthesis of the second strand cDNA relied on the hairpin formation at the 3' terminus of the first single strand cDNA and acted as a primer (Maniatis et al., 1976; Rougeon and Mach, 1976). This reaction requires an initial 3' to 5' exonucleolytic digestion of unpaired nucleotides on the 3' side of a partially mis-matched loop. It has been demonstrated that the second strand cDNA can be synthesized by the Klenow fragment of DNA polymerase I but not by avian myeloblastosis virus reverse transcriptase, which does not possess the 3' to 5' exonuclease activity (Rougeon and Mach, 1976). However, when using this method, it is necessary to digest the single strand hairpin loop of the double strand cDNA with S1 nuclease. This invariably results in the loss of sequences corresponding to the extreme 5'-terminal region of the mRNA (Maniatis et al., 1976;

Sippel et al., 1978; Humphries et al., 1977; Ullrich et al., 1977; Seeburg et al., 1977).

To overcome this difficulty Zain et al. (1979) inserted cDNA-mRNA hybrids into a plasmid vector and were able to clone a complete copy of the leader segments of adenovirus 2 fiber mRNA. However, this technique gave only low yields of recombinants.

Another method designed to obtain the complete 5'-end sequence complementary to the mRNA was using artificial priming on sequences generated by terminal transferase at the 3'-end of the first single strand cDNA. This was first demonstrated by Rougeon et al. (1975) using terminal transferase to add a homopolymeric dT tail at the 3'-end of the first strand cDNA. Oligo(dA) was subsequently used as a primer to synthesise the second strand cDNA. Later Cooke et al. (1980) and Land et al. (1981) varied this procedure slightly by adding a homopolymeric dC tail at the 3'-end of the first strand cDNA and subsequently using oligo(dG) as a primer to synthesis the second strand cDNA. The artificial priming methods described above require the generation of a single strand cDNA by alkaline hydrolysis of the mRNA template. This procedure might nick the first strand cDNA. In addition, even highly purified preparations of terminal transferase may be contaminated with single-strand-specific nucleases, which might also

nick the first single strand cDNA. These two problems can be circumvented by tailing the first cDNA strand as a DNA-RNA hybrid and sizing the double-strand cDNA before insertion into the plasmid vector.

Recently a very powerful vector-primer cDNA cloning system was developed by Okayama and Berg (1982) and modified by Alexander et al. (1984). In this system the vector used (pARC7) was a derivative of a dimer of p8V. The vector was linearized and a dT tail added to each end. Each dT tail of the linearized vector was used as a primer for the synthesis of first strand cDNA. The cDNA-mRNA hybrid was then dG tailed. This hybrid plasmid was then cut into half by BamHI restriction endonuclease. A linker with BamHI "sticky end" and a dC tail at the other end was used to join up the linearized hybrid plasmid. RNaseH, E. coli DNA polymerase I and E. coli DNA ligase were used to replace the mRNA with DNA and to covalently close the remaining nicks. This system generated full length cDNA with intact 5'-ends with the high efficiency of $> 10^5$ transformants per μg of mRNA (Alexander et al., 1984).

(iii) Insertion of double stranded cDNA into the cloning vector.

Apart from the vector-primer cDNA cloning system, the other methods of cDNA synthesis require the

double-ended cDNA to be inserted into the vector. For this step several different methods have been used. The double strand cDNA has been cloned as a blunt-ended fragment (Cochet et al., 1979) or after adding synthetic linkers containing a specific endonuclease recognition sequence (Heyneker et al., 1976; Bolivar and Backman, 1979). To overcome the problem of the double strand cDNA being cut during the cleaving of linkers by the restriction endonuclease, the double strand cDNA can be protected by site-specific methylation (Maniatis et al., 1978). In addition, the background of nonrecombinant plasmids caused by self-ligation of the vector can be easily overcome by dephosphorylation of the linearized vector.

The third method, which has been widely used, involves the addition of dG tails to the plasmid and complementary dC tails to the double strand cDNA (Villa-Komaroff et al., 1978; Rowekamp and Firtel, 1980; Land et al., 1981) or addition of dT tails to the plasmid and complementary dA tails to the cDNA (Wensink et al., 1974). However, it was found that the efficiency of cloning using poly dG-poly dC tails of 10-20 nucleotides in length was 10-100 fold higher than that using poly dA-poly dT tails of 50-100 nucleotides in length (Nelson and Brutlag, 1979). A reasonably high cloning efficiency, 100 to 200 recombinant transformants per ng of cDNA, can be

obtained by this method (Maeda, 1981).

(iv) Transformation of the hybrid plasmid into E. coli.

For a very low abundance mRNA a cDNA library may need to consist of 100,000 clones. The transformation efficiency of the hybrid plasmids, therefore, can govern the success of construction of a cDNA library. The most commonly used method has been calcium chloride treatment of the cells (Mandell and Higa, 1970). More recently, a high efficiency transformation method was developed (Hanahan, 1983) which gives 1×10^8 to 5×10^8 transformants per μg of plasmid DNA.

(v) Screening of the cDNA library.

The choice of methods used to screen the cDNA library for a specific gene is mainly dependent on the abundance of the mRNA and the available information concerning the gene or gene product.

(a) Hybridization-translation method.

This method is a positive screening method suitable for use with cDNA libraries made from an enriched mRNA source. The cloned cDNA is immobilized on a nitrocellulose filter and hybridized to mRNA in solution. After extensive washing, the mRNA is

released from the hybrid and translated either in cell-free protein-synthesizing system (Goldkerg et al., 1979; Parnes et al., 1981) or by microinjection into Xenopus laevis oocytes (Devos et al., 1982; Taniguchi et al., 1983). The appropriate protein can be detected by immunoprecipitation (Parnes et al., 1981; Lomedico et al., 1984) or by biological assays (Devos et al., 1982; Taniguchi et al., 1983).

(b) Hybrid-arrested translation method.

This is a negative selection method which is also suitable for cDNA libraries made from an enriched mRNA source. It relies on the observation that mRNA-DNA hybrid is not translated in an in vitro translation system (Paterson et al., 1977). The cDNA clone of interest is detected by the reduction of a translation product as a result of hybridization with the plasmid DNA. It is a rapid method because there was no step designed to remove unhybridized mRNA. However, this method is not well suited to short cDNA or those with AT rich sequences (Hentschel et al., 1979).

(c) Screening by specific hybridization with synthetic oligodeoxynucleotides.

The hybridization-translation or hybrid-arrested screening methods are tedious and time consuming. When a partial or complete amino acid sequence of the protein of interest is available, a very powerful

method is to use synthetic oligodeoxynucleotides based on the protein sequence as probes to screen a large cDNA library (Wallace et al., 1981b; Suggs et al., 1981). The nucleotide sequence of suitable synthetic oligodeoxynucleotides can be deduced from short protein sequences for areas rich in amino acids specified either by a single codon (e.g., methionine, AUG; tryptophan, UGG) or by two codons (e.g., histidine, CAU, CAC; phenylalanine, UUU, UUC; tyrosine, UAU, UAC). A mixture of oligodeoxynucleotides that represents all possible codon combinations can be chemically synthesized and used as a probe to screen the cDNA library by colony hybridization (Grunstein and Hogness, 1975). Using hybridization conditions of appropriate stringency only the perfectly matched duplex will be stable (Wallace et al., 1979; Wallace et al., 1981b). A human β_2 -microglobulin cDNA clone was isolated using this approach (Suggs et al., 1981).

(d) Differential hybridization.

Some genes are inducible by drugs, hormones, or heat shock. The mRNA preparations from non-induced and induced cells would contain many sequences in common but are different from each other in the presence and absence of a few species of interest. Thus the cDNA clone for an inducible gene can be isolated from a cDNA library constructed from mRNA extracted from the

induced cell population by screening with probes made from mRNA of induced and non-induced cells. Examples of inducible genes cloned in this way include the galactose-inducible genes of yeast (St. John and Davis, 1979) and human fibroblast interferon (Taniguchi et al., 1980). This procedure has also been used to identify cDNA clones of developmentally regulated mRNAs from Xenopus laevis (Dworkin and David, 1980), Dictyostelium discooidum (Williams and Lloyd, 1979; Rowekamp and Firtel, 1980) and sea urchins (Laskey et al., 1980).

(e) In-situ colony screening with labelled antibodies.

The method requires an antibody that can bind specifically to the protein of interest and involves the expression of the cDNA clones in bacterial cells. The double-strand cDNA is usually cloned within a structural gene present in the plasmid and under the control of a bacterial promoter. To ensure that a proportion of the cDNA will be joined in phase with the reading frame, the double-strand cDNA can be joined to the vector using the oligo(dG)-oligo(dC) tailing method or joined with linkers to a set of vectors that provide all 3 possible reading frames (Stanley and Luzio, 1984). This method does not require that a full-length clone be present in the cDNA library, since immunological detection techniques

require only that a polypeptide be expressed of sufficient length to constitute an antigenic determinant. Recently, some vectors were constructed for this in situ immunological screening method, like λ gt11, λ gt11-Amp3 (Kemp et al., 1983) and pEX1, pEX2, pEX3 (Stanley and Lugio, 1984).

(f) Directed expression of cDNA clones in mammalian cells and bacteria.

Recently several eukaryotic cloning vectors have been developed which can express full-length cDNAs in mammalian cells to give biologically active polypeptides (Gheysen and Fiers, 1982; Okayama and Berg, 1983). In theory the cDNA of interest can be identified in the cDNA library by screening transfected cells for the production of the protein of interest, or by gene complementation in a defective cell line (Jolly et al., 1983). Recently a mouse IL-2 cDNA clone was isolated by direct expression of the cDNA clones in monkey COS cells (Yokota et al., 1985). This is a very powerful direct screening approach for the cDNAs of rare mRNAs.

In addition, cDNAs cloning for mammalian proteins that are highly conserved during evolution can also be isolated by complementation of the appropriate E. coli mutant (Goddard et al., 1983; Chang et al., 1978).

Strategy for cloning murine interleukin-3.

It was necessary to establish a very sensitive assay for IL-3 mRNA since it was expected to be a low-abundance mRNA. This was feasible since there was a very sensitive biological assay for IL-3 using the IL-3 dependent line 32Dcl-23 (Ihle et al., 1982a). What was therefore required was a translation system which would yield biologically active IL-3 and this was achieved using Xenopus laevis oocytes (Chapter 2). Therefore, the hybridization-translation screening method could be used as the cloning strategy.

II. Materials.

Reagents

1. dATP (2'-Deoxy-adenosine-5'-triphosphate, disodium salt) was obtained from Pharmacia P-L Biochemical (No. 103985).
2. dTTP (Thymidine-5'-triphosphate, tetrasodium salt) was obtained from Pharmacia P-L Biochemical (No. 104272).
3. dCTP (2'-Deoxy-cytidine-5'-triphosphate, disodium salt) was obtained from Pharmacia P-L Biochemical (No. 104043).
4. dGTP (2'-Deoxy-guanosine-5'-triphosphate, disodium salt) was obtained from Pharmacia P-L Biochemical (No. 104108).
5. ddATP (2',3'-Dideoxyadenosine-5'-triphosphate, sodium), was obtained from Pharmacia P-L Biochemical (No. P-L 27-4819-XX).
6. ddCTP (2',3'-Dideoxycytidine-5'-triphosphate, sodium) was obtained from Pharmacia P-L Biochemical (No. P-L 27-4823-XX).
7. ddGTP (2',3'-Dideoxyguanosine-5'-triphosphate, sodium), was obtained from Pharmacia P-L Biochemical (No. P-L 27-4817-XX).
8. ddTTP (2',3'-Dideoxythymidine-5'-triphosphate, sodium), was obtained from Pharmacia P-L Biochemical (No. P-L 27-4831-XX).

9. [α - ^{32}P]dATP. (Deoxyadenosine 5'-[α - ^{32}P] triphosphate triethylammonium salt) was obtained from Amersham (No. PB10204).
10. [^3H]dCTP (Deoxy[1',2',5- ^3H] cytidine 5'-triphosphate ammonium salt), 51Ci/mole, was obtained from Amersham (No. TRK625).
11. [^3H]dGTP (Deoxy[8- ^3H] guanosine 5'-triphosphate, ammonium salt), 12.3Ci/mole, was obtained from Amersham (No. TRK350).
12. Oligo(dT₁₂₋₁₈), was obtained from Pharmacia P-L Biochemical (No. P-L 7858).
13. Calf thymus DNA, was obtained from Sigma (No. D8899).
14. TCA (Trichloroacetic acid), analytical reagent, was obtained from AJAX Chemicals (No. 558).
15. DTT (DL-Dithiothreitol) was obtained from Sigma (No. D-0632).
16. AMV reverse transcriptase, was obtained from Life Science Inc.
17. Terminal deoxynucleotidyl transferase, was obtained from Pharmacia P-L Biochemical (No. 0730).
18. Oligo(dG₁₀), was obtained from Pharmacia P-L Biochemical (No. P-L 7889).
19. Tetracycline, was obtained from Boehringer Mannheim (No. 15539).
20. Lysozyme, was obtained from Sigma (No. L-6876).

21. Ribonuclease A, was obtained from Sigma (No. R4875).
22. Ethidium bromide, was obtained from Sigma (No. E8751).
23. PIPES [Piperazine-N, N'-bis(2-ethanesulfonic acid)] was obtained from Calbiochem-Behring Corp. (No. 528132).
24. Formamide, puriss, was obtained from Fluka (No. 47670).
25. E. coli tRNA, was obtained from Boehringer Mannheim (No. 109541).
26. Calf intestinal alkaline phosphatase, was obtained from Sigma (No. P5521).
27. PEG6000 (polyethylene glycol 6000), laboratory reagent, was obtained from BDH Chemicals Australia Pty. Ltd. (No. 29577).
28. Silane (Methacrylic acid 3-Trimethoxy-Silylpropyl Ester), was obtained from Tokyo Kasei (No. M725).
29. Dimethyldichlorosilane LABCHEM, was obtained from AJAX Chemical Company (No. 664).
30. TEMED (N,N,N',N'-Tetramethylethylene-diamine), was obtained from BDH Chemical Ltd. (No. 30385).
31. Ammonium persulfate, laboratory reagent, was obtained from BDH Chemicals Ltd. (No. 27195).
32. [γ -³²P]ATP (Adenosine 5'-[γ -³²P] triphosphate, triethylammonium salt), 5000Ci/mmole, was obtained from Amersham (No. PB10128).

33. T4 polynucleotide kinase, was obtained from Pharmacia P-L Biochemicals (No. P-L 0734).
34. Salmon sperm DNA, was obtained from Sigma (No. D-1626).
35. Low melting point agarose, electrophoresis grade, was obtained from Bethesda Research Laboratories Inc. (No. 55174A).
36. Partisil-10SAX, was obtained from Watman (No. 4126-010).
37. Ampicillin, was obtained from Beecham Research Laboratories.
38. Ficoll 400, was obtained from Pharmacia Fine Chemicals. (No. 17-0400-01).
39. Polyvinyl-pyrrolidone (av. MW 360,000) was obtained from Sigma (No. DVP-360).
40. Bovine serum albumin (Bovine albumin powder, fraction V from bovine plasma) was obtained from Armour-Pharmaceutical Company Ltd.
41. Acrylamide, was obtained from BDH Chemicals Ltd. (No. 44299).
42. N,N'-Methylenebisacrylamide, was obtained from BDH Chemicals Ltd. (No. 15213).

Plasmids, bacteriophages and bacteria strains.

1. pAT153 (Twigg and Sherratt, 1980).
2. M13mp8 (Messing, 1981).
3. M13mp9 (Messing, 1981).
4. pBR322 (Bolivar et al., 1977).
5. E. coli HB101 (Bolivar and Backman, 1979).
6. JM103 (Messing, 1983).

III. Solutions.

1. Scintillation fluid: 2,5-diphenyloxazole in Xylol-Triton X-100 (2:1 vol/vol).
2. High-salt buffer: 100mM NaCl, 50mM Tris-HCl pH7.5, 10mM MgCl₂, 1mM 2-mercaptoethanol.
3. Medium-salt buffer; 50mM NaCl, 10mM Tris-HCl pH7.5, 10mM MgCl₂, 1mM 2-mercaptoethanol.
4. Low-salt buffer: 10mM Tris-HCl pH7.5, 10mM MgCl₂, 1mM 2-mercaptoethanol.
5. SmaI buffer: 20mM KCl, 10mM Tris-HCl pH8.0, 10mM MgCl₂, 1mM 2-mercaptoethanol.
6. LB medium: 10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl, 1 litre H₂O, pH7.5.
7. LB agar: 10g Bacto agar in 500ml LB medium.
8. Tetracycline agar: LB agar plus 25µg/ml tetracycline.
9. Ampicillin agar: LB agar plus 50µg/ml Ampicillin.
10. STET buffer: 8% sucrose, 5% Triton X-100, 50mM EDTA, 50mM Tris-HCl pH8.0.
11. BPB dye mix: 29gm sucrose, 0.09gm Bromophenol blue, 1.86gm EDTA in 100ml H₂O, pH8.0.
12. SSC: 0.15M NaCl, 0.015M Na citrate, pH7.
13. STE buffer: 10mM NaCl, 10mM Tris-HCl pH7.5, 1mM EDTA.
14. 56 medium: 10g (NH₄)₂SO₄, 1g MgSO₄, 30.5g NaH₂PO₄, 53g K₂HPO₄ in 1 litre H₂O.

15. 56 culture medium: 1 litre 56 medium with 0.02g $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.0001% FeSO_4 , 0.03M glucose, 0.0015mM Vitamin B1 and 0.5% Casamino acid.
16. YT medium: 8g tryptone, 5g yeast extract, 5g NaCl, 1 litre H_2O , pH7.5.
17. Tris-acetate buffer: 0.04M Tris-acetate 0.002M EDTA, pH7.8.
18. TBE buffer: 0.089M Tris-borate, 0.089M Boric acid, pH8.
19. Denhardt's solution: 0.1g Ficoll, 0.1g polyvinylpyrrolidone, 0.1g BSA (brovin serum albumin) in 10ml H_2O .
20. Annealing buffer: 10mM MgCl_2 , 10mM Tris-HCl pH8.5.
21. 40% acrylamide stock: 38g acrylamide, 2g bis-acrylamide in 100ml H_2O , deionized and millipore filtered.
22. Colony storage medium: LB medium with the following compounds (g/litre): K_2HPO_4 (6.3), KH_2PO_4 (1.8), Na Citrate (0.45), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.09), $(\text{NH}_4)_2\text{SO}_4$ (0.9), glycerol (44).
23. H agar: 10g bacto-agar, 10g tryptone, 8g NaCl in 1l. H_2O , pH7.5.
24. H top agar: 7g bacto-agar, 10g tryptone, 8g NaCl in 1l. H_2O , pH7.5.

IV. Methods.

3.1. Construction of the WEHI-3B cDNA library.

a. First strand cDNA synthesis.

Synthesis of first-strand cDNA was carried out in a reaction volume of 50 μ l containing 50mM Tris-HCl, pH8.3, 10mM MgCl₂, 140mM KCl, 30mM 2-mercaptoethanol, 200 μ M each of dATP, dTTP, dCTP, dGTP, 1 μ Ci [α -³²P] dATP (~ 3000 Ci/mmole), 5 μ g poly(A)⁺RNA, 5 μ g oligo(dT₁₂₋₁₈), and 50 units reverse transcriptase at 42°C for 1 hr.

Samples were taken out at 30 min and 60 min to check the extent of incorporation. 2 μ l of reaction mixture was mixed with 28 μ l 0.02M EDTA, pH8. To determine the total count, 10 μ l of the mixture was diluted with 10ml of 50% ethanol in a scintillation vial and the radioactivity determined by Cerenkov counting. To determine TCA precipitable counts a 10 μ l sample was added to 100 μ l of sonicated calf thymus DNA (500 μ g/ml in 10mM Tris-HCl, pH7.5, 0.1mM EDTA) in a 5ml disposable plastic tube. 5ml of ice-cold 10% TCA was added and chilled on ice for 15 min. The precipitate was collected by filtering the solution through a GF/C glass-fiber disc (Whatman) using a suction filter apparatus. The filter was washed six times with 5ml ice-cold 10% TCA followed by 5ml of 95% ethanol. The radioactivity of the filter was

determined in 10ml 50% ethanol by Cerenkov counting. The reaction was stopped by adding 1/10 volume (5 μ l) of 0.2M EDTA, pH8. 5 μ l of 5M NaCl and 1 μ l of 10% SDS were added and mixed well. The solution was then extracted with an equal volume of phenol, chloroform and isoamylalcohol mixture (50:48:2). 2.5 volumes of absolute ethanol was added to the aqueous phase and mixed well. It was kept in dry-ice ethanol bath for 1 hr and centrifuged in Eppendorf centrifuge for 30 min at 4°C. The pellet was washed with cold absolute ethanol and dried under vacuum.

b. Hydrolysis of the RNA.

The pellet was dissolved in 200 μ l of fresh 0.1M NaOH and incubated at 70°C for 20 min. The solution was then neutralized with 10 μ l of 1M Tris-HCl, pH7.5, and 20 μ l of 1M HCl. The first strand cDNA was then separated from the unincorporated dNTPs and the products of alkaline hydrolysis of the template by chromatography on a 5ml Sephadex G-75 column in 10mM Tris-HCl, pH7, 1mM EDTA, 100mM NaCl. 5 drops fractions were collected and the amount of radioactivity determined by Cerenkov counting. The fractions in the excluded volume that contained radioactivity were pooled. 1/10 volume of 5M NaCl and 2.5 volumes of absolute ethanol were added and the mixture kept in a dry-ice ethanol bath for 1 hr. It was centrifuged in an SS34 rotor at 20,000rpm, -15°C

for 30 min. The DNA pellet was washed with cold absolute ethanol and dried under vacuum.

c. dC tailing of the first-strand cDNA.

The dC tailing or dG tailing of DNA was performed according to the method of Michelson and Orkin (1982).

5 μ Ci of [³H] dCTP (51 Ci/mmole; ie 98 pmoles) was dried in a siliconized Eppendorf tube under vacuum. 10 μ l of 10X TdT buffer, 1 μ l of 10mM DTT (dithiothreitol), 1 μ l of 500mM dCTP, 5 μ l of 10mg/ml BSA (bovine serum albumin), 49 μ l of first-strand cDNA (16.4ng/ μ l in 10mM Tris-HCl, pH7.5 , 0.1mM EDTA) and 31.4 μ l of water were added to the dried [³H] dCTP and mixed well. The solution was equilibrated at 12°C for 5 min. A 1 μ l sample was withdrawn for the determination of the TCA precipitable counts (see below).

The reaction was started by adding 3.6 μ l of 15 units/ μ l terminal deoxynucleotidyl transferase and incubated at 12°C. 1 μ l samples were taken out at 15 min and 30 min to measure the % incorporation of dCTP. The 1 μ l samples were diluted in 29 μ l ice cold 0.02M EDTA, pH8. 10 μ l of the diluted sample was spotted on a GF/C glass-fiber filter and dried under a lamp and counted in 10ml of scintillation fluid for the determination of total counts. Another 10 μ l of the diluted sample was added to 100 μ l sonicated calf

thymus DNA (500 μ g/ml in 10mM Tris-HCl, pH7.5, 0.1mM EDTA) in a disposable plastic tube. 5ml ice cold 10% TCA was added and chilled in ice for 15 min. The precipitate was collected by filtering through a GF/C glass-fiber filter using a suction filtering apparatus. The filter was washed 6 times with ice-cold 10% TCA and once with 5ml of 95% ethanol. The filter was dried under a lamp and counted in 10ml of scintillation fluid.

The reaction mixture was temporarily chilled on ice to stop the reaction while checking the length of the added tail. The incubation was continued until the tail length reached about 15 residues per molecule of cDNA and the reaction was stopped by adding 0.1 volume of 0.2M EDTA, pH8, followed by rapid chilling on ice. The reaction mixture was immediately extracted with an equal volume of chloroform:isoamylalcohol (48:2). The organic phase was reextracted with 20 μ l 10mM Tris-HCl, pH7, 1mM EDTA, 100mM NaCl. 0.1 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol were added to the pooled aqueous phase and mixed well. The solution was kept in a dry-ice ethanol bath for 1 hr and centrifuged in an Eppendorf centrifuge for 30 min at 4 $^{\circ}$ C. The cDNA pellet was washed with cold absolute ethanol and dried under vacuum.

d. Double-stranded cDNA synthesis (Land et al., 1981).

The primer, oligo(dG₁₀) was first annealed with the dC tailed single-strand cDNA in a volume of a 65 μ l containing 535ng of dC tailed single-strand cDNA, 3 μ g of oligo(dG₁₀), 50mM Tris-HCl, pH8.3, 30mM KCl and 10mM MgCl₂. The annealing mixture was heated at 68°C for 5 min then cooled at 43°C for 15 min and finally cooled at 0°C for 2 min.

After annealing, 1.8 μ l of 1M Tris-HCl, pH8.3, 1.1 μ l of 1M KCl, 3.5 μ l of 0.1M MgCl₂, 1 μ l of 1M DTT, 8 μ l of dNTPs (2.5mM each), 2 μ l of [α -³²P]dATP (1 μ Ci/ μ l) and 16.7 μ l of water were added to the annealing mixture and mixed well. 1 μ l of the mixture was taken out to measure the background radioactivity as before. The reaction was started by adding 2 μ l of reverse transcriptase (15units/ μ l) and the reaction mixture incubated at 42°C. 1 μ l samples were taken out at 30 min and 60 min to check the percentage incorporation of label. After the maximum incorporation was reached (usually 1 hr) the reaction was stopped by adding 10 μ l of 0.2M EDTA, pH8, 10 μ l of 5M NaCl and 2 μ l of 10% SDS. It was then extracted with an equal volume of phenol, chloroform and isoamylalcohol mixture (50:48:2).

The double-strand cDNA in the aqueous phase was separated from the unincorporated dNTPs by

chromatography on a 5ml Sephadex G-75 column in 10mM Tris-HCl, pH7, 1mM EDTA, 100mM NaCl. Fractions in the excluded volume containing radioactivity were pooled. 1/10 volume of 3M NaCl, and 2.5 volumes of absolute ethanol were added and the mixture kept in a dry-ice ethanol bath for 1 hr. The precipitated cDNA was centrifuged in an Eppendorf centrifuge for 30 min at 4°C. The pellet was washed with cold absolute ethanol and dried under vacuum.

e. dC tailing of double-stranded cDNA.

5 μ Ci of [³H]dCTP (51Ci/mmole, ie 98 pmoles) was dried down in a siliconized Eppendorf tube under vacuum. 10 μ l of 10X TdT buffer, 1 μ l of 10mM DTT, 1 μ l of 500 μ M dCTP (ie. 500 pmoles), 5 μ l of 10mg/ml BSA, 50 μ l of double-strand cDNA (718ng) and 29.6 μ l of water were added to the dried [³H]dCTP and mixed well. It was equilibrated at 20°C for 5 min. A 1 μ l sample was taken out for checking the background radioactivity by acid precipitation as in Method 3.1c. The reaction was started by adding 3.2 μ l of 15 units/ μ l terminal deoxynucleotidyl transferase (~12 units/pmole of 3' end) and incubated at 20°C. After 20 min of incubation the reaction mixture was chilled on ice. A 1 μ l sample was taken out to determine the average number of residues added per 3' end as in Method 3.1c. When the tail length reached the required size, the reaction was topped by adding 10 μ l of 0.2M EDTA, pH8,

and the mixture was immediately extracted with an equal volume of chloroform:isoamylalcohol (48:2). The aqueous phase was chromatographed on a 5ml Sephadex G75 column in 10mM Tris-HCl, pH7, 1mM EDTA, 100mM NaCl. Fractions in the excluded volume containing radioactivity were pooled. 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol were added and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in an Eppendorf centrifuge for 30 min at 4°C. The DNA pellet was washed with cold absolute ethanol and dried under vacuum. It was finally resuspended in 10mM Tris-HCl, pH7.5, 0.25mM EDTA, 100mM NaCl.

f. dG tailing of linearized pAT153.

50µg of pAT153 DNA was digested in 150µl of 1X high salt buffer containing 250 units of PstI restriction endonuclease at 37°C for 3 hr. In order to ensure the complete digestion of pAT153, 10 times excess PstI restriction endonuclease was used in the digestion. After digestion the reaction mixture was extracted with an equal volume of phenol, chloroform and isoamyl alcohol mixture (50:48:2). 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volume of absolute ethanol were added and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in an Eppendorf centrifuge for 15 min at 4°C. The pellet was washed with cold absolute ethanol

and dried under vacuum. It was resuspended in water.

In the dG tailing of PstI linearized pAT153, two parallel experiments were carried out under the same conditions. One reaction was followed by the incorporation of [³H]dGTP and the other was not. To 98μl of 0.075μg/μl PstI digested pAT153, 20μl of 10X TdT buffer, 2μl of 10mM DTT, 2μl of 500μM dGTP, 10μl of 10mg/ml BSA and 60μl of water were added and mixed well. The reaction mixture was equilibrated at 20°C for 5 min. 8μl of 15 units/μl terminal deoxynucleotidyl transferase was added to the reaction mixture and mixed well. 180μl of the mixture was transferred to another Eppendorf tube containing 1μl of 81μM dGTP and incubated at 20°C. Another 20μl of the mixture was transferred to an Eppendorf tube containing 1μCi of dried [³H]dGTP (12.3Ci/mmole) and incubated at 20°C. After 5 min of reaction both tubes were chilled on ice. A 1μl sample was taken from the tube containing [³H]dGTP to determine the length of the dG tail as in Method 3.1c. When the dG tail reached the optimal length, the reaction without [³H]dGTP was stopped by adding 18μl of 0.2M EDTA, pH8. It was immediately extracted with an equal volume of chloroform:isoamylalcohol (48:2). 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol were added and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and

centrifuged in an Eppendorf centrifuge for 15 min at 4°C. The DNA pellet was washed with cold absolute ethanol and dried under vacuum. It was resuspended in 10mM Tris-HCl, pH7.5, 0.25mM EDTA, 100mM NaCl.

g. Annealing of the dC tailed double-stranded cDNA with dG tailed pAT153.

39.1ng of dG tailed pAT153 (0.016 pmoles) and 5.29ng of dC tailed double-stranded cDNA (ie. 0.016 pmoles, assuming an average size of 500 base pair) were mixed in 90µl of 10mM Tris-HCl, pH7.5, 0.25 mM EDTA, 100mM NaCl. The annealing mixture was heated at 70°C for 10 min in a water bath then the whole water bath was transferred into a polystyrene foam box. The annealing mixture was allowed to cool down very slowly to room temperature for 24 hr.

h. Transformation (Dagert and Ehrlich, 1979).

HB101 was grown to 1×10^8 cells/ml (ie. Klett value of 40) in 30ml LB medium at 37°C. The cells were collected by centrifugation in a Sorvall SS34 rotor at 5,000rpm, 4°C for 5 min. The cell pellet was resuspended in 30ml of cold 10mM Tris-HCl, pH7.3, 50mM CaCl₂. The cells were pelleted again and resuspended in 30ml of cold 10mM Tris-HCl, pH7.3, 50mM CaCl₂, and kept in ice for 30 min. They were pelleted again and resuspended in 1ml of cold 10mM Tris-HCl, pH7.3, 50mM CaCl₂.

After the annealing mixture had cooled to room temperature it was chilled in ice for 2 min. 310 μ l of CaCl₂ treated HB101 were added to 90 μ l of the annealing mixture and kept in ice for 1 hr with gentle mixing every 10 min. The cells were then heat shocked at 39° for 2 min. The transformed cells were plated (100 μ l/plate) on tetracycline plates and incubated at 37°C for 36 hr.

Pools of ten transformants were transferred to a sterilized vial with 1ml of colony storage medium (Gergen et al., 1979). The vials were incubated at 37°C for 24 hr and then stored at -20°C.

3.2. Screening the WEHI-3B cDNA library by the hybridization-translation assay (Parnes et al., 1981; Taniguchi et al., 1983).

a. Small scale preparation of plasmid DNA (Holmes and Quigley, 1981).

50 μ l of cells carrying the recombinant plasmids was inoculated into 40ml LB medium and grown at 37°C for 18 hr. Cells were harvested by centrifugation and resuspended in 2.8ml STET buffer at room temperature. 200 μ l of lysozyme (10mg/ml in 250mM Tris-HCl, pH8) was added to the cell suspension and kept in a boiling water bath for 2 min and centrifuged in a Sorvall SS34 rotor at 20,000rpm, 4°C, for 15 min. The supernatant

was transferred to another centrifuge tube and an equal volume of cold isopropanol added and mixed well. The tube was kept at -20°C for 30 min and centrifuged in a SS34 rotor at 10,000rpm, 0°C for 10 min. The pellet was washed with ether and dried under vacuum. The pellet was finally resuspended in 400 μl 10mM Tris-HCl, pH7.5, 0.1mM EDTA and insoluble material removed by centrifugation in an Eppendorf centrifuge for 5 min at 4°C . The clear supernatant was then stored in a siliconized Eppendorf tube at -20°C .

b. Binding of the plasmid DNA on nitrocellulose filters.

The plasmid DNA was first linearized with BamHI restriction endonuclease. 400 μl of crude plasmid DNA (equivalent to 40ml culture) was added to 400 μl of 2X high salt buffer containing 50 units of BamHI restriction enzyme and was incubated at 37°C for 20 hr. Digestion of the plasmid DNA was checked by gel electrophoresis. A 10 μl sample from the digestion mixture was treated with 1 μl RNAaseA (10mg/ml in 0.1M sodium acetate, pH5, pre-boiled for 10 min) at 37°C for 15 min. 5 μl of BPB dye mix was added to the RNAaseA treated samples and loaded to a 1% agarose gel containing ethidium bromide 0.5 $\mu\text{g}/\text{ml}$. Electrophoresis was carried out in Tris-acetate buffer containing 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide for 1 hr at 100mA constant current.

The BamHI digested crude plasmid DNA was extracted with an equal volume of phenol, chloroform and isoamyl alcohol mixture (50:48:2). 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol was added to the aqueous phase and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr, then centrifuged in an SS34 rotor at 20,000rpm, -15°C for 15 min. The DNA pellet was washed with cold absolute ethanol and dried under vacuum.

The dry DNA pellet was dissolved in 1ml of fresh 0.1M NaOH and kept at 70°C for 20 min. It was then diluted into 20ml of 2M NaCl (20ml for 2.5cm diam. filter, 10ml for 1.3cm diam.) and filtered through a nitrocellulose filter (Schleicher and Schuell BA85, 0.45mm pore) as shown in Figure 3.1. The filter was washed with 10ml 6XSSC and blotted dry between two sheets of sterilized Whatman No. 1 filter paper. It was then baked at 80°C in a vacuum oven for 2 hr.

c. Hybridization and elution of mRNA.

Hybridization was carried out in a sterilized and siliconized glass vial which was just larger than the nitrocellulose filters used.

Hybridization fluid (1ml for 2.5cm diam. filter, 250µl for 1.3cm diam.) containing 50% deionized formamide, 20mM PIPES, pH6.4, 0.4M NaCl, 2mM EDTA,

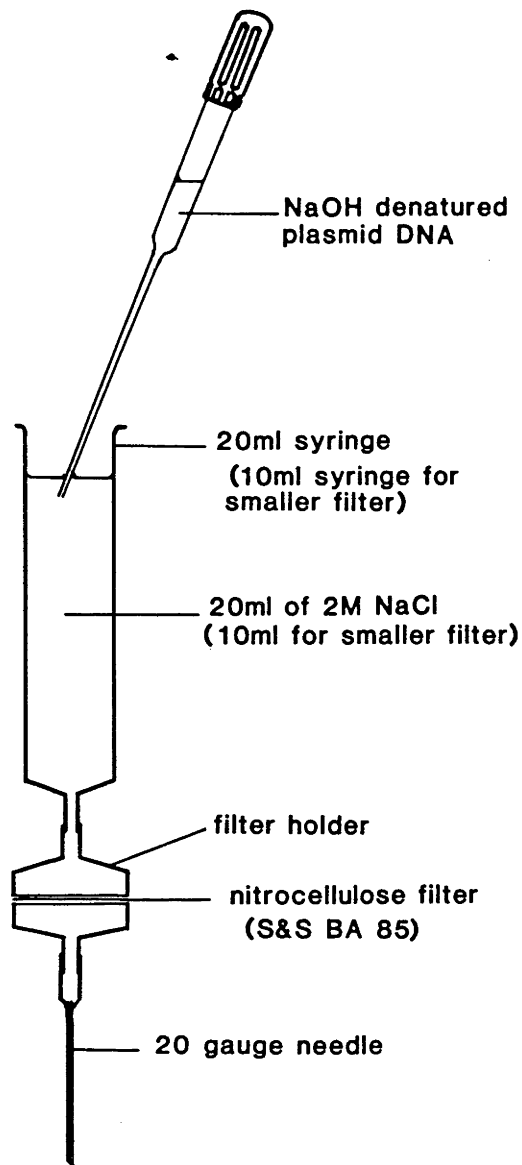


Figure 3.1 Binding of denatured plasmid DNA on to the nitrocellulose filter. The apparatus was set up without the gauge 20 needle. The nitrocellulose filter was wet by filtering 5ml of sterilized distilled water and then with 5ml of 2M NaCl. The outlet of the filter holder was sealed with parafilm and the apparatus was filled with 20ml (10ml for small filter) of 2M NaCl. 1ml (or 500 μ l for small filter) of NaOH denatured plasmid DNA was then added to the 2M NaCl and mixed with a pasteur pipette. The solution was filtered slowly (0.5ml/min) through the nitrocellulose filter by connecting a gauge 20 needle to the outlet of the filter holder. The filter was then washed with 10ml (5ml for small filter) 6XSSC.

0.1% SDS and 400µg of WEHI-3B mRNA was heated at 70°C for 10 min then chilled in ice. 10 filters were then immersed in the hybridization fluid. The glass vial was sealed and incubated at 37°C for 18 hr.

The filters were transferred to a 500ml sterilized and siliconized glass bottle and washed 3 times with 200ml (50ml for small filters) washing buffer I (10mM PIPES, pH6.4, 0.15M NaCl, 1mM EDTA, 0.2% SDS) at 65° by shaking and aspiration. They were then washed another 3 times with 50ml (10ml in a 20ml sterilized and siliconized glass bottle for small filters) washing buffer II (1mM PIPES, pH6.4, 10mM NaCl) at room temperature by vortex mixing and aspiration.

After washing, the filters were transferred separately into sterilized siliconized glass vials (or sterilized and siliconized Eppendorf tubes for small filters). 300µl of water and 4µg E. coli tRNA were added. The glass vials (or Eppendorf tubes) were placed in a boiling water bath for 90 seconds, quick frozen in a dry-ice ethanol bath, and thawed at room temperature. The 300µl water with eluted mRNA was transferred to a sterilized and siliconized Eppendorf tube. 12µl of 5M NaCl and 800µl of absolute ethanol were added and mixed well. The tubes were kept in a dry-ice ethanol bath for 2 hr and centrifuged in an Eppendorf centrifuge for 15 min at 4°C. The mRNA

pellet (hardly visible) was washed with cold absolute ethanol and dried under vacuum.

The mRNA pellet was resuspended in 5 μ l 10mM Tris-HCl, pH7.5, 0.1mM EDTA. The mRNA samples were assayed by translation in Xenopus laevis oocytes as described in Method 2.6.

3.3. Digestion of DNA with restriction endonucleases.

In a typical reaction 1 μ g of DNA was digested in 10 μ l containing 1X the appropriate digestion buffer and 1 unit of restriction enzyme at the appropriate temperature for 1 hr or longer in a sterilized siliconized Eppendorf tube. Since the manufacturers' units are based on the digestion of lambda DNA the number of units used was varied according number of restriction sites in the DNA to be digested.

The reaction was stopped by addition of 1/10 volume of 0.2M EDTA, pH8. If the DNA was to be analyzed directly by electrophoresis, 5 μ l of BPB dye mix was added, and the mixture loaded onto the gel. If the restricted DNA was to be purified, it was extracted once with phenol, chloroform and isoamylalcohol mixture (50:48:2) and precipitated with ethanol.

Most of the restriction enzymes were purchased from New England Biolabs. The restriction enzymes

were used at the temperature recommended by the manufacturers and in either high, medium or low salt buffer (see Solutions) according to their NaCl requirement. However, a particular SmaI buffer was used for the SmaI restriction enzyme (see Solutions).

3.4. Dephosphorylation of DNA fragments.

Dephosphorylation of DNA fragments was carried out in 50 μ l containing 50mM Tris-HCl, 1mM MgCl₂, 5 μ g (up to 10 μ g) linear DNA and 5 units of calf intestinal alkaline phosphatase. To dephosphorylate protruding 5' termini, the reaction mixture was incubated at 37°C for 30 min. Another 5 units of calf intestinal alkaline phosphatase was added and the reaction mixture incubated for a further 30 min.

To dephosphorylate DNA fragments with blunt ends or recessed 5' termini, the reaction mixture was incubated for 15 min at 37°C and 15 min at 56°C. Then another 5 units of calf intestinal alkaline phosphatase was added and the incubation repeated at both temperatures.

After incubation, 2 μ l of 0.5M EDTA, pH8, was added and the reaction mixture was extracted with phenol, chloroform and isoamylalcohol mixture (50:48:2). 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol were added and

mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in an Eppendorf centrifuge for 15 min at 4°C. The DNA pellet was washed with cold absolute ethanol and dried under vacuum. It was then resuspended in 10mM Tris-HCl, pH7.5, 0.1mM EDTA.

Since the calf intestinal alkaline phosphatase was supplied as a suspension in ammonium sulfate, the enzyme was pretreated before use. An aliquot of calf intestinal alkaline phosphatase was centrifuged in an Eppendorf centrifuge for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in sterile distilled water.

3.5 Ligation of DNA.

cDNA or DNA fragments were ligated to the vector DNA in a molar ratio of 1:1 in 66mM Tris-HCl, pH7.5, 6.6mM MgCl₂, 50µg/ml BSA, 1mM ATP, 20mM DTT and 1 unit of T4 DNA ligase. Reaction mixtures were incubated at 16°C, 15 hr for sticky ends and 20 hr for blunt ends. After ligation, the ligated DNA in the reaction mixture was directly used for transformation without any purification.

3.6 DNA sequencing by the chain termination method (Sanger et al., 1977; Sanger et al., 1982).

a. Preparation of the template.

A drop of overnight culture of JM101 was added to 25ml of 2XYT medium. A single plaque of the recombinant M13 phage was picked with a sterile toothpick and transferred to 1ml of the diluted JM101 culture in a 5ml disposable plastic tube. The tube was shaken at 37°C for 6-9 hr. The culture was centrifuged in an Eppendorf centrifuge for 5 min at 4°C. 800µl of the supernatant was carefully transferred to another Eppendorf tube. 200µl of 2.5M NaCl, 20% PEG 6000 was added, mixed well and kept at room temperature for 15 min. The mixture was centrifuged in an Eppendorf centrifuge for 5 min at 4°C. Supernatant was carefully removed and the tube was recentrifuged briefly and the supernatant again removed. The PEG pellet was resuspended in 100µl 10mM Tris-HCl, pH6.5, 0.1mM EDTA. It was extracted with 50µl of neutralized phenol. The aqueous phase was extracted with 0.5ml diethyl ether. 10µl of sodium acetate, pH5.5, and 250µl of absolute ethanol were added and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in an Eppendorf centrifuge for 15 min at 4°C. The pellet was washed with cold absolute ethanol and dried under vacuum. It was resuspended in 50µl of 10mM Tris-HCl,

pH7.5, 0.1mM EDTA.

b. Preparation of 6% acrylamide sequencing gel.

The acrylamide gels were covalently bonded to one of the glass plates to facilitate subsequent drying of the gel prior to autoradiography. Before setting up the gel apparatus, one glass plate was treated with Silane and the cut-out glass plate was siliconized. 5ml of Silane mixture (50 μ l Silane, 10ml ethanol and 330 μ l 10% acetic acid) was spread evenly on the clean back glass plate using a tissue and allowed to dry for 3 min. The plate was then washed with ethanol and dried. 5ml of siliconizing solution (2% of dimethyldichlorosilane in CCl₄) was spread evenly on the cut-out glass plate and dried for 3 min. The cut-out plate was then rinsed with distilled water. The Silane treated back glass plate was then taped together with the siliconized cut-out glass plate and appropriate spacers. For a 36.5cm x 20cm and 0.4mm thick gel, 40ml of urea-acrylamide mix was used. 19.2g of ultra pure urea was dissolved in 6ml 40% acrylamide stock, 2ml 10X TBE and 10ml distilled water at 37°C. The solution was made up to 40ml with distilled water. 40 μ l of TEMED was added and then filtered through a millipore filter (Schleicher and Schuell, BA85, 0.45mm). 240 μ l of 10% fresh ammonium persulfate was added to the solution and mixed well and the mixture immediately dispensed into the cavity

between the two glass plates using a 60ml syringe. The gel was allowed to set for 1 hr. The gel could be prepared a day before the DNA sequencing experiment.

c. Preparation of 2X N termination mix and N* mix.

The 2X N termination mix was prepared according to the following table:

<u>2X N termination mix</u>				
	2XdTTP mix (μ l)	2XdCTP mix (μ l)	2XdGTP mix (μ l)	2XdATP mix (μ l)
Content				
dTTP (1mM)	4	80	80	80
dCTP (1mM)	80	4	80	80
dGTP (1mM)	80	80	4	80
2 x dNTP buffer	20	20	20	20
ddTTP (0.8mM)	184	-	-	-
ddCTP (0.2mM)	-	184	-	-
ddGTP (0.2mM)	-	-	184	-
ddATP (0.1mM)	-	-	-	184
total	368	368	368	368

stored at -20°C

2 x dNTP buffer: 0.1mM Tris-HCl, pH8, 2mM EDTA.

The N* was made up just before use according to the following table:

content	<u>dN* mix</u>			
	dA* mix (μ l)	dG* mix (μ l)	dT* mix (μ l)	dC* mix (μ l)
2 x dATP mix	6	-	-	-
2 x dGTP mix	-	6	-	-
2 x dTTP mix	-	-	6	-
2 x dCTP mix	-	-	-	6
$[\alpha\text{-P}^{32}]\text{dATP}$ (10 μ ls/ μ l)	1.2	1.2	1.2	2.4
dATP(5mM)	1.2	1.2	1.2	1.2
sterilized distilled water	3.6	3.6	3.6	2.4
<hr/> total <hr/>	12	12	12	12

d. Sequencing reaction.

(i) Annealing.

5 μ l of M13 template, 1 μ l 10X annealing buffer, 1 μ l sequencing primer (5ng) and 3 μ l sterilized distilled water were mixed well and sealed in a capillary tube. The universal primer described by

Duckworth et al. (1981) ($5'$ GTAAAACGACGGCCAGT $3'$) was used in the sequencing reaction unless otherwise specified. The capillary was placed in a boiling water bath for 3 min then cooled to room temperature.

(ii) Preparation of the reaction matrix.

The sealed glass capillary containing the annealed primer-template was dried with tissue and cut open by a diamond pen. 0.3 μ l of Klenow (~1.5 units) was added to the capillary. The annealed template and enzyme were mixed well by sucking up and down and expelled to the bottom of a sterilized Eppendorf tube. 2 μ l of the enzyme and template mixture was distributed to the bottom of each sterilized capless Eppendorf tube using an automatic pipette with siliconized tips. The tubes were inclined at an angle of 45°. 2 μ l of N* mix (dA*, dG*, dC*, dT*) was added to the top of each corresponding Eppendorf tube.

(iii) Initiating the reaction.

The reactions were started by centrifuging the tube briefly to mix the drops and the tubes incubated at 37°C for 10 min.

(iv) dNTP chase.

The whole rack was taken out from the 37°C water bath and inclined at an angle of 45°. 2 μ l containing 0.5mM of each dNTPs was added to the top of each tube.

Drops were mixed by brief centrifugation and the tubes placed in a metal rack at 37°C for another 10 min.

(v) Termination of the reaction.

The metal rack was taken out from the 37°C water bath and inclined at an angle of 45°. 4µl of formamide dye mix was added to the top of each tube. The drops were mixed by centrifugation and the tubes placed in a boiling water bath for 5 min prior to loading on a 6% acrylamide sequencing gel.

e. Electrophoresis of the samples.

About 2.5µl of each reaction mixture was loaded onto the 6% acrylamide gel. Electrophoresis was initially carried out at 50 Watts constant power and then reduced to 30 Watts after the temperature of the gel reached to 57°C.

Electrophoresis was stopped when the bromophenol-blue dye reached the bottom of the gel. The two glass plates were separated leaving the acrylamide gel on the back glass plate. The gel was fixed in 10% acetic acid for 30 min with occasional mixing. The gel was then completely dried in a drying cupboard (~70°C) and cooled to room temperature. 'Scotch gard' (3M, Scotch gard) was sprayed evenly on top of the gel and dried for 5 min.

An X-ray film (Fuji, RX) was placed directly on

top of the dried gel and covered by a glass plate to ensure good contact. The film was exposed for several hours and developed in a Kodak X-OMAT processor.

The DNA sequence data was assembled and analyzed using the computer programs described by Standen (1980, 1982a).

3.7 Labelling restriction fragments using [α - 32 P] dATP.

DNA fragments with protruding 5' end were labelled with [α - 32 P]dATP using the Klenow fragment of E. coli DNA polymerase I.

In a typical reaction, 1 μ g of pBR322 DNA was digested in 20 μ l of 1X high salt buffer, with 5 units of HinfI restriction endonuclease at 37°C for 1 hr. The reaction mixture was diluted with 20 μ l of 1X low salt buffer. 1 μ l of [α - 32 P] dATP (10 μ Ci/ μ l) and 0.5 μ l of the Klenow fragment of E. coli DNA polymerase I (-2.5 units) were added and incubated at 37°C for 10 min. In some cases, the radioactive restriction fragments were separated from the unincorporated [α - 32 P]dATP by chromatography on a Sephadex G-75 column in 10mM Tris-HCl, pH7, 1mM EDTA, 100mM NaCl.

3.8 5'-end labelling of synthetic primers.

The reaction was carried out in 50 μ l containing 20 pmoles of the synthetic oligonucleotide. 20pmoles

of [γ - 32 P]dATP (Amersham 10218, 2pmoles/ μ l), 70mM Tris-HCl, pH7.6, 10mM MgCl₂, 5mM DTT and 5 units of T4 polynucleotide Kinase (P-L 0734) at 37°C for 1 hr. The reaction mixture was heated at 65°C for 10 min and then chromatographed on a 1ml Sephadex G-10 column in 10mM Tris-HCl, pH7, 1mM EDTA, 100mM NaCl, to separate the labelled primer from unincorporated label.

3.9. Colony hybridization.

a. Growth of the colonies on the nitrocellulose filter.

Colonies of recombinant plasmid were transferred to an agar plate containing the appropriate antibiotic in a grid pattern using sterile toothpicks. The plates were incubated at 37°C overnight and kept as a master plate. The nitrocellulose filter (Schleicher and Schuell, BA85, 401116) was marked with a black marker pen and lowered onto a second agar plate using 2 pairs of sterilized forceps and allowed to wet completely. The replica plating tool was sterilized by soaking in 70% ethanol for 5 min, in 100% ethanol briefly and the excess ethanol flamed. A print was made from the master plate to the agar plate with the nitrocellulose filter. The plate was then incubated at 37°C overnight and the master plate was stored at 4°C.

b. Binding the DNA to the filters.

The nitrocellulose filter was peeled off the agar plate and placed colony side up for 10 min on top of two sheets of 3MM paper, which were saturated with denaturing solution (0.5M fresh NaOH, 1.5M NaCl). The filter was dried briefly on a 3MM paper and placed colony side up for 10 min on top of two sheets of 3MM paper, which were saturated with neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, pH7.5). Excess liquid was removed from the filter on a 3MM paper. The filter was then placed colony side up for 10 min on top of 2 sheets of 3MM paper, which was saturated with 2XSSC. The filter was air dried, place between 2 sheets of 3MM paper and baked at 80°C in a vacuum oven for 2 hr. After baking, the filter could be stored in a vacuum dessicator for at least 2 weeks at room temperature prior to use.

c. Prehybridization.

The filters still placed between 2 sheets of 3MM paper were wet with 6XSSC. Filters were then separated from the paper, placed in 300ml prewashing solution (50mM Tris-HCl, pH8, 1M NaCl, 1mM EDTA, 0.1% SDS) and incubated at 42°C for 2 hr with shaking. The prewash step was to remove from the filter any absorbed medium, fragments of agarose or loose bacterial debris.

After the prewash, the filters were prehybridized in 10ml prehybridization fluid (5XSSC, 5X Denhardt's solution, 200µg/ml of denaturated sonicated Salmon sperm DNA) in a sealed plastic bag (up to 10 filters/bag) at the hybridization temperature for 4 hr.

The hybridization temperature was dependent on the probe size. For probe size larger than 200 base pairs the hybridization temperature was 65°C. For synthetic oligonucleotide probes the hybridization temperature was 5 to 10° below the melting temperature (T_m).

Approx. T_m of a synthetic primer = (2X no. of AT + 4X no. of GC)
(up to 20-mer)
(Singer-Sam et al., 1983)

d. Hybridization.

The prehybridization fluid was removed from the plastic bag. 5ml of hybridization fluid (5XSSC, 5X Denhardt's solution) was added to the bag. Double-stranded probes were denatured by heating at 100°C for 3 min then quick chilled on ice-water. The denatured probe was added to the hybridization fluid to a final concentration of 15-20ng/ml. The plastic bag was sealed carefully avoiding the trapping of any air bubbles inside the hybridization fluid. Hybridization was carried out at the particular hybridization temperature for 12 to 16 hr.

Time of hybridization = $3 \times \text{Cot}1/2$

number of hr to achieve $\text{Cot}1/2 = \frac{1}{X} \times \frac{Y}{5} \times \frac{Z}{10} \times 2$

where X = the weight of probe added (in μg)

Y = its complexity (in Kb)

Z = the volume of the reaction (in ml)

e. Washing of the filters.

The filters were washed briefly in 300ml 5XSSC at room temperature then washed in 1l. of 5XSSC at the hybridization temperature with agitation for 1 hr. The background was checked by the hand monitor (Mini-instrument Ltd. model 5-10E). If the count was over 50 counts per sec, the filters were washed for a longer time until the background dropped to ≤ 10 counts per sec. The filters were blotted dry between 3MM papers and covered with Saran wrap. The filters were then exposed to X-ray film (Kodak, XAR-5 or Fuji, RX) at -70°C with an intensifying screen overnight. The X-ray films were developed in a Kodak X-OMAT processor.

3.10. Isolation of DNA fragments from low melting point agarose gels.

DNA fragments were separated by gel electrophoresis using low melting agarose. Electrophoresis was carried out at 4°C in a cold room.

After electrophoresis the agarose gel was stained with 0.5 μ l ethidium bromide in fresh Tris-acetate buffer. The DNA bands were visualized using a 300mm UV transilluminator with a plastic sheet placed under the gel. The DNA band of interest was cut out from the gel with a sterilized scalpel blade and transferred into a graduated centrifuge tube. The gel volume was measured by the displacement method. An equal volume of 10mM Tris-HCl, pH7.5, 0.1mM EDTA was added to the gel fragment. The gel was melted at 70°C for 2 min with brief shaking. The solution was extracted 4 times with phenol and then 4 times with diethyl ether, 1/10 volume of 5M NaCl (and 1/100 volume of 1M MgCl₂ for the DNA fragment < 200bp), 2.5 volumes of absolute ethanol were added and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in a SS34 rotor at 20,000rpm, -15°C for 30 min. The DNA pellet was washed with cold absolute ethanol and dried under vacuum. It was finally resuspended in 10mM Tris-HCl, pH7.5, 0.1mM EDTA.

3.11. Deoxyoligonucleotide synthesis.

All the synthetic primers used in this thesis were prepared by Dr. I.G. Young and Mr. G. Mayo.

Deoxyoligonucleotides were synthesized by the phosphite phosphotriester route (Tanaka and Letsinger, 1982; McBride and Caruthers, 1983). After cleavage

from the silica supports the oligonucleotides were purified by HPLC using a Partisil-10SAX ion exchange column. The column was packed and equilibrated in buffer A (1mM KPO_4 , pH6.3, 60% formamide v/v). Samples were loaded and eluted using a linear gradient from 100% buffer A, 0% buffer B (0.3 M KPO_4 , pH6.3, 60% formamide v/v) to 30% buffer A, 70% buffer B, at a flow rate of 1ml/min. The elution was monitored by measuring the absorbance at 270nm. Peak fractions were pooled, diluted 1 in 2 in buffer A, reloaded on the column, and eluted using a steep linear gradient from about 20% to 70% of buffer B. The peak fraction was collected and desalted using a Sephadex G25 column. 1.5ml fractions were collected and absorbance at 270nm was measured. The peak was collected and evaporated to dryness by rotary evaporation. The sample was finally resuspended in 0.1ml 10mM Tris-HCl, pH7.5, 0.1mM EDTA. All glassware was siliconized and all plastic apparatus was sterilized.

3.12. Size-fractionation of DNA on Sepharose CL-4B column.

A Sepharose CL-4B column was prepared in a disposable 1ml plastic pipette plugged with sterile siliconized glass wool. The column was equilibrated with 10mM Tris-HCl, pH8, 1mM EDTA, 0.3M NaCl.

DNA sample (about 60 μ l) was applied to the

column. After the DNA sample had completely run into the column, the reservoir was topped up with the above buffer. A 6 drop fraction was collected for the first fraction and then thirty 1 drop fractions were collected. The radioactivity of each fraction was measured by Cerenkov counting of the whole tube inside a scintillation vial.

The same column was calibrated using as size marker HinfI restriction fragments derived from PBR322 and labelled with [α -³²P]dATP. The fractions were collected in the same way as before and electrophoresed on a 1.4% agarose gel. The gel was dried on a glass plate and covered with Saran wrap. The gel was then exposed to X-ray film (Kodak XAR-5) at room temperature for 5 hr. The X-ray film was developed using a Kodak X-OMAT processor.

V. Results.

1. Preparation of WEHI-3B cDNA library.

The myelomonocytic leukemia line WEHI-3B (Warner et al., 1969) was chosen as the source of mRNA which was prepared from the WEHI-3B cells by the guanidine thiocyanate method. The mRNA preparation was purified by chromatography on oligo(dT)-cellulose and then fractionated on a sucrose density gradient. The fractions were assayed by microinjection into Xenopus oocytes. RNA from the peak fraction was used as template to synthesize double-stranded cDNA.

a. First-strand cDNA synthesis.

4.6 μ g of IL-3 mRNA from fraction 8 of the sucrose density gradient was used as template to synthesize the first-strand cDNA using oligo(dT₁₂₋₁₈) as primer. The percentage of incorporation of dNTPs reached the maximum of 10.3% after 30 min reaction (Table 3.1) and it was calculated that 1.35 μ g of first-strand cDNA was synthesized.

b. Hydrolysis of RNA.

The RNA was hydrolyzed in 200 μ l of freshly prepared 0.1M NaOH at 70°C for 20 min. It was then neutralized and the cDNA separated from the unincorporated dNTPs and the products of alkaline hydrolysis by chromatography on a Sephadex G-75

Table 3.1 First-strand cDNA synthesis

Time of reaction	% of incorporation of [³² P]dATP	cDNA synthesised	
30 min	10.3	1.35μg	29%
60 min	10.3	1.35μg	29%

First strand cDNA was synthesized from partially purified IL-3 mRNA as described in Method 3.1a. 1μl of sample was taken out after 30 min and 60 min of reaction and assayed for the incorporation of [α -³²P]dATP by TCA precipitation.

column. The cDNA was then ethanol precipitated giving a recovery of 820ng of first-strand cDNA.

c. dC tailing of the first-strand dNA.

The method of Land et al. (1981) was used for second strand synthesis and cloning in order to try and obtain a cDNA carrying a complete copy of the original mRNA. 804ng of single-strand cDNA was used as a template for dC tailing using terminal deoxynucleotidyl transferase. It was assumed that the average size of the cDNA was 500 base pairs long. The enzyme to substrate ratio used was about 11 units per pmole of 3' ends. After 30 min the length of the dC tail | reached an average of 11.1 residues per 3' end of cDNA (Table 3.2). About 590ng cDNA was recovered after the tailing reaction.

d. double-strand cDNA synthesis.

Oligo(dG₁₀) was hybridized to the dC tailed single-strand cDNA and second strand synthesis carried out using reverse transcriptase. The percentage of dNTPs incorporated reached a maximum after 30 min and about 630ng of second-strand cDNA were synthesized. After phenol extraction and chromatography on Sephadex G75 about 718ng of double-stranded cDNA was recovered.

e. dC tailing of double-strand cDNA.

The cDNA was then tailed with dC residues using

Table 3.2 dC tailing of the first-strand cDNA

Reaction time	% of [³ H]dCTP incorporated	pmoles of dCTP incorporated (minus background)	number of dC residues per 3' end
0 min	1.5(background)	-	-
15 min	5.7	25.0	5.1
30 min	10.6	54.2	11.1

The dC tailing reaction was carried out using terminal transferase as described in Method 3.1c.

terminal deoxynucleotidyl transferase. After 20 min the average size of dC tail was about 11 residues per 3' end of double-stranded cDNA. After chloroform extraction and chromatography on Sephadex G-75 about 464ng of dC tailed double-stranded cDNA was recovered.

e. dG tailing of pAT153.

pAT153 was converted into a linear form by digestion with PstI restriction endonuclease. 7.4µg of the linearized pAT153 (i.e. 6 pmoles of 3' ends) was tailed with dG residues using terminal deoxynucleotidyl transferase. A parallel experiment was conducted using [³H] dGTP to determine the length of the dG tails. After 5 min of reaction the length of the dG tail was about 12 residues per 3' end and the reaction was terminated by the addition of EDTA. Following chloroform extraction the dG tailed vector was recovered by ethanol precipitation.

f. Annealing and transformation.

The dC tailed double-strand cDNA and dG tailed pAT153 were annealed in a molar ratio of 1:1 and transformed into HB101 (Method 3.1g and h). In the transformation, three different heat shock temperatures were tested (37°C, 39°C and 42°C). The transformation efficiency was almost the same using heat shock temperatures of 39°C and 42°C but was very low using 37°C (Table 3.3). Most of the

Table 3.3 Transformation efficiency at different heat shock temperatures

heat shock temperature	transformation efficiency (transformants/ng of cDNA)
37°C	5.7
39°C	66.2
42°C	61.9

dC tailed double-strand cDNA was annealed to dG tailed PstI linearized pAT153 as in Method 3.1g. To each 90µl of annealed mixture, 310µl of CaCl₂ treated HB101 was added. After standing on ice for 60 min with gentle mixing every 10 min, the cells were heat shocked at 37°C, 39°C or 42°C. The transformed cells were then plated on agar plates containing tetracycline. Number of transformants were counted after incubation at 37°C for 36 hr.

transformation experiments were therefore carried out using 39°C. The average transformation efficiency was 65 transformants per ng of cDNA. The transformants (6720) were grown as a pool of ten colonies in glycerol broth (colony storage medium) and stored at -20°C. A small sample of 57 individual transformants were tested for sensitivity to ampicillin. About 90% were sensitive indicating that they contained cDNA inserts.

2. Screening the WEHI-3B cDNA library by hybridization-translation .

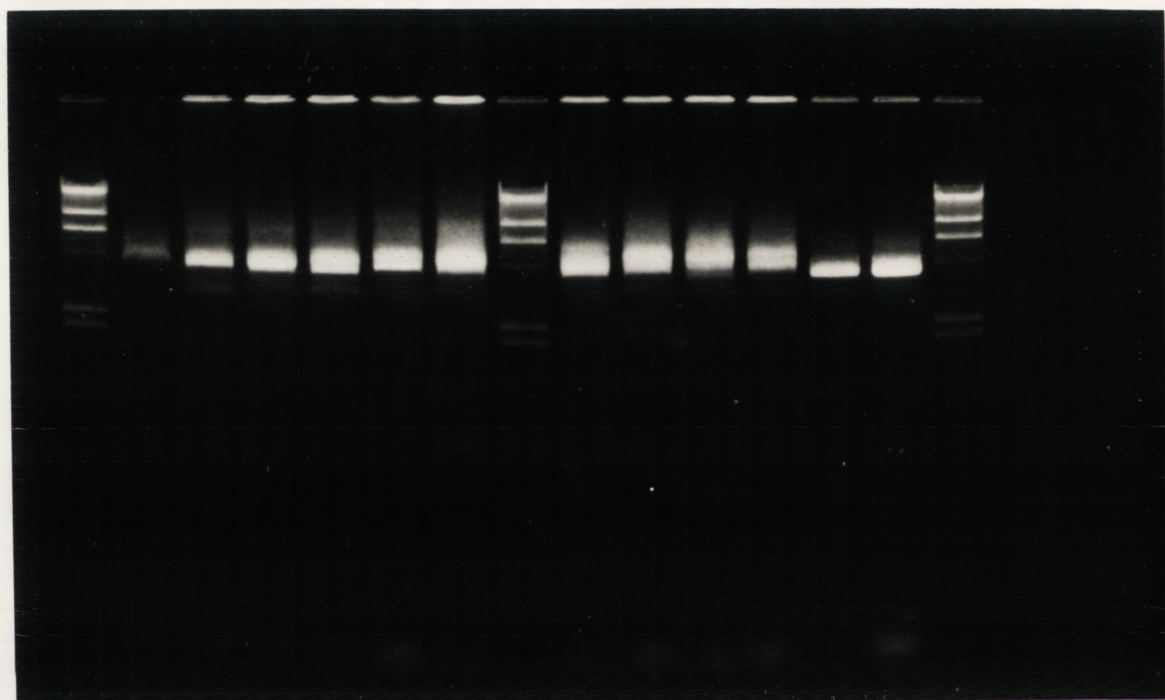
a. Preparation of plasmid DNA.

Recombinant plasmids were grown in pools of 10 on a small scale and crude plasmid DNAs were prepared by the method of Holmes and Quigley (1981) generally yielding about 30µg of plasmid DNA per 40ml of culture.

The plasmid DNA was linearized using BamHI restriction endonuclease and checked by electrophoresis on 1% agarose gel (Figure 3.2). After phenol extraction and ethanol precipitation the DNA preparations were treated with 0.1M NaOH at 70°C for 20 min to hydrolyse contaminating RNA.

Figure 3.2 Plasmid DNAs prepared from hybrid plasmid pools. Plasmid DNAs were prepared on a small scale, digested with BamHI restriction endonuclease and checked by gel electrophoresis as described in Method 3.2b. Lanes 1, 8 and 15, λ -HindIII standards; lanes 2-7 and 9-14, plasmid DNAs. The direction of electrophoresis is from top to bottom.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



b. Binding of plasmid DNA to nitrocellulose.

Following alkaline hydrolysis to DNA preparations were diluted with 2M NaCl giving a final pH of about 9 and slowly passed through 2.5cm diameter nitrocellulose filters to allow binding (Figure 3.1). The filters were then washed with 10ml 6XSSC, blotted dry and baked at 80°C in a vacuum oven for 2 hr.

The binding efficiency was studied by adding [α -³²P] dATP labelled linear pBR322 DNA to the crude plasmid DNA prior to hydrolysis of the RNA. The binding efficiency for the procedure above was 72%. The binding experiments established that the efficiency was much lower if protein was not removed by phenol extraction, or if the phenol itself was not removed by ethanol precipitation and washing. In addition, the binding efficiency reduced to about half if the DNA preparation was neutralized after alkaline hydrolysis.

c. Hybridization-translation.

The recombinant plasmids were firstly screened by the hybridization-translation assay in pools of 10. 10 nitrocellulose filters each containing plasmid DNA from 10 recombinant clones were used in each hybridization-translation assay. Therefore 100 different clones could be screened in each experiment. The 10 filters were hybridized with 400 μ g of WEHI-3B

mRNA and the bound mRNA subsequently eluted and recovered by ethanol precipitation using 4 μ g of E. coli tRNA as carrier. The mRNAs were then translated in oocytes (Method 2.6). The E. coli tRNA does not interfere with translation of WEHI-3B mRNA in Xenopus laevis oocytes.

One positive pool of 10 clones was detected among the screening of 45 pools. This positive pool was verified by repeating the hybridization-translation assay 3 times. The activity observed in translates derived from this positive pool was quite low but was consistently 2 to 3 times above the background and the values of the other negative clone pools.

DNA was therefore prepared from the individual clones of the positive pool and the hybridization-translation assay repeated. In screening the individual clones, half the amount of plasmid DNA (~15 μ g) was used and bound to 1.3cm diameter nitrocellulose filters. 10 of these small filters were hybridized with 100 μ g of WEHI-3B mRNA in 250 μ l of hybridization fluid in a small glass vial. From the hybridization-translation assay one of the ten individual clones (pILM1) was clearly positive (Table 3.4). The IL-3 activity detected showed the same titration curve as oocyte translates of WEHI-3B mRNA (Figure 3.3).

Table 3.4 mRNA hybridization translation assay for detection of IL-3 cDNA clones

DNA sample	IL-3 activity (units per ml)
1	< 1
2	< 1
3	< 1
4	< 1
5	< 1
6 (pILM1)	121, 106, 139
7	< 1
8	< 1
9	< 1
10	< 1

DNA samples (~15 μ g) were prepared from each clone by the rapid boiling method and were bound to the nitrocellulose filters as described in Method 3.2. The 10 filters were hybridized with 100 μ g of WEHI-3B mRNA and the bound mRNA was eluted as described in Method 3.2c. The eluted mRNA was translated in oocyte as in Method 2.6. A unit of IL-3 activity is that required to give 50% of the maximum incorporation of ³H-thymidine in the assay.

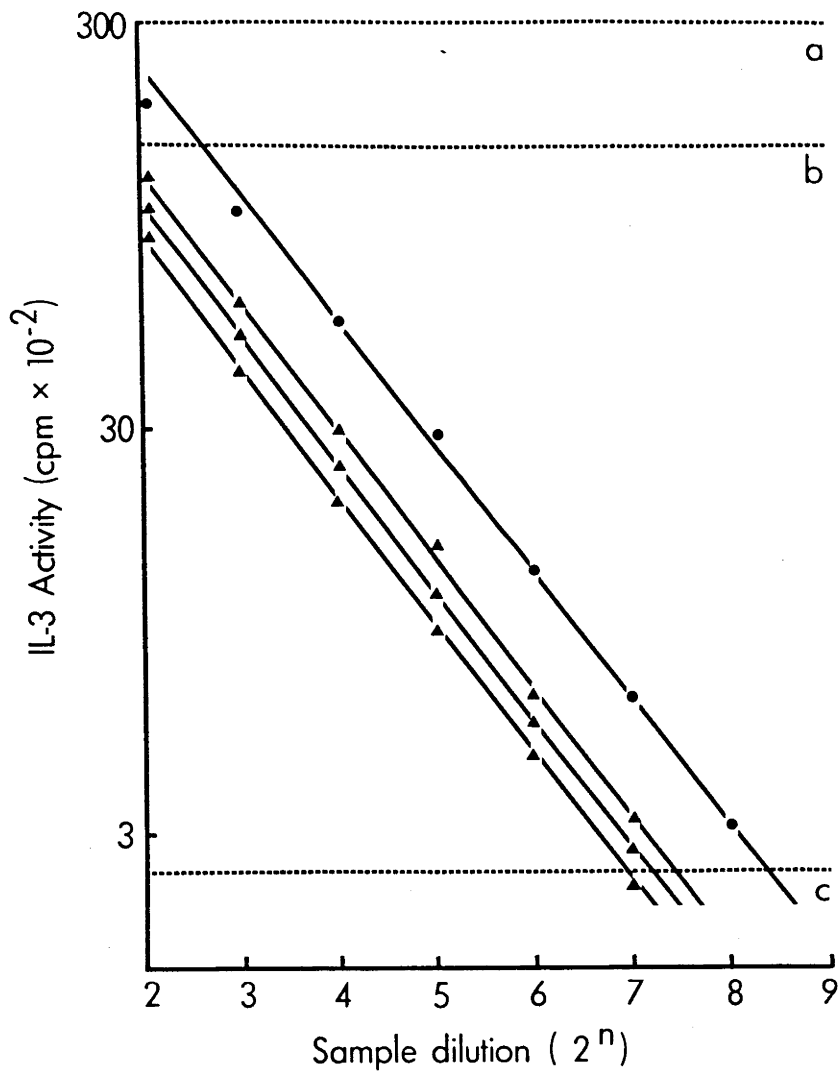


Figure 3.3. Titration curves for the IL-3 activity present in oocyte translates. \blacktriangle , Translates of mRNA hybridizing to pILM1; \bullet , translates of WEHI-3B mRNA. Twofold dilutions of the oocyte translates were made in microtitre trays and the IL-3 activity present was measured using the IL-3 dependent cell line 32D c1-23 as described in Method 2.7. The stimulation of growth of the indicator cells is expressed in terms of ^3H -thymidine incorporation. The dotted lines designated a, b and c represent incorporations of 100%, 50% and background plus three standard deviations respectively.

3. Characterization of pILM1.

a. Size of pILM1 cDNA insert.

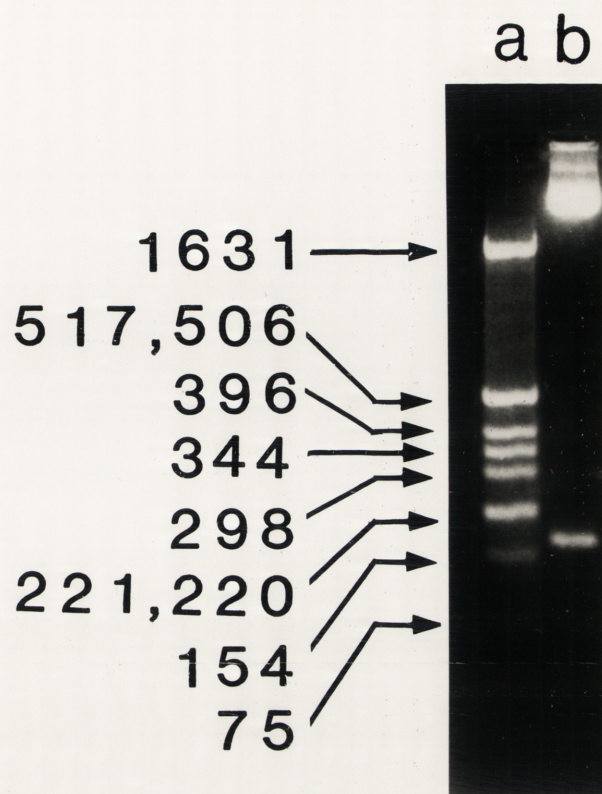
Since even short cDNA clones would be active in the hybridization-translation assay, the cDNA used to construct the library was not size fractionated and was expected to contain some short cDNA. Indeed the size of the pILM1 cDNA insert was found to be about 160 to 170 base pairs (Figure 3.4).

b. subcloning of the pILM1 cDNA insert into M13mp8 and M13mp9.

pILM1 plasmid DNA was digested with PstI restriction endonuclease and the mixture fractionated by electrophoresis on a low melting agarose gel. The gel was stained with ethidium bromide and the insert DNA was recovered by phenol extraction and ethanol precipitation.

M13mp8 (or M13mp9) was completely digested with PstI restriction endonuclease and dephosphorylated with calf intestinal alkaline phosphatase (Method 3.4). The pILM1 cDNA insert was ligated with the M13mp8 (or M13mp9) using T4 DNA ligase and transfected into CaCl₂ treated competent cells of JM103 (Method 2.1h). The cells were plated on H top agar containing X-gal and IPTG so that the recombinant phage (colorless plaques) could be distinguished from the parental phage (blue plaques).

Figure 3.4 Size of the cDNA insert in pILM1. 10 μ g of pILM1 plasmid DNA was digested with 30 units of PstI restriction endonuclease in 30 μ l of 1X high salt buffer at 37 $^{\circ}$ C for 1 hr. It was treated with 20 μ g of RNAaseA at 37 $^{\circ}$ c for 15 min before being loaded to a 2.5% agarose gel. The gel was electrophoresis at 70mA for 3.5 hr and then was stained with 0.5 μ g/ml ethidium bromide and photographed. Lanes (a) HinfI-digested pBR322; (b) PstI-digested pILM1. The direction of electrophoresis is from top to bottom. The sizes of standards are indicated in base pairs.



c. Nucleotide sequence analysis.

The nucleotide sequence analysis was carried out in collaboration with Dr. H.D. Campbell. Single stranded DNA was prepared from the templates of M13mp8 and M13mp9 recombinants and the inserts sequenced (Method 3.6) using the chain termination method.

This yielded the sequence of both strands of the cDNA insert in pILM1 (Figure 3.5). The cDNA insert in pILM1 was quite short. It was only 139 base pairs excluding the G-C tails. There were 12 G-C pairs at one end at 19 G-C pairs at the other end. Since the 3' end of the first-strand cDNA was dC tailed twice in making the cDNA library, the end with the longer G-C tail should represent the 5'-end of the original mRNA.

4. Construction of the second cDNA library.

To isolate a clone carrying the entire IL-3 coding sequence, a second cDNA library was prepared, but in this case the double-stranded cDNA was size-fractionated using Sepharose CL-4B to enrich for cDNA fragments larger than 500 base pairs.

2.9 μ g of WEHI-3B mRNA from the fraction 8 of the sucrose gradient (see above) was used as a template to synthesize the first-strand cDNA. 665ng of first-strand cDNA was synthesized (i.e. 22.9%) after 60 min

5' G₍₁₉₎ AACGAGACAATGGTTCTTGCCAGCTCTACCACCAGCATCCACACCATGCTGCTCCTGCTCCT
30 40 50 60 70 80

GATGCTCTTCCACCTGGGACTCCAAGCTTCAATCAGTGGCCGGGATACCCACCGTTTAAC₍₁₂₎ 3'
90 100 110 120 130

Figure 3.5 Nucleotide sequence of the cDNA insert in pILM1. The cDNA insert in pILM1 was subcloned into the PstI site of M13mp8 and M13mp9. DNA sequencing was carried out by the chain termination method.

of reaction.

After hydrolysis of the mRNA template and purification of the first-strand cDNA on a Sephadex G-75 column (Method 3.1b). 380ng of single-strand cDNA was recovered.

A homopolymeric dC tail was added to the 3' end of the first single-strand cDNA (Method 3.1c). After 45 min of reaction, the tail length reached an average of 11.6 residues per 3' end of the cDNA, assuming the average size of the cDNA to be 500 base pairs. 311ng of dC tailed first single-strand cDNA was recovered after chloroform extraction and ethanol precipitation.

The second-strand of the cDNA was synthesized using Oligo(dG₁₀) as primer yielding 219ng of the second-strand cDNA from 311ng of the dC tailed template. 310ng of the double-stranded cDNA was recovered after phenol extraction, chromatography on a Sephadex G-75 column and ethanol precipitation.

This was then used as template for the dC tailing reaction and after 30 min of reaction, it was found that the dC tail had reached an average size of 15.4 residues per 3' end of the cDNA. After phenol extraction, the sample was chromatographed on a Sepharose CL-4B column and 30 fractions were collected (Figure 3.6).

The same column was calibrated using as size

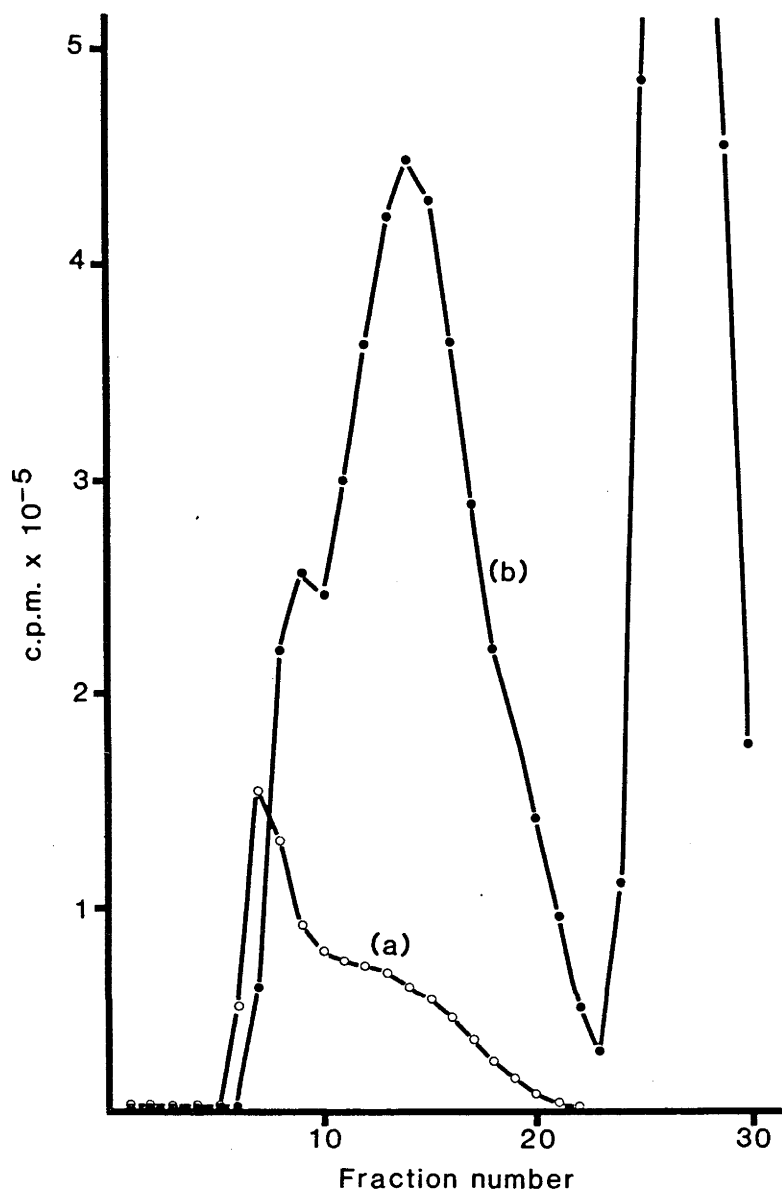


Figure 3.6 Size fractionation of the dC-tailed double stranded cDNA on a Sepharose CL-4B column. (a) Column profile for size fractionation of [³²P]-labelled double stranded cDNA (Method 3.11). (b) Column profile for size fractionation of [³²P]-labelled HinfI digested pBR322 size standards (Method 3.7). The individual fractions from this separation were analyzed by agarose gel electrophoresis (see Figure 3.7).

markers HinfI fragments derived from pBR322 and labelled with [α - 32 P]dATP. The fractions were collected and electrophoresed on a 1.4% agarose gel (Figure 3.7). The dC tailed double-stranded cDNA from fractions 5 to 8 which were expected to contain cDNA fragments of sufficient size were pooled and ethanol precipitated. 57ng of DNA was recovered, annealed with dG tailed pAT153 and transformed into HB101. Approximately 32 transformants per ng cDNA were obtained and 92% of these were ampicillin sensitive.

5. Screening of the second cDNA library by colony hybridization.

An oligonucleotide primer ($5'$ TTGAAGCTTGGAG-TCCCAGGT $3'$) corresponding to nucleotides 92-112 of the short cDNA insert of pILM1 was synthesized and labelled using [γ - 32 P] dATP and polynucleotide kinase (Method 3.8). The labelled primer with a specific activity of 7.6×10^8 cpm/ μ g, was used to examine the remaining clones in the first cDNA library and those in the second cDNA library by colony hybridization at 55°C (Method 3.9).

No other clones containing this sequence were detected from the first library. One clone, pILM3, in the second library hybridized strongly to the labelled primer (Figure 3.8).

Figure 3.7 Autoradiograph of the calibration of the Sepharose CL-4B column with [^{32}P]-labelled size markers. [^{32}P]-labelled HinfI digested pBR322 fragments were size-fractionated on a Sepharose CL-4B column (see Figure 3.6) and the fractions analyzed by electrophoresis on a 1.4% agarose gel. The gel was dried and autoradiographed. The lane numberings correspond with the fraction numbers in Figure 3.6(b). The direction of electrophoresis is from top to bottom.

pBR322/
Hinf I

Fractions

7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

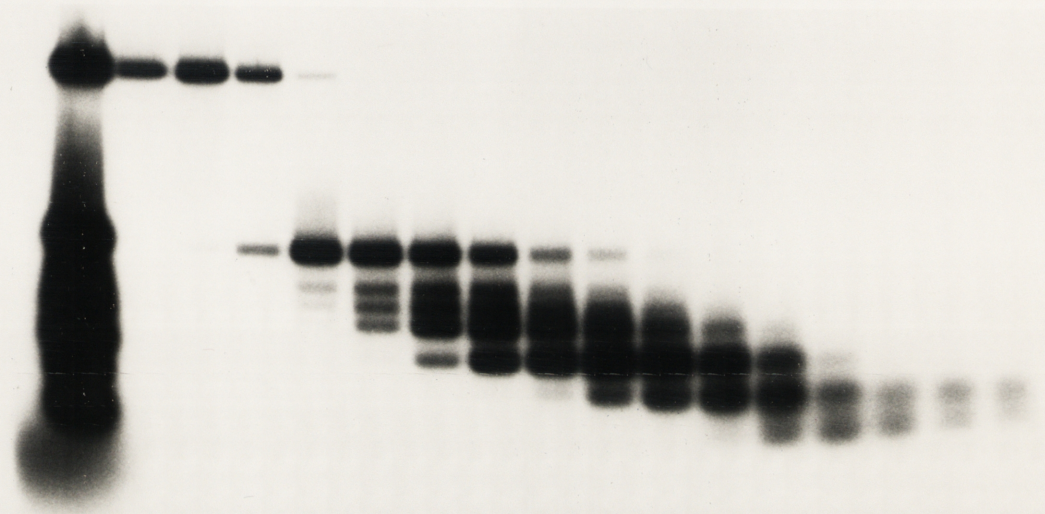
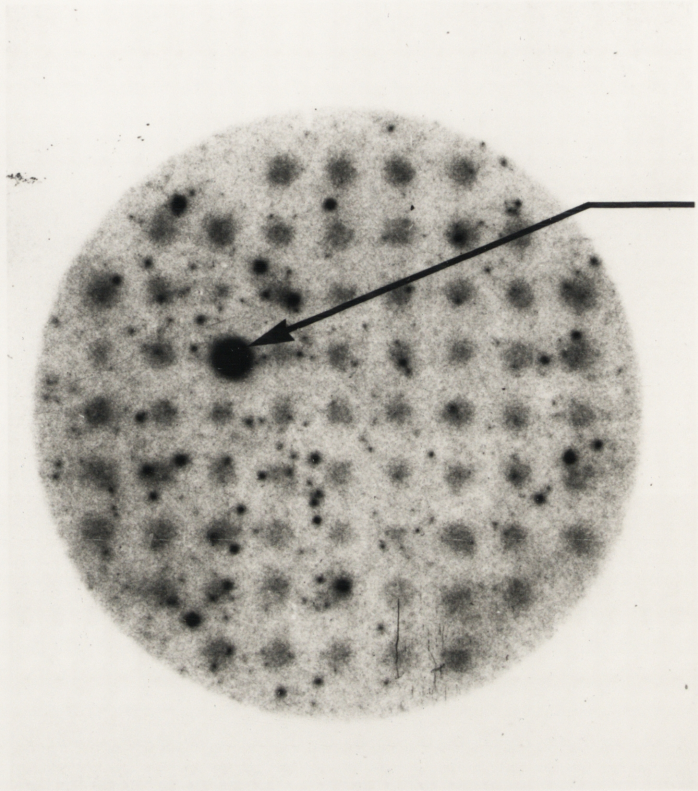


Figure 3.8 Autoradiograph of colony screening, of IL-3 cDNA library. The oligonucleotide primer (⁵TTGAAG-CTTGAGTCCCAGGT³) complementary to portion of the IL-3 cDNA sequence of pILM1 was labelled using [γ -³²P] ATP and T4 polynucleotide kinase (Method 3.8). The labelled probe had a specific activity of 7.6×10^8 cpm/ μ g was used for screening the IL-3 cDNA library by colony hybridization as described in Method 3.9. A positive clone (pILM3) is indicated.



pILM3

6. Characterization of pILM3.

Plasmid DNA was prepared from pILM3 on a small scale and a partial restriction map prepared (Method 3.3). As shown in Figure 3.9, one of the PstI cloning sites was lost. The cDNA insert was about 600 base pairs long with one site for both HindIII and BamHI restriction endonucleases. There were no PvuI, EcoRI or PstI sites in the cDNA insert.

7. Nucleotide sequence of the cDNA insert in pILM3.

The DNA sequence of clone pILM3 was determined on both strands using the chain termination method (Method 3.6) after subcloning into M13mp8 and 9. The sequencing strategy is given in Figure 3.10a. The subcloning was complicated by the fact that one of the PstI sites flanking the cDNA insert had been lost due to exonucleolytic removal of one nucleotide during the G tailing of the vector.

First the 2.8 Kb BamHI fragment from pILM3 was isolated using low melting point agarose gel electrophoresis (Method 3.10) and subcloned into M13mp9. The sequence of the cDNA from the BamHI site to the G-C tail was determined by the chain termination method (Method 3.6). An oligonucleotide primer, P2 ($5'$ TAGATAAATTGATAAGA $3'$) was synthesized (Method 3.11) and used with the M13 templates to

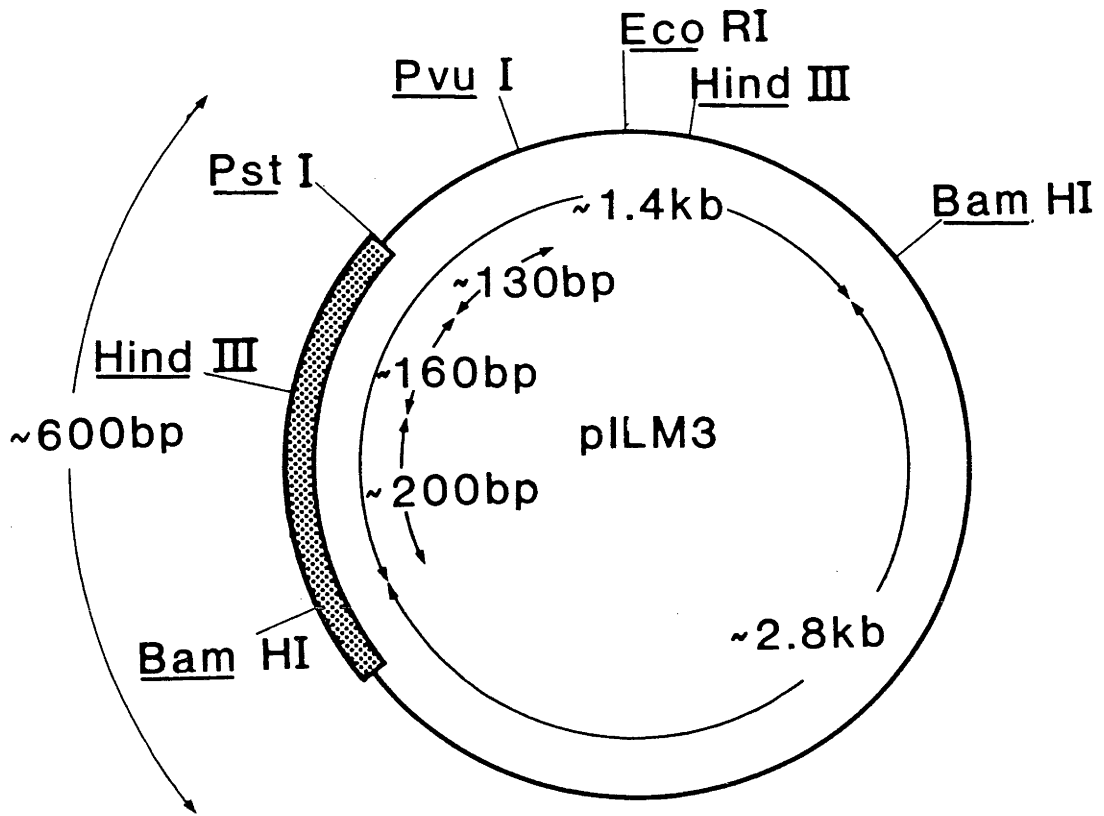
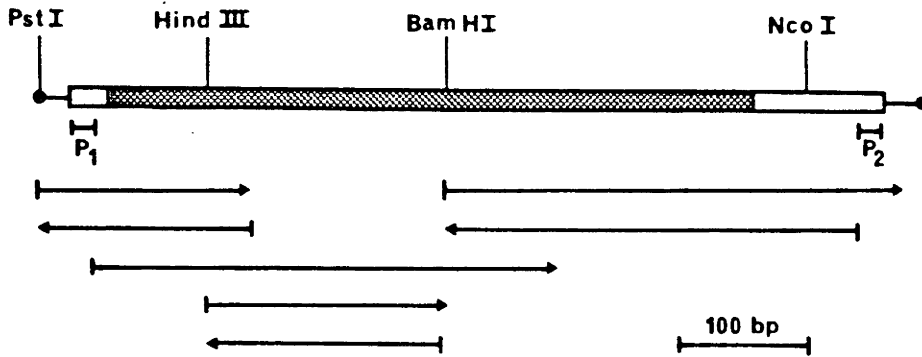


Figure 3.9 The restriction map of the pILM3. The solid line depicts the vector sequence (pAT153) and the hatched section the cDNA insert. The map is based on the results of single and double digestions with PstI, HindIII, BamHI, PvuI and EcoRI restriction endonucleases (Method 3.3). The sizes of the resultant fragments were determined by agarose gel electrophoresis.

a



b

M V L A S S T T S I H T M L L L L L M L F
AACCCTTGGAGGACCAGAACGAGACAATGGTTCTTGCCAGCTCTACCACCAGCATCCACACCATGCTGCTCCTGCTCTGATGCTCTTC
10 20 30 40 50 60 70 80 90

↓
D T H R L T R T L X X S S I V
H L G L Q A S I S G R D T H R L T R T L N C S S I V K E I I
CACCTGGGACTCCAAGCTTCAATCAGTGGCCGGGATACCCACCGTTAAACCAGAACGTTGAATTGCAGCTCTATTGTCAAGGAGATTATA
100 110 120 130 140 150 160 170 180

G K L P E P E L K T D D E G P S L R N K S F R R V N L S K F
GGGAAGCTCCCAGAACCTGAACTCAAACTGATGATGAAGGACCCTCTCTGAGGAATAAGAGCTTTCGGAGAGTAAACCTGTCCAATTC
190 200 210 220 230 240 250 260 270

V E S Q G E V D P E D R Y V I K S N L Q K L N C C L P T S A
GTGAAAGCCAAGGAGAAGTGGATCCTGAGGACAGATACG^TIATCAAGTCCAATCTTCAGAACTTAACTGTTGCCTGCCTACATCTGCG
280 290 300 310 320 330 340 350 360

N D S A L P G V F I R D L D D F R K K L R F Y M V H L N D L
AATGACTCTGCGCTGCCAGGGTCTTCATTCGAGATCTGGATGACTTTCGGAAGAACTGAGATTCTACATGGTCCACCTTAACGATCTG
370 380 390 400 410 420 430 440 450

E T V L T S R P P Q P A S G S V S P N R G T V E C
GAGACAGTGTAACTCTAGACCACCTCAGCCCGCATCTGGCTCCGTCTCTCCTAACCGTGAACCGTGGAATGTTAAAACAGCAGGCAG
460 470 480 490 500 510 520 530 540

AGCACCTAAAGTCTGAATGTTCTCATGGCCCATGGTCAAAGGATTTTACATTCCTTTATGCCATCAAATGTCTTA1CAATTTATCTA
550 560 570 580 590 600 610 620

Fig. 3.10 (a) The cDNA insert in pILM3 showing the relevant sites of restriction endonuclease cleavage. The reading frame is delineated from the untranslated regions by hatching and the flanking G-C tails are shown as short bars. One of the expected PstI sites was lost during G-tailing of the vector pAT153. The sequencing strategy is summarized beneath the cDNA insert. DNA sequencing was carried out by the chain termination method after subcloning fragments of pILM3 into M13mp8 and M13mp9. The computer programs described by Staden (1978, 1980) were used to assemble and analyse the sequence data. To avoid interference from the longer G-C tails, which was encountered using the universal primer of Duckworth et al. (1981), two internal primers (P₁ and P₂) 17 nucleotides in length were synthesized and used with the M13 templates. (b) Nucleotide sequence of the cDNA insert of pILM3 and predicted amino acid sequence of murine IL-3. The N-terminal sequence of murine IL-3 (Ihle et al., 1983) is boxed in above the predicted amino acid sequence. Potential sites for N-glycosylation are underlined. The arrow indicates a possible site of cleavage for removal of the signal peptide.

determine the DNA sequence of the opposite strand. An NcoI restriction site was found in the DNA sequence close to the G-C tail of the 2.8 Kb BamHI fragment. Therefore the PstI-NcoI fragment of the cDNA was isolated using low melting agarose gel electrophoresis (Method 3.10), made blunt-ended using the Klenow fragment of E. coli DNA polymerase I (Method 4.2) and subcloned into SmaI digested M13mp9. An oligonucleotide primer, P1, (5'AACCCCTTGGAGGACCA^{3'}) was synthesized (Method 3.11) and was used with the M13 template to determine the DNA sequence from the PstI site to BamHI site. The DNA sequence from PstI site to the HindIII site was determined using plasmid sequencing method (Method 4.7). Finally, the fragment from the HindIII to the BamHI site of pILM3 was isolated using low melting agarose gel electrophoresis (Method 3.10), made blunt-ended using the Klenow fragment of E. coli DNA polymerase I (Method 4.2) and subcloned into SmaI digested M13mp9. The DNA sequences of both strands of this fragment were determined by the chain termination method.

Using the above strategy the DNA sequence of both strands of the cDNA insert in pILM3 was determined. The use of the internal oligonucleotide primers solved the interference with the chain termination method by the G-C tails. This was a problem when copying the G strand of the tail and has been observed previously. The complete DNA sequence of the cDNA insert in pILM3

is shown in Figure 3.10b. There were 25 G-C base pairs at the 5' end and 11 G-C base pairs at the 3' end of the consensus sequence of the cDNA.

Clone pILM3 was found to extend beyond pILM1 at the 3' end only, both clones possessing the same 5' end. The DNA sequence shows a single large open reading frame (Figure 3.10b). Assuming that the first ATG from the 5' end serves as the initiation codon, the reading frame extends from nucleotides 28-525 inclusive and specifies a polypeptide of 166 amino acids. The 5' untranslated region is therefore quite short (27 bp). The method of Land et al. (1981) which was used to generate the cDNA clones, is designed to produce a high proportion of cDNA clones with complete 5' termini. Since both cDNA clones isolated in the course of this work had the same 5' end, it is possible that pILM3 carries a close to full length copy of the 5' untranslated region of IL-3 mRNA. Clone pILM3, however, does not extend to the 3' end of the IL-3 mRNA and is probably missing a significant portion of the 3' untranslated region.

The reading frame specifies a protein of MW 18,540. The strongly hydrophobic stretch of 9 amino acids (residues 13-21) is indicative of the presence of a signal peptide. In the cases studied to date, the last residue of the signal sequence is invariably an amino acid with a small uncharged side chain.

Analysis of the signal peptide region suggests a number of potential sites for cleavage by the signal peptidase. Application of the empirical rules proposed by Von Heijne (1983) predicts the end of the signal peptide at residue 27 (Ala). This potential cleavage site is shown with an arrow in Figure 3.10b.

As this work was proceeding a short stretch of N-terminal amino acid sequence was published for murine IL-3 (Ihle et al., 1983a). This sequence is derived from IL-3 purified to apparent homogeneity from the WEHI-3B cell line and is ⁱⁿ complete agreement with the reading frame and extends from residues 33 (Asp) to 47 (Val). It seems most unlikely that the signal peptide would end at residue 32 (Arg) and this suggests that the murine IL-3 isolated from the WEHI-3B cell line has been further processed.

The protein sequence of mature processed IL-3 indicates a MW of 15,102 with four potential N-glycosylation sites (Asn-X-Ser or Asn-X-Thr). The four sites are underlined in Figure 3.10b. One of these is in the region of the published N-terminal sequence and glycosylation at this site would explain the inability to identify the Asn residue during automated Edman degradation. When the amino acid composition figures for murine IL-3 (Ihle et al., 1983a) are normalized to the size predicted by the DNA sequence they give good agreement with the predicted

composition (Table 3.5).

The amino acid sequence of murine IL-3 was compared with the sequences of human interleukin-2 (Taniguchi et al., 1983) and human immune interferon (Gray et al., 1982) both of which are also produced by antigen-activated T lymphocytes. No significant sequence homology was detected. Although human IL-2 is of very similar size to IL-3 it carries no potential sites for N-glycosylation and does not appear to be closely related.

Table 3.5 Amino acid composition of murine IL-3

	Predicted	Determined
Phe	5	4.2
Leu	15	16.7
Ile	5	4.8
Met	1	1.2
Val	10	8.3
Ser	13	11.3
Pro	10	7.1
Thr	8	7.1
Ala	3	4.2
Tyr	2	1.8
His	2	2.4
Glu, Gln	12	13.7
Asp, Asn	18	16.1
Lys	9	9.5
Cys	4	ND
Trp	0	ND
Arg	11	9.5
Gly	6	8.3

The amino acid composition data are taken from Ihle et al. (1983). The composition was originally calculated for a MW of 23,722 and has been normalised to the size of mature IL-3 which is predicted from the DNA sequence. The normalization was done using the values for Glu and Asp. ND, not determined.

IV. Discussion.

The demonstration that IL-3 mRNA can be efficiently translated in oocytes (see Chapter 2) to give biologically active factor provides a very sensitive and useful assay for this mRNA. This assay has enabled the identification of a cDNA clone carrying the entire coding sequence for murine IL-3 using the mRNA hybridization translation method.

Sequence analysis of the cloned cDNA has shown that it codes for a polypeptide of 166 amino acids including a signal peptide of possibly 27 amino acids. Mature IL-3 has been purified to apparent homogeneity (Ihle et al., 1983a) from the same WEHI-3B cell line as was used as the source of the IL-3 mRNA in the present work. The N-terminal sequence of the mature IL-3 is in complete agreement with the reading frame but starts at residue 33 suggesting an additional proteolytic cleavage has occurred apart from the cleavage of the signal peptide. The proposed proteolytic cleavage could be catalysed by a number of serine-type proteases such as a member of the kallikrein family or plasmin and could provide another level of regulation if processing is required to generate biologically active IL-3. Whether this processing step occurs in vivo or simply represents adventitious proteolysis during purification is not clear at present.

Apart from the agreement with the N-terminal sequence, the amino acid composition data for mature IL-3 also gives good agreement with the predicted sequence. Since it appears that WEHI-3B only produces one member of the group of colony stimulating factors (that is, IL-3), there seems no doubt that the cDNA clone encodes IL-3.

Chapter 4 Expression of the IL-3 cDNA in eukaryotic and prokaryotic systems.

I. Introduction.

A convenient way to confirm the identification of a cloned cDNA encoding a particular protein is to express this cDNA in eukaryotic cells (Gray et al., 1982; Devos et al., 1982; Taniguchi et al., 1983) or in E. coli cells (Gray et al., 1982; Devos et al., 1983) to produce biologically active protein. Several shuttle vectors have been developed for the expression of the cloned cDNAs in mammalian cells (Ringold et al., 1981; Okayama and Berg, 1983; Southern and Berg, 1982; Gheysen and Fiers, 1982).

These shuttle vectors are composed of (i) a pBR322 origin of replication together with an antibiotic resistance gene which allows the vector to replicate in E. coli and provides a selectable marker, (ii) an SV40 origin which allows the vector to replicate in eukaryotic cells, (iii) a viral promoter like the SV40 early promoter (Okayama and Berg, 1983), mouse mammary tumor virus promoter (Ringold et al., 1981) or the SV40 late promoter (Gheysen and Fiers, 1982) which controls the expression of the cloned cDNA, (iv) an intervening sequence and a polyadenylation signal from the SV40 genes which allow the proper processing of the mRNA transcribed from the

cloned cDNA. The direct expression of a full length cDNA in eukaryotic cells using shuttle expression vectors is more convenient than that in E. coli since the G-C tails or A-T tails do not prevent the expression of the cDNA in the mammalian cells (Okayama and Berg, 1983). In addition, the cDNA does not have to be specifically engineered to allow expression to occur and processing of signal peptides and glycosylation are expected to occur normally.

The cDNA-expression vector can be constructed and produced in quantity in bacterial cells and can be transfected into eukaryotic cells using either the calcium phosphate precipitation technique (Graham and Van de Eb, 1973), DEAE-dextran (McCutchen and Pagano, 1968), or by microinjection (Graessmann and Graessmann, 1976; Capecchi, 1980). It has also been reported that plasmid DNA can also be transferred into mammalian cells with high efficiency by fusion with bacterial protoplasts containing the shuttle vector (Rassoulzadegan et al., 1982).

An alternative system for the expression of eukaryotic cDNAs is that using microinjection into the nucleus of Xenopus oocytes. This system has been studied in detail by Gurdon and his colleagues (Gurdon and Melton, 1981). Although some eukaryotic promoters like those for chick ovalbumin (Wickens et al., 1980) do not function in Xenopus oocytes, many other

eukaryotic promoters like those for SV40 (Wicken and Gurdon, 1983), thymidine kinase (McKnight and Kingsbury, 1982), and sea urchin histone (Probst et al., 1979) function quite well. The cDNA inserted into the shuttle vector can therefore be expressed in Xenopus oocytes after microinjection into the nucleus of the oocyte to yield biologically active proteins (Krieg et al., 1984). The oocyte system is not really suitable for the production of sufficient quantities of proteins for biological studies but provides a valuable system for studies on gene expression.

Although high levels of expression of foreign genes in mammalian cells has been achieved (Diamaio et al., 1984; Kopchick et al., 1985), the expression of recombinant DNA in microorganisms is still an inexpensive way of obtaining large amounts of useful proteins (Jay et al., 1984; Ikehara et al., 1984). In the case of expression in E. coli, a number of problems are encountered due to the fact that the mechanism of gene expression in eukaryotic cells is different from that in prokaryotic cells. These problems, however, have not been fully studied and the rules governing the expression of cloned genes are still largely empirical.

Since there are no introns in prokaryotic genes and no splicing enzymes present, genomic DNA cannot be generally used as a source of genes for expression in

bacterial cells. cDNAs coding for mature proteins (de Boer et al., 1983) or synthetic genes (Jay et al., 1984) are therefore used.

In order to achieve high-level of expression of the genes, strong prokaryotic promoters are used to drive transcription of the cloned eukaryotic genes. The more commonly used promoters are trp (Edman et al., 1981), tac (Russell and Bennett, 1982), lac (Slocombe et al., 1982) and PL (Simons et al., 1984) promoters. Each of these promoters is repressed under normal conditions and can either be induced by chemicals in the case of the trp, lac and tac promoters (Edman et al., 1981; Slocombe et al., 1982; Russell and Bennett, 1982) or by heat shock at 42°C for the PL promoter (Simons et al., 1984). The repression of these strong promoters is believed to be important for maintaining the stability of the plasmid in E. coli cells (Russell and Bennett, 1982). A very strong promoter, the T5 early promoter, was recently synthesized and used for the expression of human IFN- γ (Jay et al., 1984). This promoter is not repressed under normal conditions, however, in this case the promoter was inserted in front of a tetracycline resistance gene whose promoter had been removed, allowing selection in a tetracycline containing medium. Termination of transcription can be ensured by placing a transcription termination sequence after

the cloned gene (Nakamura and Inouye, 1982), although whether this is necessary for the maintenance of high levels of transcription is not yet clear.

Translation of the prokaryotic mRNA requires the presence of a ribosome binding site. The expression of a mature eukaryotic protein can be achieved by placing the gene together with an initiation codon downstream of a ribosome binding site. However, the spacing between the ribosome binding site and the initiation codon can have a great influence on the efficiency of the expression of the gene (Shepard et al., 1982; Itoh et al., 1984). In addition, the primary and secondary structure of the mRNA may also play an important role in expression (Tessier et al., 1984). It has been suggested that the presence of stem-loop structures can in some cases block translation initiation signals and drastically decrease the efficiency of expression (Tessier et al., 1984). The stability of the expressed mRNA can be increased to a certain extent using bacterial strains lacking polynucleotide phosphorylase (Hautala et al., 1979; Talmadge and Gilbert, 1982). Apart from varying the length of spacing between the ribosome binding site and initiation codon or varying the coding sequences to give more favourable secondary structures of the expressed mRNA, another way to overcome the translation barrier is to fuse the foreign gene to a prokaryotic gene so that the bacterial ribosome

binding site and initiation codon and the first few bacterial codons are used for translation (Goeddel et al., 1979). The translation efficiency of the expressed mRNA is also governed by the codon usage (Robinson et al., 1984). The codons used in mRNA coding for highly expressed prokaryotic genes appear not to be random and there appears to be a marked preference for particular codons for some amino acids (Grantham et al., 1981; Grosjean and Fiers, 1982). This preference appears to correlate with the abundance of different tRNA species (Ikemura, 1981). The codon preference can be optimized by synthesizing coding sequences employing the codons most frequently used in highly expressed E. coli genes (Ikehara et al., 1984).

The stability of the expressed eukaryotic protein is less easy to control, largely because the structural features governing protein stability in E. coli are not well understood. There is some evidence that short foreign polypeptides are unstable in E. coli (Itakura et al., 1977; Goeddel et al., 1979). The use of bacterial strains lacking one or more of the normal complement of proteases in E. coli (Gottesman et al., 1981; Goldberg et al., 1981) is one way of reducing the degradation of eukaryotic proteins. In addition, this problem can often be alleviated by fusing the peptide to a larger E. coli

protein from which the peptide is then cleaved (Nagai and Thogersen, 1984; Germino and Bastia, 1984).

In this chapter, the cDNA insert in pILM3 was expressed in monkey COS-1 cells, Xenopus oocytes and E. coli. The shuttle vector pSV₂-neo (Southern and Berg, 1982) was used for the expression of the cDNA in both monkey COS-1 cells and Xenopus oocytes. The recombinant IL-3 produced by the transformed monkey COS-1 cells was used to study of the wide range of biological activities of IL-3. In an attempt to produce a large amount of recombinant IL-3 in E. coli, an expression plasmid, pDR540 (Russell and Bennett, 1982), which carried a strong tac promoter was used. A fusion protein strategy was also tried using M13mp9.

II. Materials.

Reagents

1. Bromocresol green, was obtained from BDH Chemical Ltd.
2. T4 DNA polymerase, was obtained from New England Biolabs (No. 203).
3. DNA-polymerase I (large fragment, Klenow enzyme), was obtained from Boehringer Mannheim (No. 1094523).

Plasmids and cell strains

1. pSV₂-neo, was obtained from Dr. P. Berg (see Figure 4.1).
2. pDR540, was obtained from Pharmacia P-L Biochemical Inc. (see Figure 4.2).
3. E. coli RB791 (=W3110 lacI^QL8), was obtained from Dr. M. Ptashne.
4. E. coli SG1606 F'traD36, proAB, lacI^Q, lacZΔM15/Δ(lac pro) lon :: Tn101D46Δgal, was obtained from Dr. S. Gottesman.
5. E. coli SG1095 (lon::Tn101D46), was obtained from Dr. S. Gottesman.
6. JM101 (Messing, 1983).

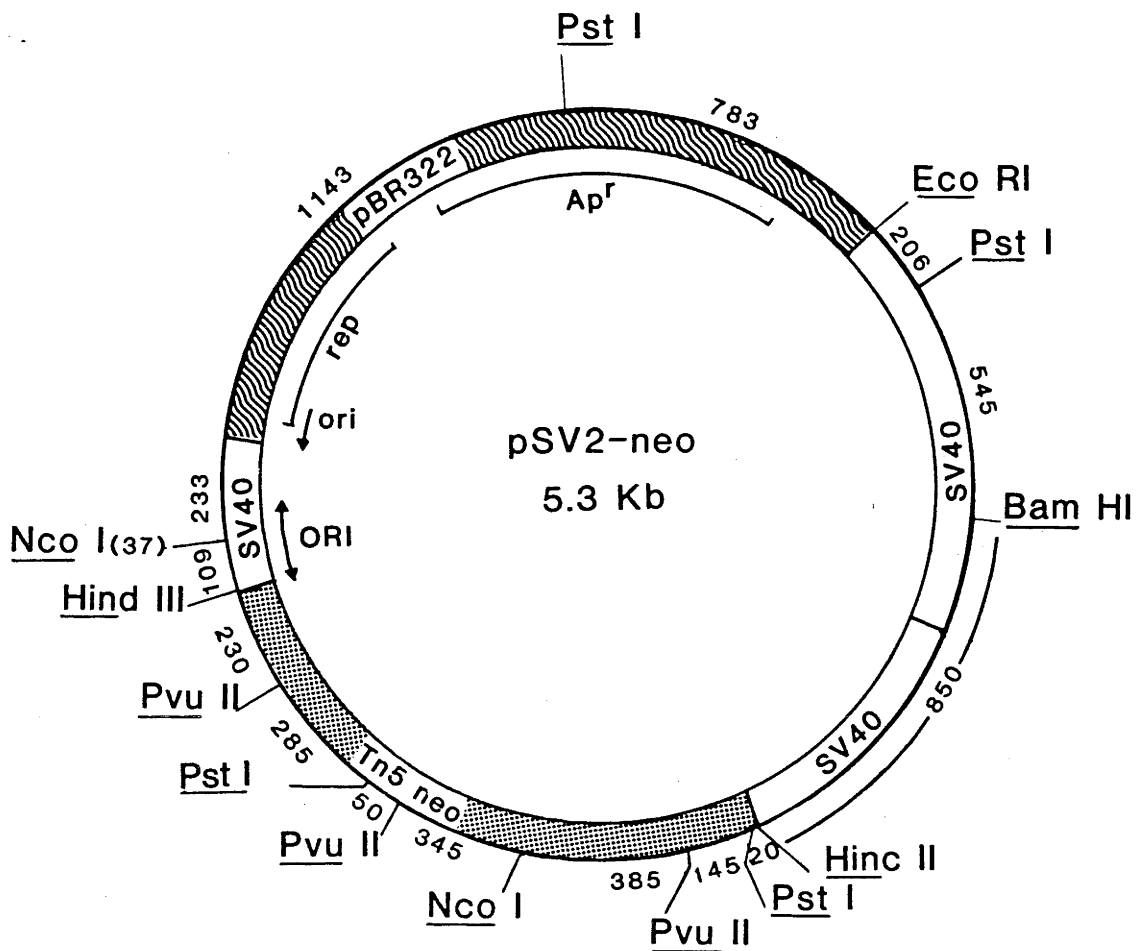


Figure 4.1 The restriction map of the shuttle vector pSV₂-neo. The restriction map of pSV₂-neo was constructed from published information (Southern and Berg, 1982) and restriction endonuclease digestion followed by agarose gel analysis of the plasmid. The nic region of pBR322 has been deleted in the vector so the PvuII site between the junction of the SV40 origin and the pBR322 origin is not present. The HincII restriction site between the Tn5neo gene and SV40 fragment was removed during construction. The hatched area denotes sequence derived from pBR322; the dotted region is the neo gene from Tn5 and the open box is from SV40. The size of the restriction fragments in base pairs are indicated.

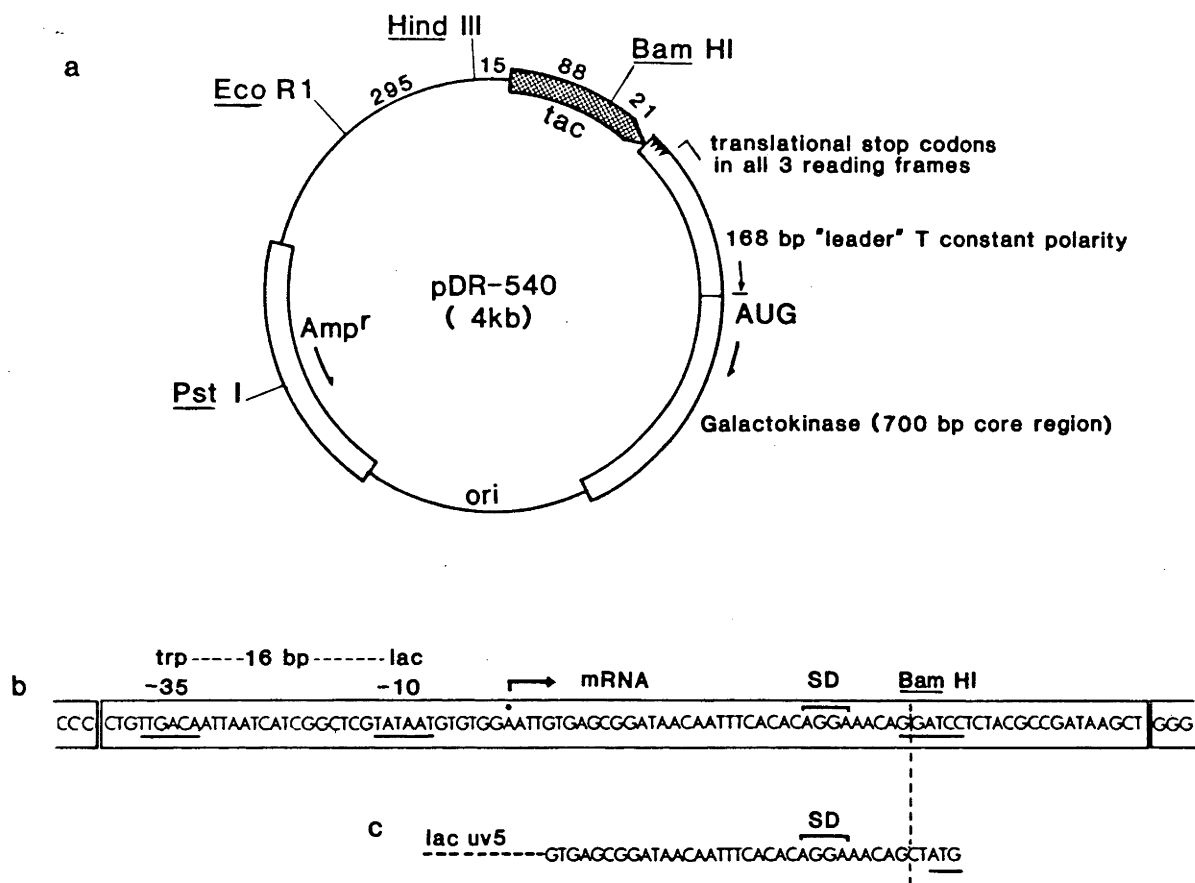


Figure 4.2 The restriction map of the expression vector pDR540. (a) The restriction map of pDR540 was constructed from published information (Russell and Bennett, 1982; McKenney et al., 1981). The galactokinase gene is under the control of the tac promoter. (b) DNA sequence of the tac promoter region. (TTGACA), the -35 consensus sequence of trp promoter. (TATAAT), the -10 consensus sequence of lac promoter. (AGGA), Shine-Dalgarno sequence. (c) Partial sequence of lac UV5 promoter showing the Shine-Dalgarno sequence and the first amino acid codon.

III. Solutions.

1. High salt NET buffer: 1.0M NaCl, 0.1mM EDTA, 20mM Tris-HCl pH8.
2. NET buffer: 0.15M NaCl, 0.1mM EDTA, 20mM Tris-HCl pH8.
3. STE buffer: 10mM NaCl, 10mM Tris-HCl, pH7.5, 0.5mM EDTA.
4. Z medium: LB medium (10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl, 1 liter H₂O, pH7.5), plus 2.5mM CaCl₂.
5. Z agar: 10g Bacto-agar in 500ml Z medium.

IV. Methods.

4.1 Isolation of DNA fragments from agarose gel using DEAE membrane.

The DEAE membrane (Schleicher and Schuell NA-45) was cut into strips of suitable size for the width of the DNA band to be recovered. The DEAE membrane was washed for 10 min in 10mm EDTA, pH7.6, 5 min in 0.5M NaOH, followed by several rapid washes in distilled water. Membranes prepared in this way could be stored in water at 4°C for several weeks.

After electrophoretic separation of the DNA fragments in a horizontal slab agarose gel, a strip of pretreated DEAE membrane was placed in a slot cut just ahead of the band of interest. The slot was filled with electrophoresis buffer, and electrophoresis continued (approximately 5 min) until binding was complete as judged by ethidium bromide fluorescence. The DEAE membrane strip was freed of residual agarose by thorough shaking in a scintillation vial containing NET buffer. The DEAE membrane strip was submerged in 250µl high salt NET buffer (for double stranded DNA) in a siliconized quick-fit tube. The tube was shaken at about 65°C for 35 min. The buffer was transferred into a sterilized and siliconized Eppendorf tube and the membrane was washed with another 50µl of high salt NET buffer. The buffer containing the eluted DNA was extracted twice with 3 volumes of water-saturated n-

butanol, 2.5 volumes of absolute ethanol were added and mixed well. The mixture was kept in a dry-ice ethanol bath for 3 hr and centrifuged in an Eppendorf centrifuge for 30 min at 4°C. The pellet was washed with cold absolute ethanol and dried under vacuum. The DNA was re-precipitated from 0.3M sodium acetate, pH5.5, to remove any residual NaCl.

4.2 Filling recessed 3' ends of double-stranded DNA using Klenow fragment of E. coli DNA polymerase I.

The reaction was carried out in a 25µl reaction volume containing the DNA fragment (\leq 1µg, 50mM Tris-HCl, pH7.2, 10mM MgCl₂, 0.1mM DTT, 0.2mM of each dNTPs, 1 unit of Klenow fragment of E. coli DNA polymerase I, at room temperature for 30 min. The reaction was stopped by adding 1µl of 0.5M EDTA, pH8. The reaction mixture was extracted with an equal volume of phenol: chloroform:isoamylalcohol mixture (50:48:2). 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol were added to the aqueous phase and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in Eppendorf centrifuge for 15 min at 4°C. The DNA pellet was washed with cold absolute ethanol and dried under vacuum. It was finally resuspended in 10mM Tris-HCl, pH7.5, 0.1mM EDTA.

4.3 Conversion of protruding 3' termini to blunt ends using T4 DNA polymerase.

The reaction was carried out in a 20 μ l reaction volume containing up to 5 μ g of DNA fragment with protruding 3' ends, 66mM Tris-HCl, pH8, 6.6mM MgCl₂, 1mM DTT, 0.5mM of each dNTPs and 10 units of T4 DNA polymerase, at 15 $^{\circ}$ C for 2 hr. The reaction was stopped by adding 1 μ l of 0.5M EDTA, pH8, and extracted with phenol:chloroform:isoamylalcohol mixture (50:48:2). 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol were added to the aqueous phase and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in an Eppendorf centrifuge for 15 min at 4 $^{\circ}$ C. The DNA pellet was washed with cold absolute ethanol and dried under vacuum. It was then resuspended in 10mM Tris-HCl, pH7.5, 0.1mM EDTA.

4.4 Large scale preparation of plasmid DNA using the rapid boiling method (Holmes and Quigley, 1981).

11 of 56 culture medium was inoculated with the plasmid carrying strain and shaken at 37 $^{\circ}$ C until the culture reached late logarithmic phase (about 4 hr). 0.15g of chloramphenicol was added and the culture shaken at 37 $^{\circ}$ C for another 16 hr. Cells were collected by centrifugation in a Sorvall GS3 rotor at 5000rpm for 10 min at 0 $^{\circ}$ C and resuspended in 70ml

STET buffer. 10ml of lysozyme (10mg/ml in 250mM Tris-HCl, pH8) was added and mixed well. It was then aliquoted into SS34 centrifuge tubes (3ml per tube) and the tubes heated at 100°C for 2 min then centrifuged at 20,000rpm, 0°C for 30 min. The clear supernatants were pooled and extracted twice with an equal volume of phenol:chloroform:isoamylalcohol mixture (50:48:2). An equal volume of isopropanol was added to the aqueous phase and mixed well. The mixture was kept in a dry-ice ethanol bath for 15 min and centrifuged in an SS34 rotor at 15,000rpm, 0°C for 10 min. The pellet was washed with ether and dried under vacuum. It was resuspended in 16ml of 10mM Tris-HCl, pH7.5, 0.1mM EDTA. 16g of CsCl was added and mixed gently until all of the salt was dissolved. 0.8ml of ethidium bromide (10mg/ml) was added and mixed well. The solution was divided into two 50 Ti centrifuge tubes and centrifuged in a 50 Ti rotor at 43,000 rpm, 16°C for 46 hr. The lower band of plasmid DNA was withdrawn using a syringe with an 18 gauge needle. The DNA solution was extracted 5 times with 2 volumes of CsCl-saturated isopropanol. The plasmid DNA was then dialyzed against three changes of STE buffer.

4.5 Microinjection of plasmid DNA into the nucleus of Xenopus laevis oocytes.

The microinjection of plasmid DNA into the

nucleus of the oocyte was essentially the same as for the injection of mRNA into oocytes in Method 2.6 except that the oocyte was orientated with a pair of watch-maker forceps so that the needle was placed at the center of the black pigmented hemisphere and was perpendicular to the equator of the oocyte. The needle was then punched into the oocyte until the tip of the needle was one-third of the way from the top to the opposite pole of the oocyte. About 30nl of the plasmid DNA was injected per oocyte.

4.6 Synthesis of single-stranded radioactive probes using M13 templates.

mILM5 is a recombinant phage of M13mp9 (Figure 4.3) containing the PstI-NcoI fragment from pILM3 inserted in the SmaI site so that the IL-3 coding sequence is in the opposite direction to β -galactosidase. A synthetic oligonucleotide primer, P1, ($5'$ AACCCCTTGGAGGACCA $3'$) was used to copy the mILM5 insert.

2 μ g of primer P1 was annealed to 34 μ g of the mILM5 template in 50 μ l annealing buffer containing 10mM Tris-HCl, pH8.5, 10mM MgCl₂. The mixture was sealed in a capillary tube and boiled at 100°C for 3 min then cooled to room temperature. The annealed DNA was added to another 50 μ l reaction mixture containing 10mM Tris-HCl, pH8.5, 10mM MgCl₂, 0.05mM of each dTTP,

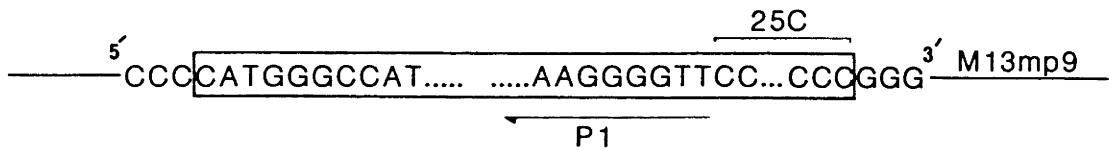


Figure 4.3 DNA sequence of the mILM5 template. The boxed region represents the PstI-NcoI fragment from plasmid pILM3. This fragment was made blunt-ended and inserted into the SmaI site of M13mp9. Only the junction sequences are shown. The orientation of the IL-3 coding sequence is opposite to that of the β -galactosidase. A synthetic oligonucleotide primer, P1, used for making probes complementary to the insert is also shown above.

dCTP, dGTP, 1.3 μ M of dATP, 20 μ Ci of [α -³²P]dATP (~3000 Ci/mmole) and 50 units of Klenow fragment of E. coli DNA polymerase I. The reaction mixture was incubated at 37° for 7 min then stopped by adding 10 μ l of 0.5M of EDTA, pH8. 2 μ l of 1M NaOH, 4 μ l of 0.25% bromocresol green and 20 μ l glycerol were added. The synthesized cDNA was separated from the template by electrophoresis on a 1% alkaline agarose gel. 3 μ g of HinfI digested pBR322 was run in parallel as a size marker. The 1% alkaline agarose gel was prepared by melting 1.3g agarose in 125ml distilled water. 3.9ml 1M NaOH and 0.52ml of 0.5M EDTA were added to the melted agarose, mixed well and the solution poured into the agarose gel apparatus. The gel was electrophoresed in the alkaline buffer containing 30mM NaOH, 2mM EDTA. The gel was run at 150mA constant current for 2 hr. The gel was neutralized in 200ml of 100mM Tris-HCl, pH7.5, 100mM NaCl for 30 min. The marker lane was cut out and stained with 10 μ g/ml of ethidium bromide in water for 30 min. The single-stranded radioactive DNA corresponding to the region of 300 base pair to 600 base pairs was excised from the gel and melted in the minimum volume of 100mM Tris-HCl, pH7.5, 100mM NaCl by boiling at 100°C for 5 min in an Eppendorf tube. The melted gel containing the radioactive single-stranded DNA probe could then be added directly to the hybridization fluid in the colony hybridization as in Method 3.9.

4.7 Plasmid sequencing by the chain termination method (Wallace, et al., 1981c).

(a) Preparation of plasmid DNA for DNA sequencing.

The plasmid DNA was prepared on a small scale by the rapid boiling method (Method 3.2a). The final DNA pellet was resuspended in 50 μ l 10mM Tris-HCl, pH8, 1mM EDTA and 50 μ g/ml of pretreated RNAaseA (Method 3.2b) and incubated at 37 $^{\circ}$ C for 10 min. It was then extracted five times with phenol:chloroform:isoamylalcohol mixture (50:48:2). 1/10 volume of 3M sodium acetate, pH5.5, and 2.5 volumes of absolute ethanol were added and mixed well. The mixture was kept in a dry-ice ethanol bath for 1 hr and centrifuged in Eppendorf centrifuge for 15 min at 4 $^{\circ}$ C. The DNA pellet was washed with cold absolute ethanol and dried under vacuum.

The plasmid DNA was linearized with a suitable restriction endonuclease. The linearized plasmid DNA was adjusted to 1 μ g/ μ l.

(b) Denaturation of the double-stranded template.

1 μ l of template (1 μ g), 1 μ l of primer (10ng) and 7 μ l of water were mixed well and sealed in a capillary tube. The tube was heated at 100 $^{\circ}$ C for 3 mins then

quickly chilled in a beaker of cold ethanol which was held in a dry-ice ethanol bath.

(c) Initiating the reaction.

The N* mix were prepared as in method 3.6c. 2 μ l of N* mix (dA*, dG*, dC*) was added to the bottom of a sterilized and siliconized capless Eppendorf tube placed in a rack which was inclined at an angle of 45°.

The capillary was thawed under running distilled water and dried with tissue. The capillary was cut open and put into a cold Eppendorf tube in an ice bath. 1 μ l 10X annealing buffer and 0.5 μ l Klenow fragment of E. coli DNA polymerase I were added to the capillary tube. The annealed DNA and Klenow were mixed well and expelled into the cold Eppendorf tube. 2 μ l of the annealed DNA and Klenow mixture was added to the top of each Eppendorf tube with N* mix. The tubes were briefly centrifuged to mix the drops and placed in a metal rack at 37°C for 10 min.

The dNTP chase reaction, termination of reaction and electrophoresis of the samples were the same as in Method 3.6d.

4.8 Screening for the orientation of M13 templates by annealing (Barnes and Bevan, 1983).

The template of the recombinant M13 phage to be

tested was annealed to a known template. 1 μ l of the known template (mILM5), 1 μ l of the template to be tested, 1 μ l of 10XSSC and 2 μ l of water were mixed well and sealed in a capillary tube. The tube was boiled at 100°C for 3 min then kept at 67°C for 1 hr. The capillary tube was cut open and the annealed template expelled into 5 μ l of BPB dye mix. The sample was analyzed by agarose gel electrophoresis.

4.9 Induction of pILM5 in E. coli.

40ml LB medium was inoculated with plasmid pILM5 and shaken at 37°C to a Klett value of 80 ($\sim 2 \times 10^8$ cells/ml). 20ml of the culture was transferred to another culture flask as a control. 20 μ l of 1M IPTG was added to one flask of 20ml culture. Both flasks were shaken at 37°C for the required time (1 hr, 2 hr or 4 hr).

1ml of culture from each flask was transferred into an Eppendorf tube. The tubes were centrifuged in an Eppendorf centrifuge for 5 min. Supernatant was removed by aspiration. The cell pellet was resuspended in 90 μ l of lysis buffer. 10 μ l of hot 20% SDS was added and mixed well. The sample was boiled at 100°C for 5 min before loading onto a 15% polyacrylamide-SDS gel (Laemmli, 1970).

The rest of the culture was prepared for the

biological assay of IL-3. 20ml of induced or uninduced culture was centrifuged in an SS34 rotor at 5000rpm, 0°C for 5 min. The supernatant was collected in a small bottle and retained for the biological assay of IL-3. The cell pellet was resuspended in 1ml of 10mM Tris-HCl, pH7.2, 30mM NaCl, 1mg/ml BSA and 1mg/ml lysozyme. The sample was transferred to a 10ml Sorvall centrifuge tube and kept on ice for 30 min. The sample was sonicated until clear in an ice-water bath and centrifuged at 20,000rpm, 0°C for 30 min. The supernatant of the cell extract was collected in an Eppendorf tube for the biological assay of IL-3.

4.10 Induction of mILM6 in E. coli.

A phage stock of mILM6 was prepared with a titer of $-5 \times 10^{11} \phi$ /ml. The host, SG1606, was grown in 40ml LB medium to a Klett value of 80 (-2×10^8 cells/ml). mILM6 phage stock was added to the culture to a multiplicity of infection equal to 100. The phage was allowed to adsorb the host at 37°C for half an hour. The infected culture was then divided into 2 flasks. 20 μ l of 1M IPTG was added to one flask. Both flasks were shaken at 37°C for the required time (1 hr, 2 hr or 4 hr).

Samples for analysis on 15% polyacrylamide-SDS gel and for biological assay of IL-3 were prepared as in Method 4.9.

4.11 Construction of IY103 by transduction.

SG1095 was grown in Z medium overnight. 0.1ml of P1 phage ($\sim 10^9 \phi$ /ml) was mixed with 0.2ml of overnight culture of SG1095 and plated in 3ml of Z top agar. After incubation at 37°C overnight, the top agar harvested from 5 plates was resuspended in 10ml Z medium with 0.2ml chloroform. It was vortexed for 5 min and centrifuged in an SS34 rotor at 15,000rpm, 4°C for 30 min. The supernatant was stored over a drop of chloroform.

RB791 was grown in Z medium overnight. 20ml of the overnight RB791 culture was centrifuged in an SS34 rotor at 5000rpm, 4°C for 5 min. The cell pellet was resuspended in 2ml Z medium. For the transduction experiment, 0.2ml of concentrated RB791 cells, 1ml P1 phage prepared from SG1095 and 4ml Z medium were mixed well and kept at 37°C for 20 min. The control experiment was done without the P1 phage. After incubation the cells were centrifuged in an SS34 rotor at 5,000rpm, 4°C for 5 min and the cell pellet was resuspended in 1ml of 1M sodium citrate. 9ml LB medium was added and the cells were grown at 37°C for 2 generations (~4 hr). The cells were centrifuged in an SS34 rotor at 5,000rpm, 4°C for 5 min and the cell pellet was resuspended in 1ml of (1X)56 medium. 0.1ml of the cells was plated on each agar plate containing

tetracycline (25 μ g/ml) and incubated at 37°C overnight. Tetracycline resistant and mucoid colonies were isolated.

V. Results.

1. Construction of pILM4.

pSV₂-neo is a shuttle vector which can replicate in both eukaryotic cells and in E. coli (Southern and Berg 1982). Any cDNA cloned into the HindIII site of pSV₂-neo can be expressed in the COS cell system under the control of the SV40 early promoter.

The IL-3 cDNA insert in pILM3 was therefore cloned into the HindIII site of the pSV₂-neo as shown in Figure 4.4. The IL-3 coding sequence was resected from plasmid pILM-3 by digestion with the restriction endonucleases PstI and NcoI (Method 3.3), the fragment purified by agarose gel electrophoresis and recovered using DEAE membrane (Method 4.1). The fragment was made blunt-ended using the Klenow fragment of E. coli DNA polymerase I (Method 4.2) and ligated with pSV₂-neo which had been linearized by digestion with HindIII restriction endonuclease, made blunt-ended (Method 4.2) and dephosphorylated with calf intestinal alkaline phosphatase (Method 3.4). The ligated DNA was transformed into E. coli HB101 and ampicillin resistant colonies were selected. Small scale plasmid preparations of ampicillin resistant clones were prepared (Method 3.2a), the DNA digested with BamHI restriction endonuclease and examined by agarose gel electrophoresis. Clones giving the expected 2 fragments were double digested with HindIII and PstI

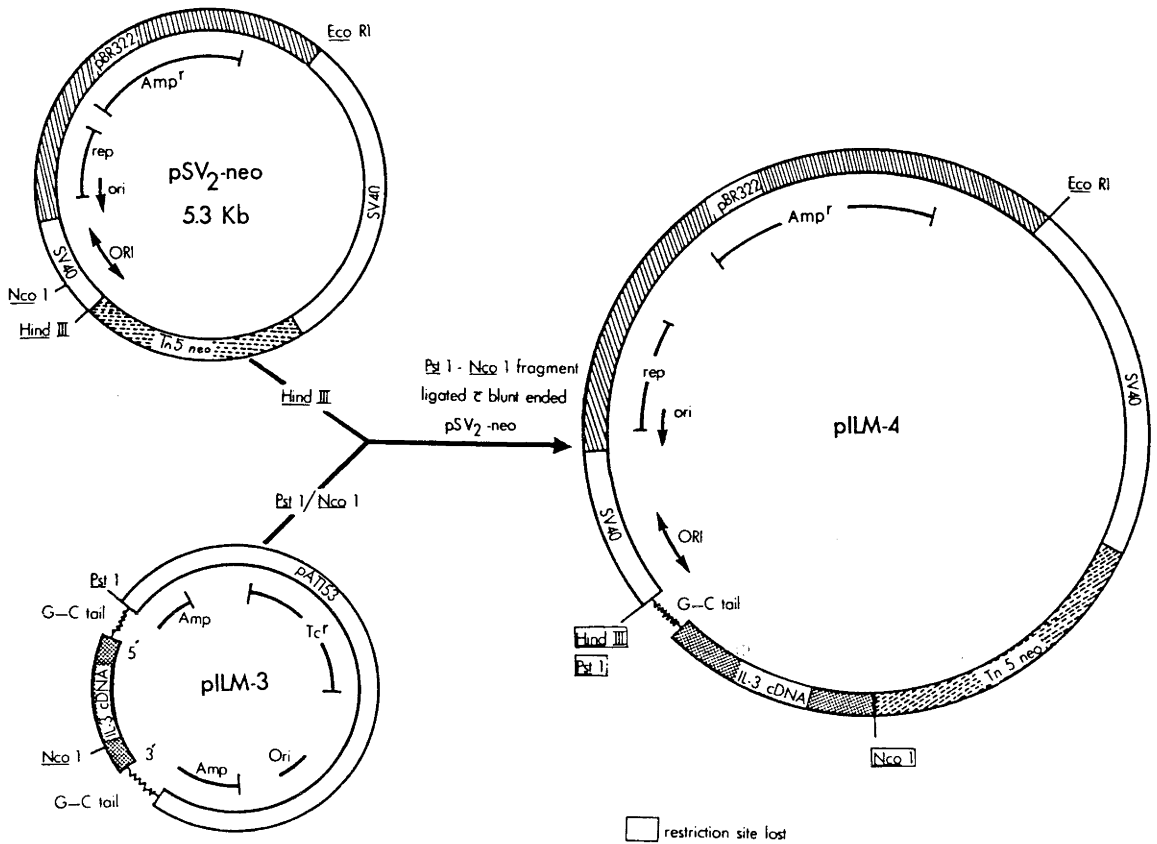


Figure 4.4 Construction of SV40 expression vector carrying the IL-3 gene. IL-3 cDNA insert was resected from pILM3 by digestion with PstI and NcoI restriction endonucleases. The PstI-NcoI fragment was made blunt-ended and ligated with pSV₂-neo which had been digested with HindIII restriction endonuclease and also made blunt-ended (See Results). After transformation into HB101, recombinant plasmids carrying the IL-3 cDNA in the correct orientation were identified by restriction analysis.

restriction endonuclease and examined by agarose gel electrophoresis. One of these clones, designated pILM4, had the IL-3 cDNA sequence arranged in the same orientation as the direction of transcription from the SV40 early promoter and showed the predicted 1615 base pair fragment.

Plasmid DNA was prepared from pILM4 on a large scale by the rapid boiling method and then purified on a CsCl-ethidium bromide gradient (Method 4.4). The purified pILM4 plasmid DNA was used in the experiments on the expression of IL-3 in Xenopus laevis oocytes nuclei and in monkey Cos-1 cells.

2. Microinjection of the pILM4 DNA into the nucleus of Xenopus laevis oocytes.

It was of interest to determine if pILM4 could be expressed in the oocyte nucleus. 30nl of 1µg/µl pILM4 DNA was injected into the nucleus or injected into the cytoplasm of Xenopus laevis oocytes (Method 4.5). 20 oocytes from each injection were incubated in 10µl of oocyte incubation medium at 20° for 48 hr. The oocyte incubation medium was then centrifuged for 15 min at 4°C. The supernatant was assayed for IL-3 activity as described in Method 2.7. The results (Table 4.1) showed that the IL-3 cDNA in pILM4 could be transcribed and translated to give biologically active IL-3 when it was injected into the nucleus of Xenopus

Table 4.1 Nuclear injection of pILM4 into X. laevis oocytes.

site of injection	IL-3 activity* (c.p.m.)
nucleus	24,800
cytoplasm	139

Purified plasmid, pILM4, DNA was injected into the nucleus or the cytoplasm of the Xenopus laevis oocytes (Method 4.5). After 48 hrs the incubation medium was harvested and assayed for IL-3 activity using an IL-3 dependent cell line (Method 2.7) * Maximum activity in the IL-3 assay was 42,250 c.p.m. using WEHI-3B conditioned medium.

laevis oocytes, but not when injected into the cytoplasm. The expression of the IL-3 gene in the oocyte nucleus will be of interest in connection with future experiments on the regulation of IL-3 gene expression (see Discussion).

3. Expression of the IL-3 gene in monkey cells and biological properties of recombinant IL-3.

These experiments were done in collaboration with Dr. Andrew Hapel, Department of Medicine and Clinical Science, JCSMR and Drs. G. Johnson and D. Metcalf, Walter and Eliza Hall Institute for Medical Research, Melbourne.

The expression of the IL-3 cDNA clone in monkey COS cells was the most convenient way of producing moderate amounts of a 'recombinant' IL-3 which was closely related to authentic mature IL-3. The 'recombinant' IL-3 secreted from COS cells would be expected to be correctly processed with respect to the signal peptide and to be appropriately glycosylated. This material could therefore be used to establish the range of biological activities possessed by IL-3.

The expression plasmid pILM4 was transfected into monkey COS-1 cells to allow replication and expression of the IL-3 gene. Culture supernatants were collected periodically after transfection and assayed for IL-3

activity using the IL-3 dependent cell line 32Dcl-23. Maximal activity was achieved 72 hr after transfection (Figure 4.5). No activity was detected when COS-1 cells were transfected with the parent vector pSV₂-neo. The culture supernatants, collected 72 hr after transfection with pILM4, were used in a variety of biological assays as described below. Analogous material collected 72 hr after transfection with pSV₂-neo was used as a control.

In some cases assays were also carried out using partially-purified expressed IL-3 which was partially purified by chromatography on DEAE-Sephacel and phenyl-Sepharose. The expressed IL-3 showed the same chromatographic properties as the IL-3 from the WEHI-3B cell line and the procedure resulted in a purification of at least 10,000 fold compared with the starting material.

Expressed IL-3 supported the growth of the IL-3 dependent cell lines 32Dcl-23 and FDC-P1. The 32Dcl-23 line consistently gave a 4 to 8 fold higher titre with the expressed IL-3 than the FDC-P1 cell line. This has also been observed with IL-3 from WEHI-3B conditioned medium (Hapel et al., 1984).

Expressed IL-3 also 'induced' 20 α -SDH in splenic lymphocytes from nu- ν mice in a standard assay as previously described (Ihle et al., 1981).

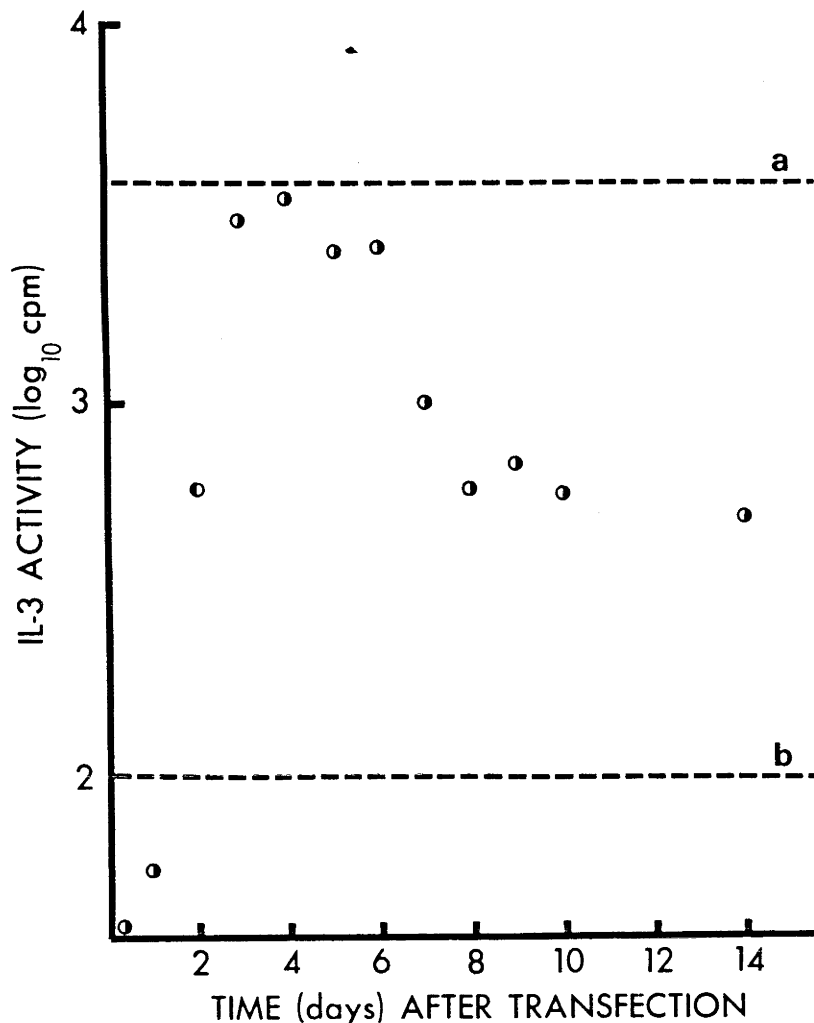


Figure 4.5 Production of IL-3 by COS-1 cells transfected with pILM4. COS-1 cells were seeded at 5×10^6 /60mm petri dish in DMEM 10% FCS and transfected with pILM4 using the calcium phosphate precipitation and glycerol shock technique. Cells were washed, fed with DMEM 10% FCS (5mls/dish) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Media were replaced every 24 hours and tested for IL-3 activity (proliferation of 32D cl-23 cells). The data plotted were obtained at a dilution of 1/8. The dotted lines designated (a) and (b) represent incorporation of 100% and background plus three standard deviations respectively.

Supernatants from COS-1 cells transfected with the parent vector pSV₂-neo showed no detectable activity in assays with the IL-3 dependent cells or in the induction of 20 α -SDH. The titres (ED₅₀/ml) for 20 α -SDH induction were the same as for growth factor activity on FDC-P1 cells whereas the titre for growth factor activity on 32Dcl-23 was some 4 fold higher.

The expressed IL-3 also supported the growth of 'P' cells (Schrader 1981).

Medium from COS cells transfected with pILM4 was able to stimulate the formation from fetal liver cells of typical day 2 erythroid colonies. This conditioned medium was able to sustain and/or elevate levels of erythroid (BFU-E) and non-erythroid (GM-CFC) progenitor cells in suspension cultures of fetal liver cells, a property of materials containing IL-3.

Titration of the tenfold concentrate of pILM4-transfected COS cell conditioned medium on cultures of adult marrow cells established that the CSF levels were 400 units per 0.1ml, based on granulocyte-macrophage colony formation. In the analysis of the colonies developing in adult marrow cultures containing 0.1ml of concentrated conditioned medium, it was observed that granulocyte, granulocyte/macrophage and macrophage colonies developed and in addition, low numbers of typical megakaryocyte and eosinophil colonies. Also developing were the

dispersed colonies of candidate NK-like cells (Claeson et al., 1982) that are characteristically stimulated by WEHI-3B conditioned medium and by SCM (pokeweed mitogen-stimulated spleen conditioned medium) (Djeu et al., 1983; Metcalf et al., 1969; Metcalf et al., 1974). In cultures containing 400 units of IL-3 purified from WEHI-3B conditioned medium, an essentially similar distribution of colony types was observed.

Addition of medium conditioned by peritoneal cells has been reported to enhance megakaryocyte colony formation stimulated by IL-3 (Williams et al., 1982). When 0.1ml of peritoneal cell conditioned medium was added to cultures of marrow cells, no megakaryocyte colonies developed but in cultures containing a mixture of peritoneal cell conditioned medium and pILM4-COS cell conditioned medium, megakaryocyte numbers rose to 8% of total colonies and were increased in average size.

In agar cultures of 12 day CBA fetal liver cells, pILM4 transfected COS cell conditioned medium stimulated the formation of granulocyte and/or macrophage, eosinophil, megakaryocyte and pure and mixed erythroid colonies. The various colony types were similar to those stimulated by IL-3 purified from WEHI-3B conditioned medium. A detailed comparison of the erythroid cell content of mixed colonies and of

the hemoglobinization of the erythroid cells in pure and mixed-erythroid colonies indicated that purified IL-3 from WEHI-3B conditioned medium was a relatively weak stimulus for erythroid cell proliferation and hemoglobinization compared with the size and hemoglobinization of the erythroid colonies stimulated by SCM. This characteristic has been noted previously in a comparison of IL-3 purified from WEHI-3B conditioned medium versus that purified from SCM (Cutler, R.L., Metcalf, D., Johnson, G.R. and Nicola, N.A., unpublished data) and it was of interest that the erythroid colonies stimulated by pILM4-transfected COS cell conditioned medium also were of small size and exhibited incomplete hemoglobinization.

Thus the recombinant IL-3 possessed the range of biological activities appropriate for a multi-lineage haemopoietic growth factor and these activities were in broad agreement with those claimed for biochemically purified IL-3 (see Discussion).

4. Construction of pILM5.

Although the pILM4 transfected monkey COS-1 cells yielded sufficient 'recombinant' murine IL-3 for initial determination of the biological properties of IL-3 it was of interest with respect to future research to try and obtain expression of IL-3 in E. coli.

The IL-3 cDNA was first expressed in E. coli under the control of the tac promoter using the pDR540 expression vector. The strategy of constructing the IL-3 expression plasmid is shown in Figure 4.6. The 570 base pair HpaII fragment containing the IL-3 coding sequence starting with the amino acid number 32 (Arginine), was isolated after digestion of pILM3 with HpaII and HphI ^{restriction} endonucleases using agarose gel electrophoresis and DEAE membrane (Method 4.1). Two complementary oligonucleotide primers, P22 (5' GATC ATG TCA ATC AGT GGC³¹) and P23 (5' CG GCC ACT GAT TGA CAT³¹) were synthesized. They were annealed to form a short linker with BamHI and HpaII "sticky ends". The HpaII fragment was then ligated with this short linker and the BamHI digested pDR540 in a molar ratio of 1:100:1 respectively and transformed into JM101. The use of large amount of linker was to try and ensure the isolation of constructs having both the linker and the IL-3 insert.

620 ampicillin resistant clones were screened by colony hybridization (Method 3.9) using a single-stranded DNA probe copied from mILM5 template (Method 4.6). mILM5 is a recombinant of M13mp9 containing the PstI-NcoI fragment of IL-3 cDNA (Figure 4.3). 25 clones were found to contain the IL-3 cDNA sequence. Plasmid DNA preparations of these clones were prepared on a small scale by the rapid boiling method (Method

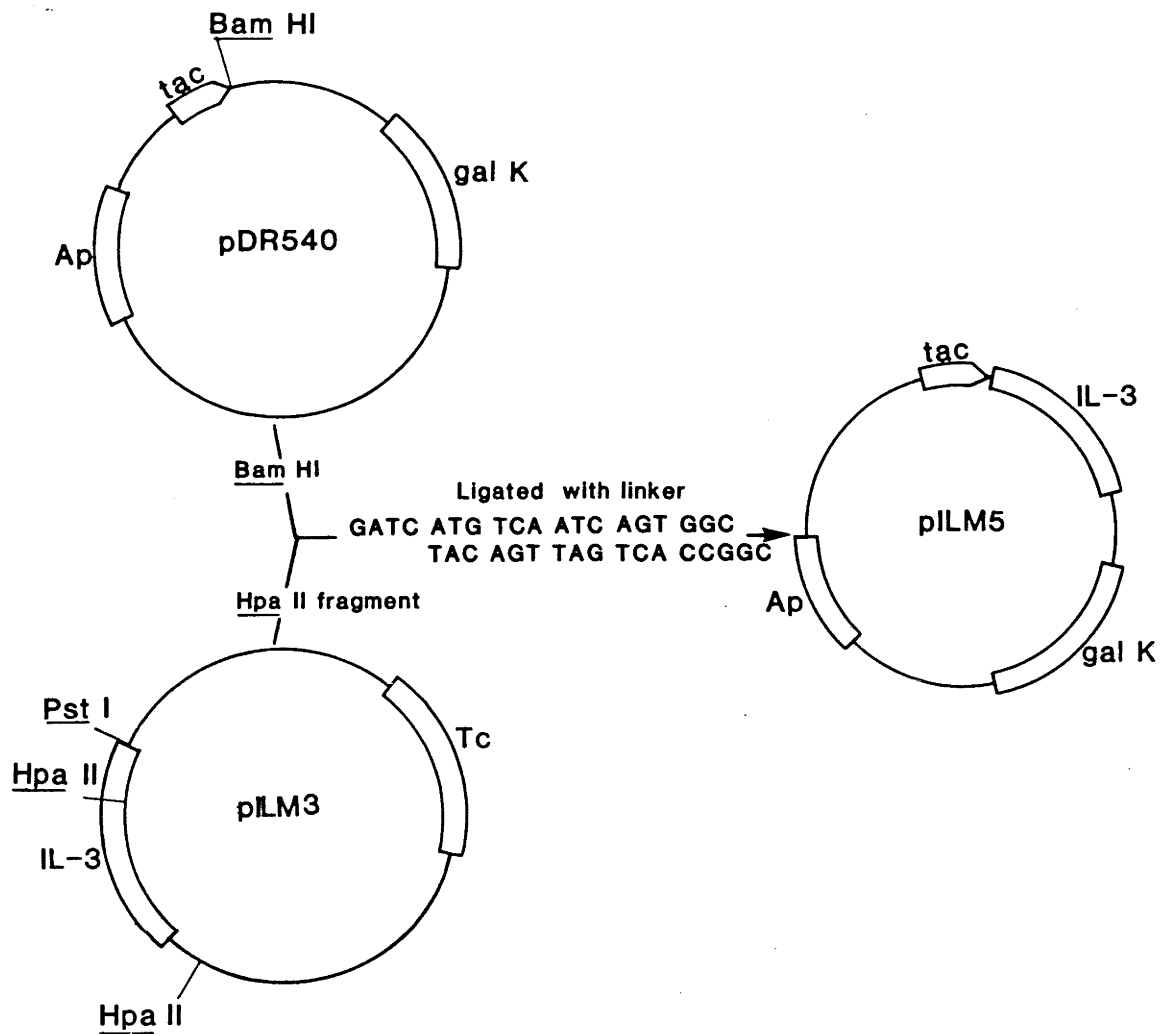


Figure 4.6 Construction of pILM5. The HpaII fragment containing the complete IL-3 coding sequence was isolated from pILM3 and joined to the BamHI linearized pDR540 with a synthetic linker. After transformation into JM101, recombinant plasmids carrying the IL-3 insert were identified by colony hybridization. The positive clones carrying the IL-3 insert in the correct orientation were identified by restriction analysis.

shown in Figure 4.7. In this construct the IL-3 coding sequence starts with methionine and then residue 28 (Serine, Figure 4.7). The spacing between the ribosome binding site and the starting codon ATG was 2 base pairs more than the original spacing in the lac UV5 promoter (Figure 4.2).

6. Construction of mILM6.

IL-3 was also expressed in bacteria as a fused polypeptide by cloning the IL-3 sequence in PstI site of M13mp9 in phase with the β -galactosidase reading frame. The strategy is shown in Figure 4.8. The IL-3 coding sequence starting with amino acid number 27 (Alanine, Figure 4.8) was resected from plasmid pILM3 by digestion with HindIII and NcoI restriction endonucleases and the fragment purified by agarose gel electrophoresis and DEAE membrane (Method 4.1). The fragment was then made blunt-ended using the Klenow fragment of E. coli DNA polymerase I, giving one extra base pair before the codon for Alanine. The vector M13mp9 was digested with PstI restriction endonuclease. The 3' protruding end was removed using T4 DNA polymerase (Method 4.3), and the M13mp9 fragment was then treated with calf intestinal alkaline phosphatase (Method 3.4). 10ng of the HindIII-NcoI IL-3 fragment was ligated with 150ng of the M13mp9 fragment and transformed into JM101. Templates were prepared from 24 of the white plaques

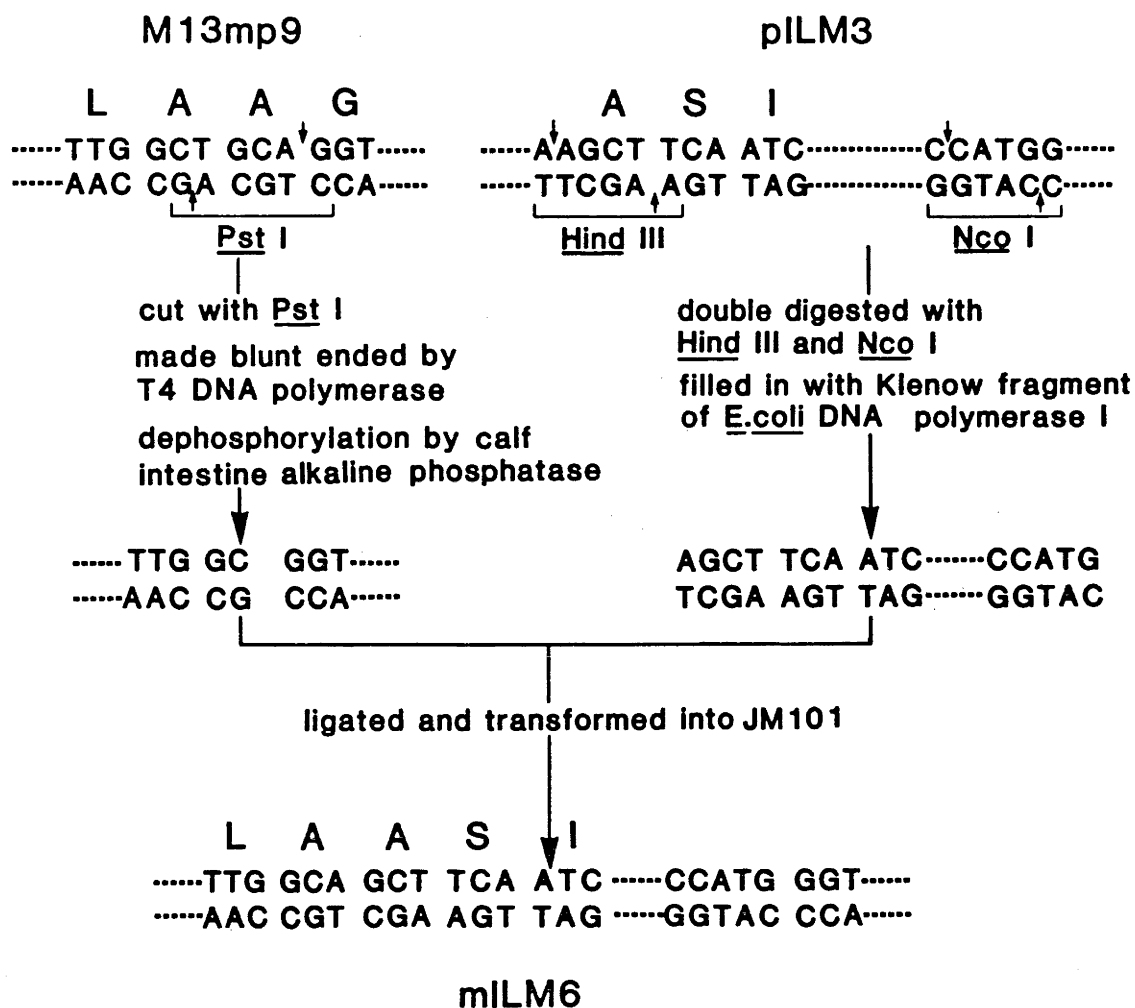


Figure 4.8 Construction of mILM6. The HindIII-NcoI fragment containing the IL-3 coding sequence was isolated from pILM3 and made blunt-ended using the Klenow fragment of E. coli DNA polymerase I. The fragment was ligated to the PstI linearized M13mp9 which had been made blunt-ended using T4 DNA polymerase and dephosphorylated using calf intestinal alkaline phosphatase. After transformation to JM101 the recombinant phages carrying the IL-3 coding sequence in the correct orientation were identified by annealing with the mILM5 template (Method 4.8).

obtained (Method 3.6a). The templates of these clones were annealed to the template of mILM5 and examined on agarose gel electrophoresis (Method 4.8). Since the IL-3 coding sequence in mILM5 is in the opposite direction to the direction of the transcription of the lac promoter in M13mp9, any clones annealing with mILM5 should have the IL-3 coding sequence arranged in the direction of the transcription of the lac promoter in M13mp9. 3 clones could be annealed with the mILM5 templated (Figure 4.9).

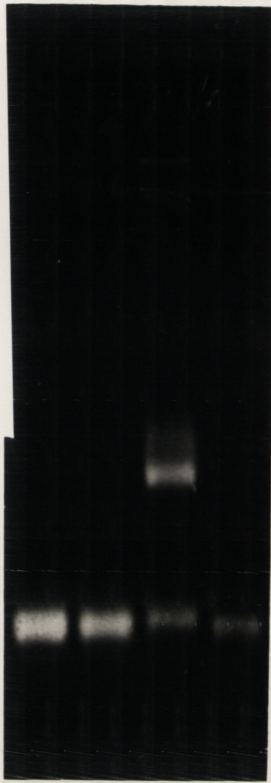
The DNA sequence of the junctions between the IL-3 coding sequence and M13mp9 of these 3 clones were determined by the chain termination method using the internal primer, P24 and the universal primer described by Duckworth et al. (1981). Only one of the clones (designate pILM6) showed a sequence in which the IL-3 coding sequence was in phase with the β -galactosidase of the M13mp9 (Figure 4.10). In this construct the IL-3 would be expressed as a fusion polypeptide with 9 extra amino acids in front of the IL-3 amino acid sequence, starting with residue 27 (Alanine, Figure 4.10).

7. Expression of IL-3 from pILM5 in different hosts.

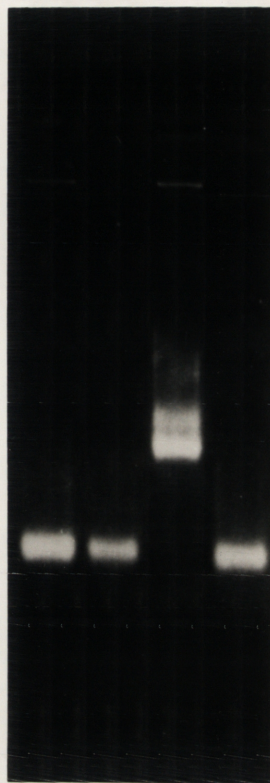
It is well established that eukaryotic proteins expressed in E. coli are often unstable due to proteolytic cleavage by endogenous proteases. In an

Figure 4.9 Test for orientation of IL-3 coding inserts by annealing with mILM5. In lanes, 1, 3, 5, 7, 9 and 11 recombinant single stranded templates were annealed with the single stranded template of mILM5 while in lanes 2, 4, 6, 8, 10 and 12 the corresponding recombinant single stranded templates were self-annealed. In lanes 3, 7 and 11 the template of the tested clones has hybridized to the mILM5 template forming slower migrating partially double-stranded DNA. The direction of migration is from top to bottom.

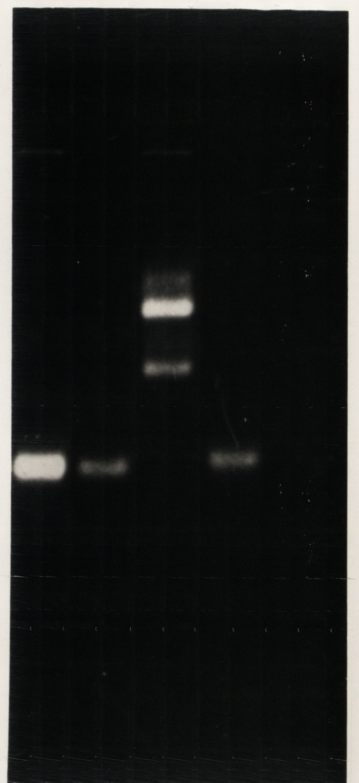
1 2 3 4



5 6 7 8



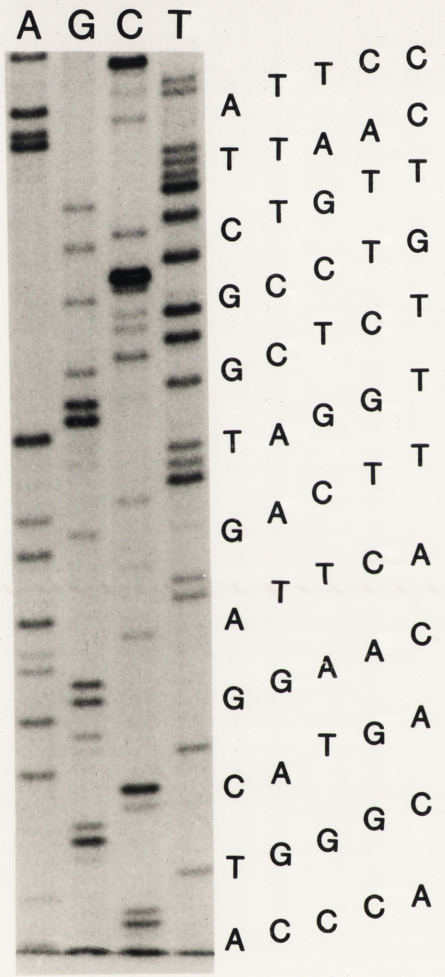
9 10 11 12



(a)



(b)



(c)

```
5'      M   T   M   I   T   P   S   L   A
-----AGCT ATG ACC ATG ATT ACG CCA AGC TTG GCA
      A   S   I   S   G   R   D   T   H   R   L
GCT TCA ATC AGT GGC CGG GAT ACC CAC CGT TTA
      T   R   T   L   N   C   S   S   I   V   K
ACC AGA ACG TTG AAT TGC AGC TCT ATT GTC AAG-----3'
```

(d)

```
5'  E C *
-----GGAATGTTAAAACAGCAGGCAGAGCACCTAAAGTCTGA
      ATGTTCCTCATGGCCCATGGGT-----3'
              Nco I
```

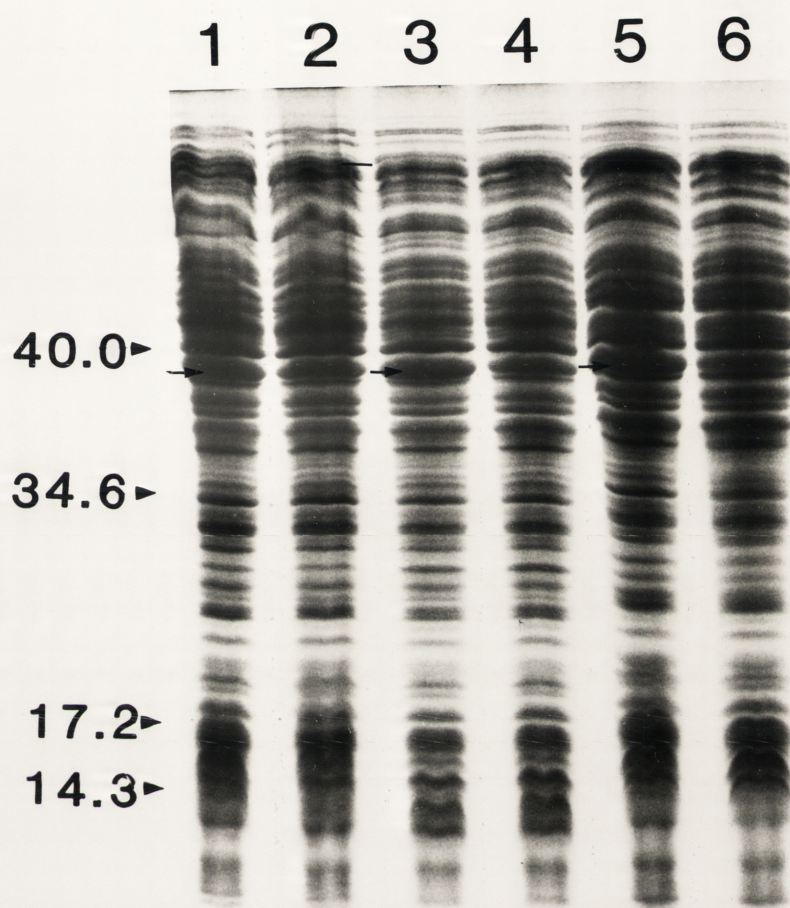
Figure 4.10 DNA sequence of the junctions between the IL-3 insert and the M13mp9 vector in the recombinant phage mILM6. (a) and (b), Autoradiographs of the sequencing gel showing the DNA sequence of the junctions at the starting codon and the termination codon of the IL-3 insert respectively. The DNA sequence was determined by the chain termination method (Method 3.6). The direction of electrophoresis was from top to bottom. (c), The DNA sequence complementary to that read from (a). The predicted amino acid sequence is shown in the one-letter code. The IL-3 coding sequence is fused with the N-terminal sequence of β -galactosidase and starts at amino acid number 10 (Alanine). (d), The DNA sequence complementary to that read from (b). Glutamate (E) and Cysteine (C) are the last two amino acids of IL-3. TAA (*) is the termination codon.

attempt to overcome this problem protease-deficient strains are often used. The most commonly employed strains are deficient in the lon protease (Gottesman et al., 1981) and in the present work a lon strain (SG1606) which carries a mini Tn10 insertion in the lon gene, was used. This strain was kindly provided by Dr. S. Gottesman, NIH. The lon ::Tn101D46 allele was also transferred to strain RB791 by transduction using P1KC phage (Method 4.11) to give strain IY103. The two lon strains, SG1606 and IY103, were used together with JM101 as hosts for the expression of pILM5. Both JM101 and SG1606 have a lacI^q mutation carried on an F prime factor (Messing, 1983). IY103 carries a chromosomal lacI^q mutation. In all three strains the tac promoter of pILM5 should be repressed by the overproduced lac repressor under normal conditions.

Induction was achieved by the addition of 1mM IPTG to cells in early log phase (2×10^8 cells/ml). The efficiency of induction was checked using the parental vector pDR540 in which expression of galactokinase is under the control of the tac promoter. In all three strains a prominent new protein band of MW approx 38,000 was evident in the cell extracts after induction (Figure 4.14) indicating efficient induction under the conditions used.

Controls were also done to check if IL-3 activity

Figure 4.14 SDS/PAGE analysis of cell extracts from different host strains carrying pDR540. Lanes are: (1) and (2), JM101; (3) and (4), SG1606; (5) and (6), IY103. The extracts in lanes 1, 3 and 5 were from cells induced with IPTG whereas those in lanes 2, 4 and 6 were from uninduced cells. For induction, 1mM IPTG was added to cells in early log phase (2×10^8 cells/ml) and growth was continued at 37°C. After 2 hr induction the cell extracts were prepared and analyzed on a 15% polyacrylamide-SDS gel (Method 4.9). The size markers are indicated by ▶ (X1000). The new band arising from the induction with IPTG is marked with an arrow. The direction of electrophoresis is from top to bottom.



could be readily detected in induced cell extracts and culture supernatants. WEHI-3B conditioned medium was mixed with an equal volume of cell extract from JM101 and IL-3 activity determined. It is clear from the results that high levels of bacterial cell extract inhibit the IL-3 assay (Figure 4.11), but that the inhibition disappears progressively with dilution. Thus, although it is not possible to measure low levels of IL-3 in bacterial cell extracts, levels of activity comparable to those found in WEHI-3B conditioned medium can be readily detected (Figure 4.12). In similar experiments it was found that bacterial culture supernatants did not inhibit the IL-3 assay (Figure 4.13).

Since the induction of the tac promoter appeared to be efficient and the assay of IL-3 in bacterial extracts was satisfactory, attempts were made to express IL-3 from pILM5 in the host strains JM101, SG1606 and IY103. pILM5 was induced by the addition of 1mM IPTG to cells in early log phase (2×10^8 cells/ml). The cells and culture supernatant were harvested at different times after induction. Cell extracts and culture supernatants were prepared (Method 4.9) and assayed for IL-3 activity (Method 2.7).

The results (Table 4.2) showed that biologically active IL-3 could be detected 2 hr after induction of

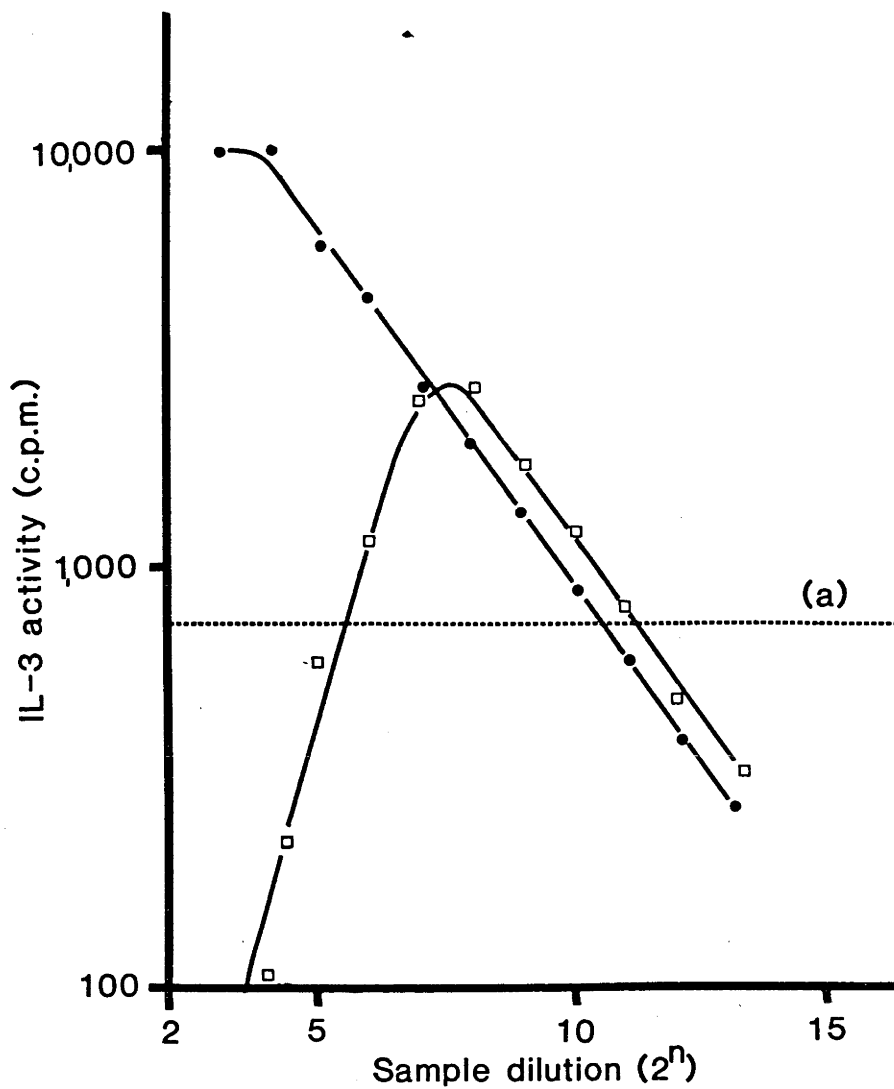


Figure 4.11 The inhibitory effect of the cell extract on the IL-3 assay. *E. coli* (JM101) cell extract was prepared as described in Method 4.9, mixed with an equal volume of WEHI-3B conditioned medium and assayed for IL-3 activity as described in Method 2.7. Symbols: ●, WEHI-3B conditioned medium + assay diluent (1:1); □, WEHI-3B conditioned medium + cell extract (1:1). The broken line (a) represents background plus three standard deviations.

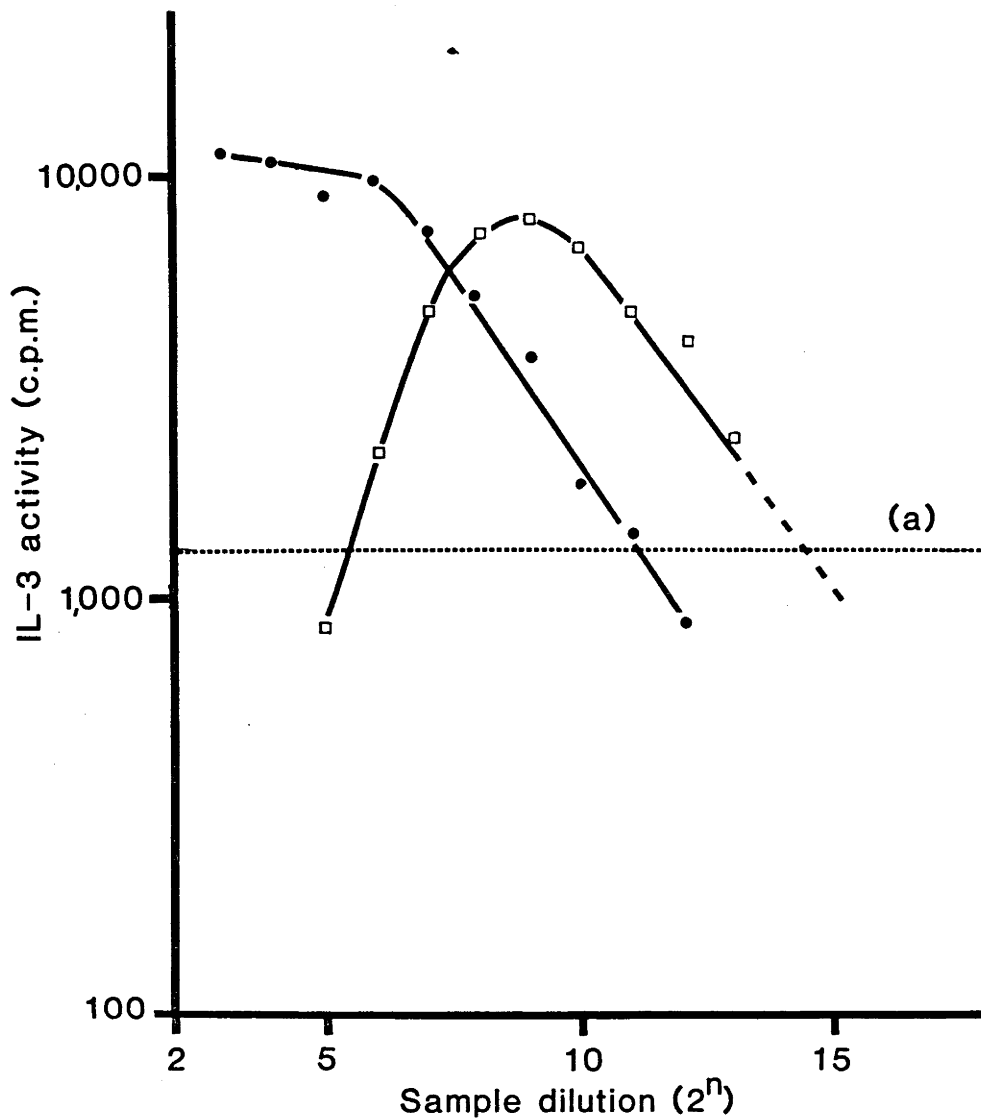


Figure 4.12 IL-3 activity assay of the cell extract from SG1606 carrying pILM5 after 2 hrs induction. The plasmid was induced by the addition of 1mM IPTG to cells in early log phase (2×10^8 cells/ml). The cell extract was prepared and assayed for IL-3 activity as described in Methods 4.9 and 2.7 respectively. Symbols: ●, WEHI-3B conditioned medium; □, cell extract from SG1606 carrying pILM5. The broken line (a) represents the background plus three times standard deviation.

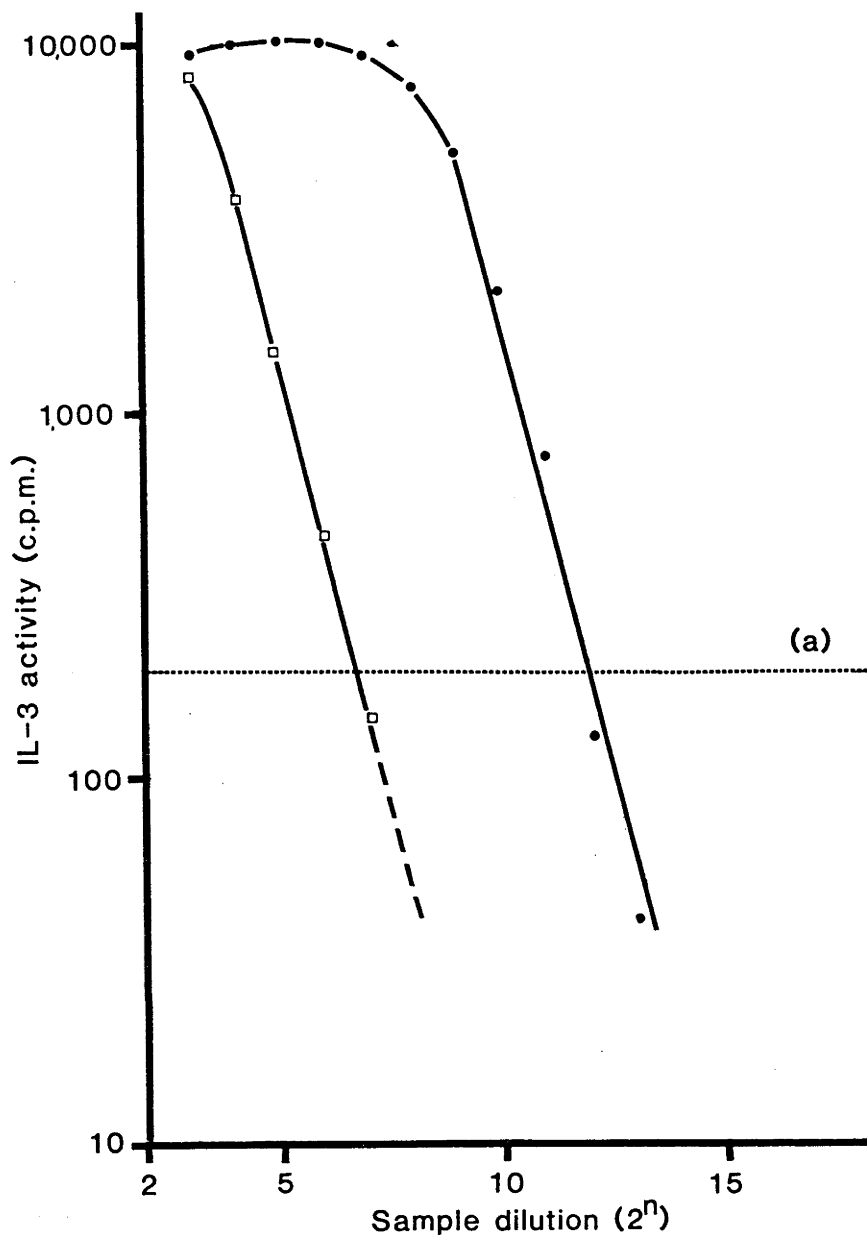


Figure 4.13 IL-3 activity of culture supernatants from IY103 carrying pILM5 after 4 hrs induction. The plasmid was induced by the addition of 1mM IPTG to cells in early log phase (2×10^8 cells/ml). The culture supernatant after 4 hr induction was prepared and assayed for IL-3 activity as described in Methods 4.9 and 2.7 respectively. Symbols: ●, WEHI-3B conditioned medium; □, culture supernatant IY103 carrying pILM5. The broken line (a) represents the background plus three standard deviations.

Table 4.2 Expression of IL-3 in different E. coli strains

Vector	host		no induction 2 hr	Time after induction		
				1 hr	2 hr	4 hr
pDR540	JM101	C.E.	ND	-	ND	ND
pDR-pH1	JM101	C.E.	ND	-	ND	ND
pILM5	JM101	C.E.	128	-	445	ND
pILM5	SG1606	C.E.	ND	-	21,620	2,350
		sup.	12	-	25	39
pILM5	IY103	C.E.	ND	28,526	24,830	1,660
		sup.	73	68	111	97
mILM6	SG1606	C.E.	ND	-	17,560	32,770
		sup.	ND	-	-	194

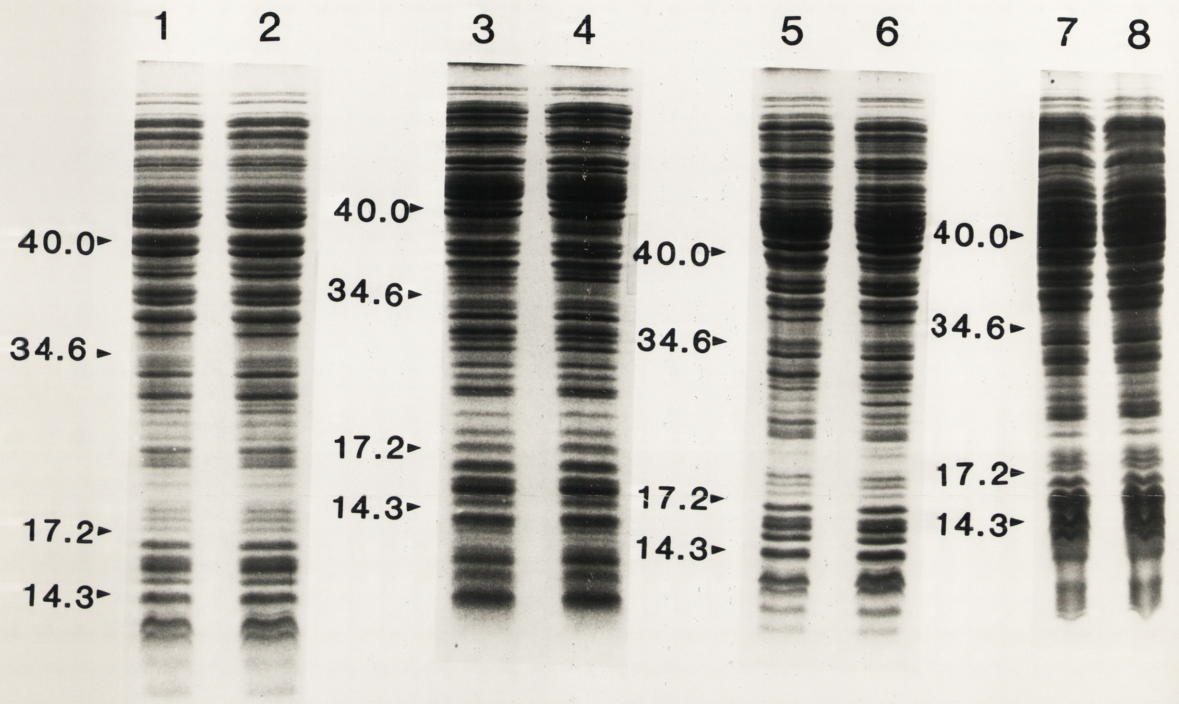
The cells were grown in LB medium to 2×10^8 cells/ml. The tac promoter in pILM5 or lacUV5 promoter in mILM6 was then induced by the addition of 1mM IPTG as described in Methods 4.9 and 4.10. Cells or culture supernatant were harvested at different times after induction and were prepared as described in Method 4.9 and assayed for IL-3 activity as described in Method 2.7. The IL-3 activity is presented in end point units. This is the reciprocal of the dilution where the titration curve of IL-3 activity meets the baseline level of background plus 3 standard deviations. For comparison the average IL-3 activity in WEHI-3B conditioned medium was 2,460 units/ml.

ND, no detectable activity. C.E., cell extract. sup., culture supernatant.

JM101. This indicated that glycosylation is not essential for biological activity and that expression in bacteria is feasible. Some expressed IL-3 was detected in uninduced cell extracts of JM101 but no expressed IL-3 could be detected after induction with IPTG in the cell extracts of JM101 carrying either the parent vector pDR540 or pDR-pH1 which carries the IL-3 gene in the wrong orientation for expression.

Much higher levels of expressed IL-3 were obtained with the lon strains SG1606 and IY103 after 2 hrs induction, presumably due to the greater stability of IL-3 in strains defective for the lon protease. However the levels of IL-3 activity after 4 hrs induction were only about one tenth of those after 2 hrs induction, suggesting that IL-3 was still being degraded in the lon strains. The levels of IL-3 activity 2 hr post induction were about 10-fold higher than typical WEHI-3B conditioned medium. If the bacterial recombinant IL-3 has a normal specific activity these levels would indicate that even at 2 hrs after induction only a small amount of IL-3 protein was produced. In agreement with this prediction, no new bands were evident after induction when the cell extracts were examined by SDS/PAGE using 15% polyacrylamide gels (Figure 4.15). However, the large number of protein bands in the region of interest make it likely that the IL-3 protein would

Figure 4.15 SDS/PAGE analysis of cell extracts from different host strains carrying pILM5 or mILM6. Lanes are: (1) and (2), pILM5/JM101; (3) and (4), pILM5/SG1606; (5) and (6), pILM5/IY103; (7) and (8), mILM6/SG1606. The extracts in lanes 1, 3, 5 and 7 were from cells induced with 1mM IPTG whereas those in lanes 2, 4, 6 and 8 were from uninduced cells. Strains carrying pILM5 were induced by the addition of 1mM IPTG to cells in early log phase (2×10^8 cells/ml) and growth was continued at 37°C. Phage mILM6 was transfected SG1606 cells in early log phase and was induced by the addition of 1mM IPTG (Method 4.10). After 2 hr induction cell extracts were prepared and analyzed on a 15% polyacrylamide-SDS gel (Method 4.9). The size markers are indicated by ▶ (X1000). The direction of electrophoresis is from top to bottom.



only be detected if it were produced in high levels. A sample of cells from the induced cultures was examined to test for loss of the plasmid after induction. The results (Table 4.3) indicate that a high proportion of the cells still possessed the plasmid pILM5 in these experiments.

The lower than expected expression of IL-3 from the tac promoter could have been due to one or a number of the following factors:- (1) degradation of the expressed IL-3, (2) poor transcription due to the presence of transcription termination signals in the IL-3 coding sequence, (3) poor translation due to codon usage, suboptimal ribosome binding site spacing or the sequence used around the initiation codon. Due to the limited time available in the present work these possibilities were not investigated in detail and it was decided to try the expression of a fusion protein.

8. Expression of IL-3 from mILM6 in SG1606.

Good levels of translation and increased stability of expressed protein can sometimes be achieved by joining the coding sequence for the protein of interest to the ribosome binding site and N-terminal coding sequence of a bacterial gene. In the M13 vector mILM6, the IL-3 coding sequence from residue number 27 (Alanine) was joined to the β -

Table 4.3 Stability of pILM5 following IPTG induction of various host strains

<u>Time after induction</u>	<u>% of cells carrying pILM5</u>		
	<u>JM101</u>	<u>SG1606</u>	<u>IY103</u>
0 hr	100%	100%	100%
2 hr	100%	92%	100%
4 hr	96%	88%	96%

pILM5 in different host strains was grown in LB medium at 37°C to 2×10^8 cell/ml. IPTG was added to 1mM final concentration and the cells grown at 37°C. Samples were taken out at 0 hr, 2 hr and 4 hr after induction, serial dilutions prepared and plated on LB agar plates with and without ampicillin.

galactosidase gene such that the first 9 residues of the expressed protein were from β -galactosidase.

SG1606 was grown to early log phase (2×10^8 cells/ml) and infected with mILM6 phage at a multiplicity of infection of 100. The phage was allowed to absorb on the cells for 30 min at 37°C and then induced with 1mM IPTG (Method 4.10). The results (Table 4.2) showed that the expressed IL-3 level in the cell extract after 4 hr post induction is roughly twice that present after 2 hr induction. This indicates that the hybrid β -galactosidase-IL-3 polypeptide is more stable in E. coli than the non-fusion IL-3 polypeptide.

9. Future possibilities for expression of IL-3.

The enhanced stability of the IL-3 fusion protein suggests that a fusion protein strategy could be the most successful for IL-3 expression in E. coli. Recent experiments by Dr. S. Clark of Biotechnology Australia have shown that good levels of activity can be obtained using a similar fusion strategy to that of mILM6 but using vector pUC9. In this case the level of expression has increased to the point where a new band of MW about 17K can be seen in cell extracts analyzed by SDS/PAGE.

Another interesting fusion approach which would yield IL-3 without a foreign N-terminal sequence would

be to use the strategy of Nagai and Thogersen (1984). In this strategy the protein of interest was fused to the λ cII protein with a factor X recognition signal peptide (Ile-Glu-Gly-Arg). The expressed hybrid protein can, therefore, be cleaved by factor Xa just after the residue Arginine to give rise to a mature protein of interest.

VI. Discussion.

To confirm the identity of the cloned cDNA as that encoding murine IL-3, the cDNA insert in pILM3 was ligated into pSV₂-neo such that it was under the control of the SV40 early promoter. This construct was then expressed in monkey COS-1 cells and in Xenopus oocytes. Biologically active IL-3 was produced in both cases. The expression of the IL-3 gene in the oocyte nucleus provides a potentially useful assay for future studies on the regulation of the IL-3 gene. Expression of the IL-3 gene in the monkey COS-1 cell system allowed sufficient recombinant IL-3 to be produced to determine its biological properties. IL-3 produced in the pILM4 transfected COS cells was indistinguishable from that of the IL-3 produced by WEHI-3B cells in terms of its chromatographic properties on DEAE Sephacel and phenylsepharose. The recombinant IL-3 not only supported the growth of the IL-3 dependent cell lines 32Dcl-23 and FDC-P1 but also induced 20 α SDH in splenic lymphocytes from nu-nu mice. The expressed IL-3 also showed a multilineage hemopoietic growth factor activity. It supported the growth of 'P' cells and stimulated proliferation and differentiation of granulocyte, granulocyte/macrophage, macrophage, eosinophil, megakaryocyte and erythroid cells.

The IL-3 cDNA was also expressed in E. coli as a

mature protein starting with the amino acid number 28 (Serine) or as a fused polypeptide with an extra 9 amino acids and starting with the amino acid number 27 (Alanine). In both cases, the expressed IL-3 was biologically active. This indicated that glycosylation is not essential for biological activity and that expression in bacteria is feasible. The IL-3 expressed from pILM5 appears to be less stable than the IL-3 fusion polypeptide produced using mILM6 (Table 4.2). The bacterial expression experiments carried out so far indicate several potentially useful future strategies for enhanced IL-3 expression.

Chapter 5 - General Discussion

5.1 Application of recombinant DNA techniques to the study of IL-3.

Lymphokines and hemopoietic growth factors are produced in minute amounts by cells and are active at very low concentrations (approx. 10^{-12} M). The purification of these factors by conventional biochemical methods is therefore quite difficult and it is not easy to completely rule out the possibility of contamination with other factors. The isolation of cDNA clones encoding these proteins is a valuable complementary approach and enables definition of factors from their protein sequences. Production of recombinant lymphokines and hemopoietic growth factors in eukaryotic and prokaryotic systems on a large scale allows wide ranging biological studies to be carried out including clinical trials in the case of human lymphokines.

The work described in this thesis is an attempt to apply recombinant DNA techniques to the study of IL-3. An assay for murine IL-3 mRNA was developed using microinjection in Xenopus laevis oocytes. This enabled purification of IL-3 mRNA, construction of a cDNA library and the isolation of a cDNA clone for IL-3. The cDNA clone was expressed in monkey COS cells using an SV40 expression vector providing recombinant

IL-3 for biological studies. Preliminary studies were also carried out concerning the expression of IL-3 in E. coli.

A short time after the work described in this thesis was published (Fung et al., 1984) a report appeared describing the isolation of a cDNA clone for a murine mast cell growth factor (Yokota et al., 1984) from a cDNA library prepared from a T cell line of C57BL/6 origin. The DNA sequences of the IL-3 cDNA clone described in the present work and that of the MCGF cDNA clone are virtually identical except for 1 nucleotide difference at position 463. It is therefore clear that the MCGF cDNA actually encodes IL-3, which is known to have MCGF activity and this was subsequently verified in biological studies of recombinant factor produced using the MCGF cDNA clone (Rennick et al., 1985; Hapel et al., 1985a; Greenberger et al., in Press).

A detailed study of the biological activity of recombinant IL-3 purified from pILM4 transfected COS-1 cells (see Chapter 4) was made in collaboration with Dr. A. Hapel and Dr. D. Metcalf and his colleagues (Hapel et al., 1985a). The studies with recombinant IL-3 provide strong support for the proposal that the biological activities previously reported under the names of IL-3, BPA, PSF, MCGF, HCGF, HCSF and multi-CSF can be attributed to a single factor and that IL-3

is a multil lineage CSF (Schrader and Hapel, 1985).

The work described in Chapter 4 on the expression of IL-3 in E. coli established that biologically active IL-3 could be produced in E. coli indicating that glycosylation is not essential for activity. This work has been extended in collaboration with Biotechnology Australia and purified bacterial recombinant IL-3 can now be prepared in mg quantities. This should greatly assist future biological studies including those of the IL-3 receptor (Palasynski and Ihle, 1984).

The availability of cDNA clones for IL-3 has also enabled the isolation of the murine IL-3 gene, both in this laboratory and elsewhere (Campbell et al., 1985; Miyatake et al., 1985). Like the genes for IL-2, γ -IFN and GM-CSF (Fujita et al., 1983; Gray et al., 1982; Gough et al., 1984), the IL-3 gene exists as a single copy in the haploid genome. The IL-3 gene contains four introns interrupting the coding sequence. A short tandem repeat is found in intron 2 which shares extensive homology with an enhancer found in human genome. Eight out of nine repeats form a 73-bp duplicated sequence and each 73-bp repeat contains sequences homologous to the core sequence suggested for enhancer elements. In addition, a G+C-rich region is found in the 5' flanking region (Campbell et al., 1985; Miyatake et al., 1985). Determination of the

structure of the murine IL-3 gene serves as a basis for further studies concerning the regulation of IL-3 expression and gene rearrangements giving altered IL-3 expression.

Interesting possibilities for future work involve gene transfer experiments using retroviral vectors. Also isolation and expression of a cDNA clone for human IL-3 should enable studies on potentially important clinical applications of human IL-3 in bone marrow transplantation.

5.2 Recent advances in the molecular biology of other lymphokines.

The isolation of cDNA clones and the respective genes for human γ -IFN and human IL-2 (Gray et al., 1982; Devos et al., 1982; Nishi et al., 1985; Taniguchi et al., 1983; Clark et al., 1984; Devos et al., 1983; Fujita et al., 1983; Degrave et al., 1983; Gray and Goeddel, 1983; Taya et al., 1982; Maeda et al., 1983) was described in the Introduction. More recently, cDNA clones have been isolated for murine γ -IFN (Gray et al., 1983), murine IL-2 (Yokota et al., 1985; Kashima et al., 1985), murine GM-CSF (Gough et al., 1984), human erythropoietin (Lee-Huang, 1984; Jacobs et al., 1985) and human lymphotoxin (Gray et al., 1984). Also cDNA clones have been isolated for the monokines, murine IL-1 (Lomedico et al., 1984) and

human IL-1 (Auron et al., 1984). Currently the field of lymphokine biology is being intensively studied using recombinant DNA methods and a considerable amount of this research is being done by biotechnology companies.

In the cases which have been examined so far there is only a single gene coding for each factor in the haploid genome. The structures of the genes for the lymphokines IL-2, IL-3 and γ -IFN show only limited homologies (Campbell et al., 1985; Degrave et al., 1983; Fujita et al., 1983) and considerably more work is needed to try and understand the regulation of lymphokine gene expression in lymphocytes.

To date no significant homologies in primary amino acid sequences have been reported between the various haemopoietic growth factors and lymphokines which have been cloned. In the present work the program "Dragon" (Staden, 1982b) was used to compare the protein sequences of murine GM-CSF, murine and human IL-2, murine and human γ -IFN, and human erythropoietin with that of murine IL-3. Little homology was detected. In the case of erythropoietin and IL-3 one short region of homology was detected as shown below.

	125		138
murine IL-3	D D F R K K L R F Y M V H L		
	* * * * *	* *	*
human erythropoietin	D T F R K L F R V Y S N F L		
	163		176

Thus even though some factors like GM-CSF and IL-3 have biological activities in common, the haemopoietic growth factors and lymphokines all appear to be unique and don't belong to a family of related protein sequences.

5.3 Recent studies on the biology of IL-3.

The biological activities of IL-3 have recently been reviewed by Iscove and Roitsch (1985). IL-3 is now considered as a multilineage hemopoietic growth factor. It is believed to act on the pluripotential hemopoietic progenitors and early committed precursors in all the hemopoietic lineages. It can induce the differentiation and maturation of neutrophilic granulocytes, macrophages, eosinophils and mast cells (Prystowsky et al., 1984; Greenberger et al., 1983; Iscove and Roitsch, 1985; Hapel et al., 1985a). However, the maturation of erythroid and megakaryocyte cells require additional factors apart from IL-3 (Iscove, 1978; Williams et al., 1981 and 1982).

The early proposal of the direct effect of IL-3 on early T-cell differentiation (Ihle et al., 1981;

Ihle et al., 1983b) seems likely to be incorrect. One source of error was the initial assumption that the enzyme, 20 α SDH, the induction of which was the definitive assay for IL-3, was a specific marker of the T-cell lineage. It has been shown that 20 α SDH is expressed, not only in T lymphocytes, but also in myeloid cells, including macrophages (Hapel et al., 1985b). Furthermore, the 20 α SDH can be induced (i) in nu/nu spleen and in normal fetal liver cells by GM-CSF, (ii) in longer term cultures of fetal liver and adult marrow by M-CSF (Hapel et al., 1985b), and (iii) in fetal liver and nu/nu marrow cells by horse serum and hydrocortisone (Garland and Dexter, 1982). It was recently reported that IL-3 enhanced the murine primary cytolytic T lymphocyte response to allogenic tumor cells and might be involved in the differentiation of cytolytic T lymphocytes (Curtsinger and Fan, 1984). However, there was no evidence that IL-3 was directly acting on the cytolytic T lymphocytes since a mixture of spleen cells was used in the assay and the reaction of IL-3 may have been an induced effect. It has been reported that IL-3 supports the growth of mouse pre-B cell clones (Palacios et al., 1984). However, the monoclonal antibody used to identify pre-B cells was known to be non-specific. Therefore the possibility of IL-3 acting directly on T or B lymphocytes needs further investigation.

Recently, Luger et al. (1985) and Frei et al. (1984) reported that keratinocytes and astroglia respectively produce a molecule with IL-3-like activities. If this can be confirmed by measurement of mRNA by Northern blots using the IL-3 cDNA probe it raises the interesting possibility that IL-3 is produced in other cell types apart from T lymphocytes and the WEHI-3B cells. However, the types of tissues that produce IL-3 in vivo is still not clear. In this connection future application of tissue hybridization methods using the IL-3 cDNA probe should greatly assist our understanding of the role of IL-3 in vivo.

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