Molecular Cloning, Characterisation and Expression of the Leukaemia Inhibitory Factor (*LIF*) Gene from the Marsupial *Sminthopsis crassicaudata*

By

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A thesis presented in fulfilment of the
requirement for the Degree of Doctor of Philosophy

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University of Adelaide

September, 1998

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2	4		Insert: Fig. 2.1 (b) Diagrammatical domain structure of LIF
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176	5	61.5% (±10.9) to 96.9% (±15.5)	61.5 % to 96.9%
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211	4		Insert: Senturk, L. M. and Arici, A. (1998) Leukemia inhibitory factor in human reproduction. *Am J Reprod Immunol.* **39**:144-151
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Declaration

I declare that this thesis contains no material which has been submitted previously for the award of any other degree or diploma in any university or any tertiary institution, and that to the best of my knowledge and belief, it contains no material previously published or written by other person except where due reference is made in the text.

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Shuliang Cui

Signed

Date: 14/9/1998

Acknowledgments

It is great honour to acknowledge with gratitude, the enthusiasm, encouragement and supervision rendered by my supervisor, **Dr. Rory Hope** throughout the course of my studies, especially the provision of this opportunity for me to return to the Department of Genetics, without which nothing would have happened. Thanks for his great efforts and patience in assistance with the preparation of this thesis.

I am deeply grateful to my unofficial supervisor **Professor Peter Rathjen** from the Department of Biochemistry, University of Adelaide for his continuous encouragement, discussion and support, particularly for his supervision at the critical final stage of my studies during the absence of Dr. Rory Hope and for his suggestions and comments on the draft of this thesis.

Many thanks to **Professor Robert Saint** for his special consideration and provision of financial support in the final year and the technical assistance from him and his group. Thanks to **Dr. Joan Kelly** for the help from her and her group, especially for provision of some experimental materials. Thank you to **Dr. Jeremy Timmis** for his support and help.

Thank you to the following people who helped me during the course of the project: **Associate Professor Bill Breed**, Department of Anatomy and Histology, University of Adelaide for his valuable advise and provision of animal tissues and organs; **Dr. Claire Roberts**, Department of Obstetrics and Gynaecology, University of Adelaide for making some old tissue section slides available for gene expression study; **Dr. Joy Rathjen,** Department of Biochemistry, University of Adelaide for her assistance with ES cell culture for the functional assays; **Drs. Stephen Donellan** and **Steven Cooper**, Evolutionary Biology Unit, South Australia Museum, for providing some experimental materials; **Dr. Lynne Selwood**, Department of Zoology, La Trobe University, for a very informative discussion and advise when she was in Adelaide; **Roger Voyle** and **Bryan Haines**, Department of Biochemistry, University of

Adelaide, for their continuous help; **Ms. Louise O'Keefe** for her valuable discussions, advice and assistance with the recombinant protein work. **Dr. Robert Shroff** for his enthusiastic help and for answering so many questions; **Mr. Clive Chesson**, a former member of Genetic Department, for his assistance in reading part of the Discussion section of this thesis.

I would like to thank my fellow postgraduate students in the department, particularly **Mi-Hye Lee** for sharing an enjoyable working environment and discussions from time to time for the whole period. Also, I would like to thank **all the members** in the Department of Genetics for their help and friendship, especially **Mr. Doug Pottrell** for his assistance with all sorts of technical aspects through the years.

The Department of Education, Employment and Training (DEET) of Australian Federal Government and **the University of Adelaide** are acknowledged for providing me with the opportunity to carry out this project and for granting the OPRS scholarship and honourable AUS scholarship for postgraduate research respectively to cover the fees and living allowance.

Finally, I would like to thank my wife **Cheng** and my son **Tongtong (Xiaokang)** and a big thanks goes to my **parents**, for their unlimited support and encouragement.

List of abbreviations

α-^{32}P-dATP	Deoxyadenosine phosphate labelled with ^{32}P
aa	amino acid
AMV	avian meyloblastosis virus
amp	ampicillin
amp^{R}	ampicillin resistant
ANGIS	Australian National Genomic Information Services
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
^{o}C	degree Celsius
cDNA	DNA complementary to RNA
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxy-nucleoside-5'-triphosphate
d/dNTP	2',3'-dideoxy-nucleoside-5'-triphosphate
ds	double strand
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
E. coli	*Escherichia coli*
EtBr	ethidium bromide
g	force of gravity

Abbreviations

GENBANK	A database of nucleic acid and protein sequences at the National Library of Medicine in the United States of America, compiled from international sources.
GST	glutathione *S*-transferase
IL	interleukin
IPTG	isopropyl-b-D-galactosidase
kan^R	resistance to the antibiotic kanamycin
kb	kilobase
kDa	kilo-Dalton
Km	kanamycin
λ	lambda phage DNA
LMT	low melting temperature
LB	Luria-Bertani medium
LIF	leukaemia inhibitory factor gene
LIF	leukaemia inhibitory factor protein
MCS	multiple cloning site(s)
mM	millimolar
M-MLV RT	moloney murine leukemia virus reverse transcriptase
m.o.i	multiplicity of infection
mw	molecular weight
μ*Ci*	microCurie
N	any nucleotide
NaAC	sodium acetate
nM	nanomolar
nt	nucleotide(s)
ODn	optical density at wavelength of n
oligo	oligodeoxyribonucleotide
ORF	open reading frame
ori	origin(s) of DNA replication
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

List of abbreviations

PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming unit
pH	hydrogen-ion exponent
PVP	polyvinylpyrolidone
PMSF	phenylmethylsulfoyl floride
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
ss	single strand(ed)
ssssDNA	sheared single-stranded salmon sperm DNA
TEMED	tetramethylethylenediamine
tet^R	resistance to the antibiotic tetracycline
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
u	unit of enzyme
UV	ultra-violet
v	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

Nomenclature used in this thesis:

The terms "monotreme", "marsupial" and "eutherian" are used to describe the three major extant groups of mammals. The term "eutherian" is preferred to "placental" because the latter could be taken to imply (incorrectly) that marsupials do not have a placenta.

The following Table shows symbols used in this thesis to identify various *LIF* genes and clones.

Symbol	Identification
mLIF, *hLIF*, *sLIF* etc.	Mouse *LIF*, Human *LIF*, *S. crassicaudata LIF* etc.
mLIF ORF, *hLIF ORF*,	Mouse *LIF* and Human *LIF* entire open reading frame cDNA
mLIF, hLIF, sLIF etc.	Mouse LIF, Human LIF, *S. crassicaudata* LIF etc.
λ-*sLIF*	λ-phage clone isolated from an *S. crassicaudata* genomic library on the basis of its hybridization to *mLIF* and *hLIF* cDNA clones.
psLIF-1 *psLIF*-1.1 *psLIF*-1.2	Sub-clones of λ-*sLIF* in the plasmid vector *pBluescript*
psLIF	*sLIF* cDNA clone in the plasmid pBluescript
pGEX2T-sLIF	*sLIF* cDNA clone in the expression vector pGEX2T

Summary

The aims of this research project were: i) to identify and characterise a marsupial homologue of the murine and human genes encoding LIF (Leukemia Inhibitory Factor), ii) to investigate marsupial *LIF* gene expression in adult and embryonic tissues, iii) to investigate the functional conservation of marsupial LIF, and iv) to investigate the evolution of LIF in mammals.

The Fat-tailed Dunnart, *Sminthopsis crassicaudata*, was used as a model marsupial species, because a laboratory colony of this marsupial was maintained in the University of Adelaide, so animals and DNA samples were readily available. Furthermore, the species is relatively well-characterised in its anatomy, histology, embryology, biochemistry, genetics and evolution.

A genomic DNA library from *S. crassicaudata* was constructed in the bacteriophage λGEM-11 and screened by hybridisation to mouse and human cDNA clones containing the entire *LIF* open reading frame. A single phage clone (λ-*sLIF*) was isolated from the library and partially characterised by restriction mapping and Southern analysis. Subsequently, a 8.75 kb fragment of this clone, that hybridised to the mouse and human *LIF* probes, was sub-cloned into the plasmid vector pBluescript II KS^+. This subclone (*psLIF*-1) was further analysed by restriction mapping and Southern hybridisation. LIF coding regions in the fragment were mapped and sequenced. *psLIF*-1 was found to contain the entire LIF exon 2, encoding the first 60 amino acids of the mature LIF protein, and the 5' coding region of exon 3 that encoded the remainder of the mature protein.

A cDNA fragment encoding the mature LIF protein from *S. crassicaudata* was isolated (using a pair of primers designed from the genomic DNA sequence) by reverse transcription PCR (RT-PCR) from its mRNA template. This fragment was then modified, using oligonucleotide-directed PCR, to remove an internal *Bam*HI restriction site, so that the *Bam*HI sites at both ends of the fragment could be used for in-frame insertion into the expression vector pGEX2T. The clone so formed

(*pGEX2T-sLIF*) was used for the production of GST-sLIF fusion protein. The fragment was also cloned into pBluescript II KS^+.

Southern hybridisation using mouse and human *LIF* cDNA probes, was carried out on genomic DNA samples from various mammalian species. The filters displayed two weakly hybridising *Bam*HI fragments from *S. crassicaudata* but failed to display hybridisation signals from other marsupial or monotreme species. In contrast, strongly hybridising bands were observed for various marsupial and monotreme species (but not from human or mouse) when *psLIF* was used as a probe. The results suggest, somewhat surprisingly, that the *LIF* coding regions of marsupials and monotremes are more similar in sequence than either is to the homologous regions in eutherian mammals. This, in turn, suggests that *sLIF* would be a useful probe for isolating *LIF* homologues either from other marsupial species, or from monotremes.

The *sLIF* cDNA sequence and the encoded LIF protein were used for evolutionary comparisons with homologues from several eutherian species including mouse, human, cow, sheep, pig and rat. A high level of identity in LIF nucleotide and amino acid sequence was shown to exist amongst seven species (six eutherians and *S. crassicaudata*). The amino acid sequence at the splice site of the LIF protein (that is, the site which is cleaved to produce mature LIF) is highly conserved. There are other examples of fully conserved amino acid residues indicating that these are likely to be important for integrity and activity of the LIF molecule.

Tests were carried out to compare the relative rates of nucleotide substitution in *LIF* along different evolutionary branches of the species phylogeny. Overall, these tests provided no evidence for significant differences in evolutionary rates, either at synonymous or non-synonymous sites. As expected, synonymous sites evolved at a higher rate than non-synonymous sites.

LIF nucleotide and amino acid sequence comparisons were used for phylogenetic analysis using maximum parsimony algorithms; *LIF* from *S. crassicaudata* was used as an outgroup. A single most parsimonious tree of the same general conformation was obtained whether DNA or protein sequences were used. Some of the nodes on

the trees had low bootstrap values reflecting, in part, the high degree of conservation and therefore small number of phylogenetically informative sites.

To investigate the tissue distribution of *sLIF* transcripts in *S. crassicaudata*, Northern blot analyses of RNA samples from adult tissues and organs were carried out using mouse and *S. crassicaudata* cDNA clones as probes. These experiments failed to detect any hybridisation signals, a result that is consistent with similar experiments that have been carried out on other species. Using the more sensitive RT-PCR technique, coupled with Southern analysis using *psLIF* as a probe, *sLIF* was shown to be expressed in a broad range of adult tissues, with the exception of liver and kidney. Strong expression was detected in the adult uterus. RT-PCR products were amplified from RNA samples isolated from formalin-fixed paraffin-embedded uterus/embryo tissue sections of *S. crassicaudata.* These sections had been isolated from pregnant females at around the time of implantation of their blastocysts. The amplified DNA fragments showed strong hybridisation to *psLIF*, indicating that *LIF* is expressed in these tissues at the time of implantation.

Over-expression of *pGEX2T-sLIF*, induced by IPTG, produced a 46 kDa fusion protein. This protein was isolated from bacterial cell lysates, purified by GST affinity chromatography, and digested with thrombin to release recombinant sLIF polypeptide as a soluble protein. After purification and quantification, the protein was used in functional assays *in vitro*. In the presence of sLIF, differentiation of cultured mouse embryonic stem cells was inhibited. Recombinant sLIF was therefore biologically active in cultured mouse cells.

In conclusion, a *LIF* gene has been identified, isolated and partially sequenced from a marsupial, *S. crassicaudata*. Evolutionary analyses have been carried out on the basis of sequence comparisons between the marsupial gene *sLIF* (and its encoded product) and homologous sequences from eutherian mammals. Marsupial *LIF* has been expressed *in vitro* and the protein product tested for its capacity to inhibit the differentiation of mouse ES cells.

Summary

This is the first report of the presence of *LIF* in a marsupial. The findings that i) the marsupial and eutherian *LIF* genes (and their protein products) are highly conserved in overall organisation and coding sequence, ii) that monotreme mammals appear to contain DNA sequences that hybridise to marsupial *LIF* cDNA probes, iii) that *sLIF* is expressed at low levels in most adult tissues and is expressed in uterus at the time of embryo implantation, and iv) that marsupial LIF functions to suppress mouse ES cell differentiation, lead to the conclusion that LIF probably plays a key role in the fertility and viability of mammals, including marsupials.

Chapter 1 Introduction

Leukemia Inhibitory Factor (LIF) is a cytokine that has been extensively studied in several eutherian mammal species, particularly in the mouse. Mouse LIF was originally characterized by its ability to suppress the proliferation of cells in the murine myeloid leukaemia cell line M1, by inducing their irreversible differentiation to macrophage cells (Metcalf *et al.*, 1988). LIF was also shown to suppress the differentiation of totipotent mouse embryonic stem cells (Williams *et al.*,1988; Smith *et al.*, 1988). These two properties are intriguing as they appear to be in contrast with one another. Further studies of LIF have shown that it has a diverse set of biological activities on different target cell types *in vitro* and on different adult and embryonic tissues *in vivo* (Gough and Williams, 1989; Hilton and Gough, 1991; Thompson and Majithia, 1998). The cellular responses to LIF are initiated through binding of the LIF molecule to a specific receptor on the cell surface (Nichols *et al.*, 1996).

LIF genes and their encoded proteins have been identified and characterized in several species of eutherian mammal, in addition to the mouse. These include human, cow, pig, sheep, rat, and mink (Gearing *et al.*, 1987; Gough *et al.*, 1988; Yamamori *et al.*, 1989; Willson *et al.*, 1992; Kato *et al.*, 1996; Piedrahita *et al.*, 1997; Song *et al.*, 1998). On the basis of their similar amino acid sequences, the LIF molecules from these species are assumed to have similar molecular conformations and functions to those of mouse LIF.

Robertson *et al.* (1994) used X-ray crystallography to determine the three dimensional structure of mouse LIF. Functionally important regions of the LIF molecule, including those concerned with binding to the LIF receptor complex, have been mapped onto the LIF 3-D structure (Robertson *et al.*, 1994; Layton *et al.*, 1994; Hudson *et al.*, 1996).

The human and murine *LIF* genes have been completely sequenced, and their intron/ exon boundaries determined (Stahl *et al.*, 1990). The coding regions of *LIF* genes, and regions of their promoters, are conserved across eutherian species (Willson *et al.*,

1992). Two alternative *LIF* transcripts, that use alternative first exons, have been identified in the mouse (Rathjen *et al.*, 1990a).

The cloning of *LIF* made it possible to investigate the expression and biochemical properties of this molecule in different biological environments. *LIF* is normally expressed at low basal levels in adult tissues and organs, and is difficult to detect by the classical Northern hybridization technique (Robertson *et al.*, 1993). The more sensitive methods such as RNase protection assays and RT-PCR, detected low levels of *LIF* transcripts in variety of adult tissues (Bhatt *et al.*, 1991; Robertson *et al.*, 1993; Charnoch-Jones *et al.*, 1994; Estrov *et al.*, 1995). The most abundant *LIF* expression in mouse occurs in the uterine endometrial glands on day 4 after plug, when implantation occurs (Bhatt *et al.*, 1991). In humans, maximal *LIF* expression occurs in the endometrium between day 19 and day 25 of the menstrual cycle, again coinciding with the time of implantation (Nachtigall *et al.* 1996). Up-regulation of LIF expression in the trophectoderm during early mouse embryogenesis is accompanied by a complementary up-regulated LIF receptor expression in the inner cell mass (Nichols *et al.*, 1996).

Direct evidence for a role of LIF in implantation was provided by Stewart *et al.* (1992), who interrupted the reading frame of *LIF* by targeted mutagenesis. The resultant LIF protein lacked C-terminal amino acids thought to be essential for *in vitro* biological activity. ES chimeras generated from homologous recombinant clones were used to produce individuals homozygous for the mutant *LIF* allele. Homozygous males and females were mated and their developing embryos studied. The morphologically normal blastocysts found in the uterine lumen of homozygous females failed to implant and develop. However, when such blastocysts were transferred to normal pseudo-pregnant females, they implanted and developed to term (Stewart *et al.*, 1992). It can be concluded that maternally expressed LIF plays a critical role in the process of embryo implantation.

There are three mammalian infra-classes, Metatheria, Prototheria and Eutheria, commonly called marsupials, monotremes and (incorrectly) "placentals" (marsupials have a placenta). The marsupials and eutherians, sometimes collectively referred to

as the subclass Theria, are thought to have separated from one another about 100 - 150 million years ago (MYA) (Air *et al.*, 1971; Beard and Thompson, 1971; Cifilli and Eaton, 1987; Kirsch *et al.*, 1997). The monotreme and therian lineages are estimated to have separated about 200 MYA (Clemens, 1989), ie. before the divergence of the marsupials and placentals, although there is some debate about the timing of these evolutionary events (Janke *et al.*, 1996; 1997). The marsupial infra-class is composed of more than 250 species that are grouped into 16 families, including 3 American families and 13 Australian families (Kirsch and Calaby, 1977).

As a result of the ancient evolutionary separation between marsupials and eutherians, comparative studies of the organisation, expression and function of *LIF* in representative marsupial species are likely to be particularly informative. Such studies would enable the evolutionary history of *LIF* to be more clearly established and would enable regions of the gene/protein to be identified, that have retained their sequence identity for at least 100 million years. Highly conserved regions would be indicative of the action of strong selective forces, and therefore help to identify conserved functional domains. The molecular structure of LIF and its receptor are expected to have evolved in parallel; it is unlikely that molecular changes to one of these components can evolve independently of changes to the other. Studies on the molecular biology of LIF in a marsupial, would be a first step towards investing the interaction between LIF and its receptor/s in marsupials and these investigations would provide valuable evidence on the co-evolution of LIF and its receptor in mammals.

A distinguishing feature of marsupials and eutherians is their different modes of reproduction and early stages of development and growth. Marsupial embryos experience a relatively short period of uterine attachment *via* a placenta, are born in a relatively undifferentiated state, and continue their development in an external pouch where they depend, initially, on resources (including milk) provided by their mother (Selwood and Woolley, 1991; Selwood, 1994).

In some marsupial species, including the Kangaroo Island Wallaby, *Macropus eugenii*, embryonic diapause (delayed implantation) occurs at the blastocyst stage of

development. Diapause is a temporary arrest of embryonic development at the unilaminar blastocyst stage. The arrest varies from total cessation of mitosis and growth to a variable degree of mitotic activity and growth (Sharman, 1963; Tyndale-Biscoe and Renfree, 1987; Shaw, 1996). *Postpartum* mating results in fertilisation, but the stimulus of the suckling young in the pouch retards the development of the newly formed embryo; the egg cleaves but does not increase greatly in size to reach the blastocyst stage. However, the embryo re-commences development if the suckling young is lost or removed from the pouch. Fertilised eggs *in vitro* are able to grow to the blastocyst stage in simple culture media but it has proved difficult to culture blastocysts over the implantation period (Tyndale-Biscoe, 1968). Blastocysts appear to require an improved nutritional environment and also a physiological trigger to release them from diapause and to synchronise their development in the uterus (Tyndale-Biscoe, 1968).

There is a paucity of information about the molecular mechanisms involved in embryonic diapause and the early stages of marsupial development and implantation. Studies on the spatial and temporal expression of *LIF* in a marsupial are likely to provide important data on these phenomena.

The treatment of cultured mouse blastocysts *in vitro* with LIF improves the efficiency of their subsequent development and implantation (Lavranos and Seamark, 1989, Robertson *et al.*, 1990;). LIF can act as a substitute for a cellular feeder cell layer in inhibiting the differentiation of cultured murine embryonic stem cells (Hooper *et al.*, 1987; Smith and Hooper, 1987; Gearing *et al.*, 1987, 1989; Williams *et al.*, 1988). These and other aspects of LIF function suggest that this protein could be an important biological agent for use in marsupial embryo manipulation. Given the findings by Stewart *et al.* (1992) that LIF expression in the pregnant mouse is essential for blastocyst implantation, it follows that manipulating or interfering with the expression of LIF could be used to block normal reproduction. Therefore, knowledge of the molecular biology of LIF from a marsupial could be used to develop strategies for artificial control of marsupial population size.

Before commencement of this project, no detailed studies of *LIF* had been carried out in a non-eutherian mammal. The author is aware of only a single reference to marsupial LIF: Spencer *et al.* (unpublished; cited by Willson *et al.*, 1992) are reported to have used Southern analysis and mouse *LIF* cDNA probes to detect the presence of LIF-like sequences in genomic DNA from "several marsupial species". The hybridisation signal was low and the background signal high. To date, no investigations of LIF have been carried out in a monotreme.

When set against the background literature on LIF (see Chapter 2), there are a number of reasons why a detailed molecular study of LIF in a marsupial is likely to be rewarding:

1) An analysis of the structure, expression and function of the *LIF* gene in a marsupial will contribute to our overall knowledge about this important gene and its product.
2) There is no evidence for the presence of LIF in non-mammalian species, yet the *LIF* gene and its protein product are conserved in sequence (and presumably function) in eutherian mammals. Therefore, if the presence of LIF can be confirmed in marsupial, it will enable the evolutionary origins of LIF to be extended back to at least 100 MYA. Furthermore, as a result of the ancient separation of the marsupial, monotreme and eutherian lineages, studies of *LIF* in all three mammalian groups are likely to be informative in an evolutionary context.
3) In the longer term, it would be of great interest to study the pattern of *LIF* expression in the marsupial tissues associated with embryonic diapause. It is possible that maternal *LIF* expression contributes to the re-activation of development of the diapaused embryo.
4) There are significant differences in the early stages of development of eutherians and marsupials. For example, the latter have a rudimentary placenta compared to the former. Differences between eutherians and marsupials in the spatial and temporal expression of *LIF* may provide important clues to LIF function.
5) The availability of marsupial LIF would help to isolate and propagate marsupial ES cells. To date, no such cell cultures have been established.

6) Cloned *LIF* DNA sequences from a marsupial would provide molecular probes of use for studying *LIF* regulation and expression in other marsupial species.
7) The availability of *LIF* DNA sequence from a species of marsupial gene would enable regions of the gene to be identified that have been conserved over very long evolutionary time spans. These regions would be prime candidates for functional domains.
8) Information on the molecular structure of LIF and its receptor in a marsupial would be useful in the study of the co-evolution of LIF and its receptor.

This project on marsupial LIF was undertaken using the small insectivorous dasyurid marsupial, the Fat-tailed Dunnart, *Sminthopsis crassicaudata* (often referred to in this thesis as the dunnart). The dunnart is a mouse-sized insectivorous marsupial, widely distributed in southern and south-western Australia. Adult animals weigh about 15g and newborns about 10mg (Bennett *et al.*, 1990). A pedigreed laboratory colony of this marsupial was established in the Department of Genetics, University of Adelaide in the 1950's (Bennett *et al.*, 1990) and is available for research. The gestation period of *S. crassicaudata* is 13.5 days; maximum litter size is 10 with about 5 animals on average surviving to weaning (about 70 days) under laboratory conditions.

The availability of this colony of dunnarts has facilitated a number of biological research projects that make use of these animals (Copper *et al.*, 1996; Soon and Breed, 1996; Roberts *et al.*, 1997; Dunlop *et al*, 1997; Hope *et al.*, 1997). It was mainly because the dunnart is relatively well characterised biologically, and animals were readily available, that this species was chosen as a model marsupial for the studies reported in this thesis.

The specific objectives of this research project were:

1. to identify and characterize a marsupial homologue of the murine and human *LIF* genes;
2. to compare the sequence of the dunnart *LIF* gene, and its encoded protein, to the *LIF* genes and proteins that have been characterised in eutherian mammals, as a means of investigating the evolution of LIF;

3. to investigate marsupial *LIF* gene expression in adult and embryonic tissues, particularly in the uterus/embryo complex during implantation;
4. to investigate the function of marsupial LIF in inhibiting the differentiation of cultured mouse ES cells.

Chapter 2 Literature Review

2.1 The Identification and Cloning of *LIF*

Communication between cells is central to a wide variety of biological processes including embryonic development. Cellular growth and differentiation are controlled, in part, by the secretion of polypeptide hormones, which exert their actions through specific receptors expressed on the surface of responsive cells (Lobie *et al.*, 1994; Morel, 1994). During embryogenesis, intracellular biochemical networks are activated in response to extracellular signals. Most of the extracellular signalling molecules, including cytokines, interact with their cognate cell surface receptor and induce a cascade of biochemical changes including changes in tyrosine phosphorylation, cyclic nucleotide metabolism, phosphoinositide levels and intracellular free calcium levels. These changes, in turn, affect gene expression.

Extracellular cytokines bind a ligand and activate receptor or ligand-receptor complexes (Clark, 1993; Clark and Brugge, 1995). The cytokine-receptor model of cell-cell communication has been experimentally verified, and the molecular characterization of cytokine receptors has progressed rapidly in recent years. This progress has resulted partly from the availability of radio-labelled cytokines, and the derivation of cultured cell lines that express specific receptors at the cell surface. The finding that some cytokines are localised within cells (usually in the nucleus) implies a more complex role for these molecules than simply the transmission of extra-cellular signals. These intracellular cytokines are frequently localised to the euchromatin-heterochromatin boundary (Lobie *et al.*, 1994), implying that they have a role in gene regulation. However, the identification of specific cell surface receptors for nuclear-localised cytokines provides evidence that these cytokines are also involved in some form of cellular signalling pathway (Morel, 1994). Despite much research on this topic, the function of intracellular cytokines remains unclear.

The process of maturation of blood cells (hemopoiesis) involves the clonal proliferation and concomitant differentiation of immature precursor cells. This process takes place continuously so that the mature blood cells, most of which are

relatively short-lived, can be replenished. The proliferation and differentiation processes are tightly coupled so that the short-lived mature cells are continually replenished with the appropriate proportions of the various cell types (Metcalf, 1984; Tomida *et al.*, 1984), indicating the involvement of some factors that induces differentiation and other factors that stimulates proliferation.

Studies on the growth and differentiation of normal haematopoietic precursor cells in culture have shown that co-cultured of such cells in the presence of a cell feeder layer can result in the formation of colonies of macrophages and granulocytes (Pluznik and Sachs, 1965); otherwise, these cells undergo proliferation without differentiation, and become leukemic cells (Sachs, 1978; 1982). The formation of these colonies is due to the secretion, by the feeder layer cells, of specific inducers of cell differentiation (Pluznik, and Sachs, 1966; Ichikawa *et al.*, 1966; Metcalf, 1969; Metcalf, 1984). One of the factors, named leukemia inhibitory factor (LIF) (Metcalf, 1984), was found to be able to inhibit the proliferation of undifferentiated haematopoietic cells.

In an attempt to clarify the identity, function and interaction of the different factors that act on specific myeloid cell types, the medium conditioned by Krebs II ascites tumour cells was fractioned (Hilton *et al*, 1988) and the fractions tested for their effect on the growth and differentiation of various cell types in culture. The results showed that two biochemically distinct but functionally similar factors were capable of inducing the differentiation of cells in the M1 mouse leukemic myeloid cell line (Hilton *et al.*, 1988). The factors were named LIF-A and LIF-B.

Since these early studies, it has become apparent that LIF is a polyfunctional cytokine with diverse effects on the growth and differentiation of cells (reviewed by Gough *et al.*, 1992; Senturk and Arici,, 1998). Because LIF has been identified on the basis of its varied function, the LIF gene and its products are known by a plethora of synonyms (Table 2.1).

Table 2.1 LIF and its synonyms

Acronym	Name	Reference
LIF	Leukemia inhibitory factor	Gearing *et al.*, 1987; Hilton *et al.*, 1988; Metcalf *et al.*, 1988
D-factor	Differentiation stimulating factor	Tomida *et al.*, 1984; Lowe *et al.*, 1989
DIF	Differentiation inducing factor	Abe *et al.*, 1989
DIA	Differentiation inhibitory activity	Smith & Hooper, 1987; Smith *et al.*, 1988; Williams *et al.*, 1988
DRF	Differentiation retarding factor	Koopman & Cotton, 1984; Williams *et al.*, 1988
HSF-III	Hepatocyte stimulating factor III	Baumaan & Wong, 1989
CNDF	Cholinergic neuronal differentiation factor	Yamamori *et al.*, 1989
HILDA	Human interleukin for DA cells	Moreau *et al.*, 1987
MLPLI	Melanoma derived lipoprotein lipase inhibitor	Mori *et al.*, 1989
OAF	Osteoclast activating factor	Abe *et al.*, 1986
MGI	Macrophage and granulocyte inducing protein	Lotem *et al.*, 1980

To identify recombinant clones containing *LIF* sequences, mouse cDNA libraries, constructed using a pool of mRNA isolated from Krebs II cells after induction with bacterial lipopolysaccharide (LPS), were screened using a degenerate oligonucleotide probe designed on the basis of the known LIF amino acid sequence. Several candidate clones were identified and subsequently characterised (Gearing *et al.*, 1987). One of these clones, *pLIF7.2b*, was sequenced and found to correspond to most of the *LIF* coding region. This clone was modified by the addition of missing 5' coding sequence and a stop codon. The modified clone was named *LIFmut2*. Southern blot

analyses of human and mouse genomic DNA, using a *LIFmut2* as a probe, revealed the existence of only a single *LIF* gene in these species; there was no evidence for the presence of pseudogenes or multiple *LIF* genes (Gearing *et al.*, 1987; Stahl *et al.*, 1990).

Human *LIF* was first cloned by Gough *et al.* (1988), and mouse *LIF* was first cloned by Stahl *et al.* (1990). *LIF* genes from the following additional eutherian mammals have now been characterised, either in the form of cDNAs or partial genomic sequences: rat (Yamamori *et al.*, 1989), sheep and pig (Willson *et al.*, 1992), cow (Kato *et al.*, 1996; Piedrahita *et al.*, 1997) and mink (Song *et al.*, 1998). The impetus for much of this work was the potential commercial use of LIF in embryo transfer and in the generation of chimeric animals using embryonic stem cells. Most research on LIF has been carried out in the human and mouse species.

Human and mouse *LIF* mRNA is about 4.8 kb in size and is derived from a three-exon single copy gene spanning about 8 kb of DNA. The first exon encodes the 5'-untranslated region of the message and the initial residues of the leader sequence of the protein. The remainder of the leader sequence and the first third of the mature LIF protein are encoded by the second exon, while the carboxyl terminal two-thirds of LIF and extensive 3'-untranslated region of the message is encoded by exon 3 (Stahl *et al.*, 1990). Three blocks of sequence, bounded by consensus splice donor and acceptor sites, which are similar in sequence to three exons in the human and murine *LIF* gene, were subsequently identified in sheep and pigs (Willson *et al.*, 1992). Comparisons of the ovine and porcine *LIF* gene sequences to those of human and mouse showed that all 4 species contain 3 exons in their *LIF* genes. The ovine and porcine *LIF* genes have bigger first introns than the mouse and human *LIF* genes (Willson *et al.*, 1992).

In the mouse, two different *LIF* transcripts have been identified, apparently produced from alternative promoters. The first of these gives rise to the "normal" (diffusible) LIF protein, LIF-D. The second transcript gives rise to a "matrix-associated" form, LIF-M. These two transcripts differ at their 5' end as shown in Fig. 2.1, with the LIF-M transcript containing an exon within intron 1 of the LIF-D transcript (Rathjen *et al.*,

1990a; 1990b). This change alters the N-terminus of the hydrophobic leader, which in turn affects the extracellular fate of the encoded LIF (Rathjen *et al.*, 1990a).

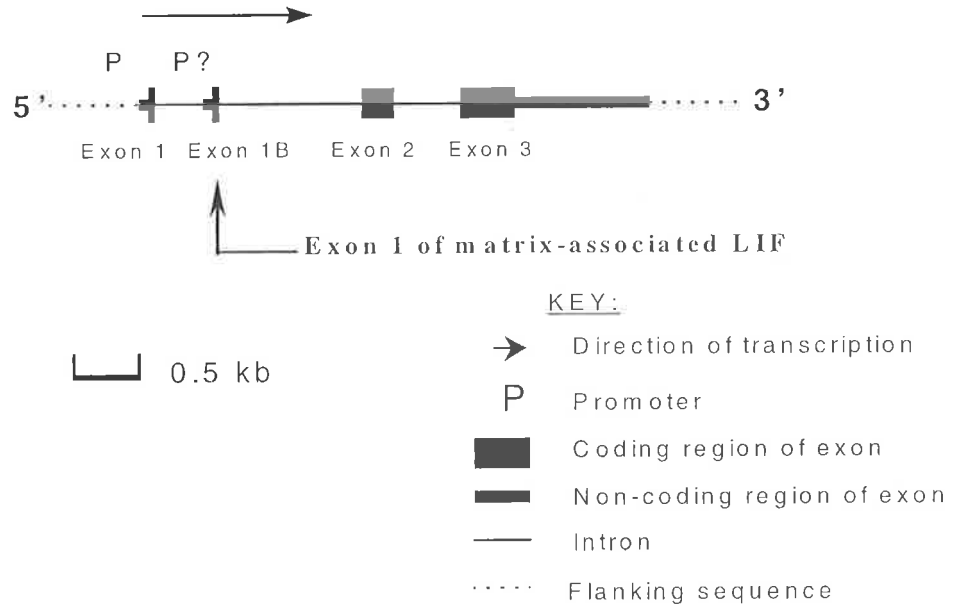


Fig. 2.1 *LIF* gene in the laboratory mouse indicating alternative transcripts.

To help identify structurally and functionally important region of *LIF* and its protein product, a study was initiated to identify regions of conserved sequence (Stahl *et al.*, 1990). Sequence comparisons of human, mouse, porcine and ovine *LIF* (Willson *et al.*, 1992) showed that the coding region is highly conserved, and that there are short conserved sequences (>75% sequence identity) within the 5' flanking region, first intron, and 3'flanking region (Stahl *et al.*, 1990; Willson *et al.*, 1992). Analysis of the promoter region of the human and mouse *LIF* genes showed an 84 % sequence identity over a region of approximately 340 bp 5'of the translational initiation codon. Within the largely non-conserved first introns, there is a block of 150 bp which is highly conserved (Stahl *et al.*, 1990). The sequence in intron 1, which corresponds to exon 1 of the matrix-associated form of mouse *LIF* (Fig. 2.1), is poorly conserved in intron 1 of human *LIF* (Stahl *et al.*, 1990). All four of the TATA-like elements previously identified in the mouse and human *LIF* genes (Stahl *et al.*, 1990) are conserved in sequence across pig and sheep (Willson *et al.*, 1992). Interestingly, apart from a few single base substitutions, sequence variation between species in *LIF* promoter regions appears to be the result of small insertions or deletions.

Three sequence elements within the 5'-flanking region from the mouse *LIF* gene, the major start-site of transcription (+1), a TATA-box (-31) and up to 72 additional 5' nucleotides (-32 to -103), are required by the essential promoter of the gene (Stahl and Gough, 1993). A negative regulatory region has been identified between position -360 and –249 (Stahl and Gough, 1993). The effect of this region can be overridden by the SV40 enhancer element and a positive control element in the *LIF* 5'-flanking region, between -860 and -661 (Stahl and Gough, 1993).

Hsu and Heath (1994) identified a GC-rich region located between the transcriptional start sites for murine D-LIF and M-LIF that, if deleted, resulted in transcripts that had not spliced out the first intron. This region also acts to suppress the expression of the *LIF* gene. They also identified a distal enhancer for *LIF*, located 1.2 - 3.2 kb upstream of the gene.

2.2 The LIF Protein

Amino acid sequence alignment of human, mouse, ovine and porcine LIF reveals a high degree of conservation (Table 2.2). All six cysteine residues found in the mouse protein are conserved at identical positions in the other three species, suggesting that intramolecular disulphide bonds are vital to the integrity and activity of the molecule. Most of the potential N-linked glycosylation sites in LIF are also conserved among species, indicating a possible role for glycosylation in normal LIF function (Willson *et al.*, 1992).

Table 2.2 Cross-species sequence identity of LIF proteins♣.

Species	Rat	Human	Ovine	Porcine
Murine	92%	79%	74%	78%
Rat		81%	75%	78%
Human			88%	87%
Ovine				84%

♣ Adapted from Willson *et al.* (1992).

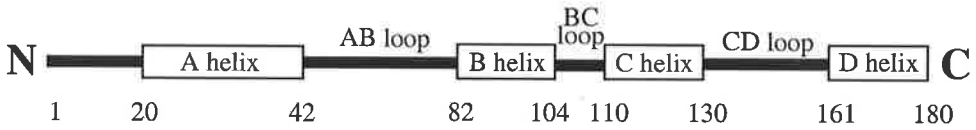


Fig. 2.1 (b) Diagrammatical representation of the domain structure proposed for LIF. Structural characteristics were predicted on the basis of X-ray crystallography and comparisons with related cytokines (Robinson *et al.*, 1994). The α helices are shown in open boxes and loops are displayed by thick lines. The numbers given at the joints of helices and loops refer to amino acids residue. **N** – amino terminus, **C** – Carboxyl terminus.

There have been a number of studies aimed at defining functionally specific regions of LIF (see below). Several of these studies have made use of species-specific differences in the binding of LIF to LIF receptors. Human LIF binds to mouse LIF receptors and effects the growth properties of mouse cells, whereas mouse LIF binds only to mouse LIF receptors; moreover, human LIF binds to mouse LIF receptors with a higher affinity than does mouse LIF (Moreau *et al.*, 1988; Smith *et al.*, 1988; Owczarek *et al.*, 1993).

Owczarek *et al.* (1993) found that the main region of human LIF involved in the interaction with the human and murine LIF receptor molecules was in the loop linking the third and fourth helices, in the predicted three dimensional structure of LIF. Their studies defined a maximum of 15 residue differences between mouse and human LIF responsible for the different properties of these molecules. In a follow-up study, Layton *et al.* (1994) defines six specific residues that contributed to the interaction of human LIF and the human LIF receptor alpha chain: Asp-57, Ser-107, His-112, Ser-113, Val-155 and Lys-158.

In a landmark paper, Robinson *et al.* (1994) describe the three dimensional structure of the mature form of murine LIF, as determined by X-ray crystallography. The LIF molecule has a compact shape comprising four main α helices (A, B, C &D) linked by two long loops (AB, CD) and a short loop (BC). The core region is strongly hydrophobic, and the N-terminal region is wrapped around the molecule and tethered to it by two disulphide bridges. The α helical bundle structure of LIF is similar to that previously described for other members of the haemopoietic cytokine family, including growth hormone and granulocyte colony-stimulating factor (reviewed by Sprang and Bazan, 1993). Human and murine LIF are the same length (180 amino acids) but differ at 39 sites. Robinson *et al.* (1994) concluded that "none (of these differences) seem likely to perturb the structure greatly".

Using an elegant series of experiments, Robinson *et al.* (1994) investigated the relationship between LIF structure and biological properties. They concluded that:

- differences in the activity of human and murine LIF result from differences in their affinity for the human LIF receptor, gp130, or both, and reside in the C-terminal region of the D helix (161 - 180);
- a second region of LIF (150 - 160) also confers species -specific activity;
- residues in both the above regions (150 - 160 and 161 - 180), are involved in the interaction of LIF with LIF receptors.

Further studies by Hudson *et al.* (1996) indicated that the region of human LIF that plays a key role in LIF receptor and gp130 binding, comprises residues from the amino terminus of the D helix, carboxyl terminus of the B helix and C-D loop. This region maps to a surface at the end of the four helix bundle of mouse LIF as determined by Robinson *et al.* (1994). The two residues that made the greatest contribution to the free energy of binding between human LIF and its receptor were identified as Phe-156 and Lys-159. Residues in the A and C helices were found to be involved in binding to gp130.

Presumably, further experiments relating structural and functional domains of LIF, will resolve, in the near future, the several inconsistencies in the findings by Owczarek *et al.* (1993); Robinson *et al.*(1994) and Hudson *et al.* (1996).

2.3 LIF Receptors

Studies of LIF and its interaction with LIF receptors suggest that each pleiotropic effect of LIF is mediated by direct interactions with the responding cells rather than by the indirect release of secondary cytokines, and that these cells display high affinity LIF receptors. Cellular responses are initiated upon binding of LIF to its cell surface receptor. The complementary tissue-specific expression of LIF and LIF-receptor protein (LIFRβ) has been demonstrated during early mouse embryogenesis (Nichols *et al.*, 1996).

The murine LIF receptor complex can have low or high affinity binding with its ligand depending on conformational changes resulting from the association between the LIF receptor protein itself and other membrane bound receptor molecules. It has been demonstrated that low and high affinity LIF receptor complexes are structurally related and inter-convertible and that high affinity receptor complexes are composed of two protein sub-units, one responsible for LIF-specific low affinity binding and the other for affinity conversion and signalling by the receptor complex (Hilton *et al.*, 1992).

For signal transduction *via* the LIF receptor complex, the interaction of LIF with its specific cell surface receptor, also known as low-affinity receptor for leukemia inhibitory factor (LIFRβ) and the formation of a dimerised complex with the common signalling sub-unit glycoprotein gp130, are needed (Taga *et al.*, 1989; Hibi *et al.*, 1990; Murakami *et al.*, 1991). The ubiquitous glycoprotein gp130 sub-unit is also shared by receptors of the other members of the haematopoietic cytokine family, including IL-6 (Van Snick *et al.*, 1988), IL-11 (Paul *et al.*, 1990; Kawashima *et al.*, 1991), CNTF (Stockli *et al.*, 1989), OSM (Malik *et al.*, 1989) and CT-1 (Pennica *et al.*,1995).

OSM, CNTF, and CT-1 initiate the hetero-dimerisation of LIFRβ and α-gp130 (Fig. 2.2) required for signal transduction. These molecules (OSM, CNTF, and CT-1) can compete with LIF, and with each other, for binding directly to LIFRβ to form LIFRβ-gp130 complexes (Boulton *et al.*, 1994). Two regions of receptor interaction exist in the fourth helix of (residues 130 - 153) and the proceeding loop of mouse LIF, human LIF, human OSM and human CNTF. These four functionally related molecules are conserved in their molecular surface characteristics (Robinson *et al.*, 1994), and disulphide bonds play a critical structural role in forming the compact core of these molecules (Robinson *et al.*, 1994).

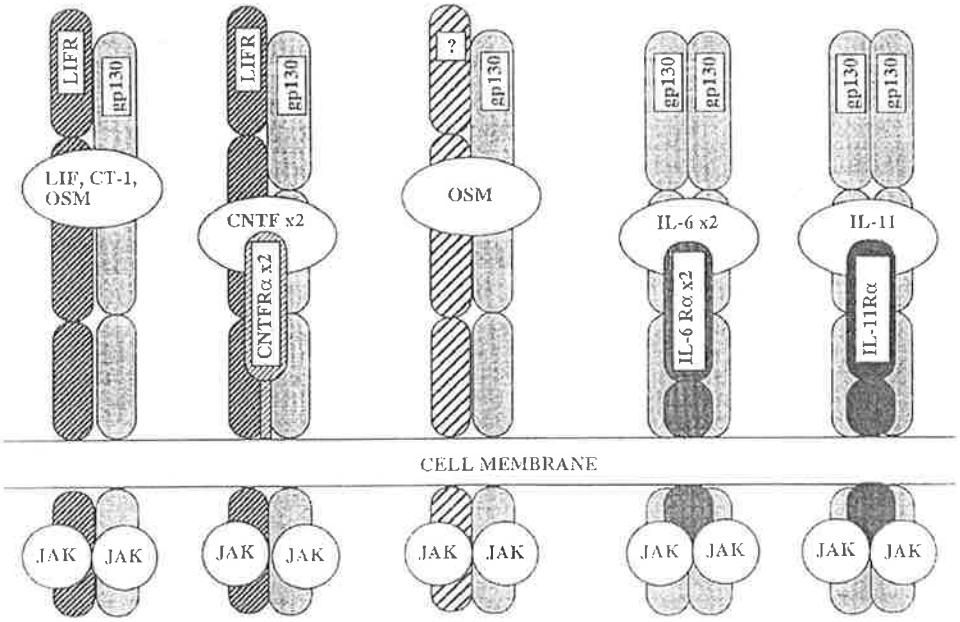


Fig. 2.2 The gp130 receptor family. The similarity between receptor sub-units of the structurally related LIF family of cytokines is illustrated. The ligands (shown inside the elliptic) bind to the extracellular cytokine-specific sub-units that include dimerised gp130 or LIFR-gp130 (top of diagram). Dimerisation activates Jak kinases which are constitutively associated with the cytoplasmic domains of receptors. The diagram (unpublished) was kindly made available by Tricia Pelton, Department of Biochemistry, University of Adelaide.

CNTF and LIF have indistinguishable effects on tyrosine phosphorylation and gene activation in neuron cell lines (Ip *et al.*, 1992), and haematopoietic cells undergo similar signalling events in response to both LIF and IL-6 (Ip *et al.*, 1992). Studies have shown that CNTF and LIF share the IL-6 signal transducing receptor component gp130 (Ip *et al.*, 1992); this accounts for the overlapping actions of these growth factors. However, an OSM receptor complex (a hetero-dimer of gp130 and OSMR) has been reported, that is activated by OSM but not by LIF (Mosley *et al.*, 1996). CNTF also can bind the cytokine-specific GPI-linked cytokine α-sub-unit to form LIFR-gp130 dimers (Ip *et al.*, 1992).

Ligand-induced dimerisation of LIFRβ is activated the Janus kinase (jak) family of kinases, and this is followed by the activation of the signal transducer and activator of transcription (STAT) family of transcription factors (Boulton *et al.*, 1994; Stahl *et al.*, 1994; Matsuda *et al.*, 1994a).

Tyrosine kinases are important intracellular signalling molecules which can be activated by the binding of a ligand to its receptor. The ligand-dependant tyrosine phosphorylation inactivates tyrosine kinase activity (Lord *et al.*, 1991; Miyajima, 1992). Tyrosine kinase Hck is normally expressed in the haemopoietic system (Holtzman *et al.*, 1987; Quintrell *et al.*, 1987; Ziegler *et al.*, 1987) but is also expressed in undifferentiated ES cells (Ernst *et al.*, 1994). Activation of Hck occurs upon binding of the ligand to the receptor, and its expression is negatively regulated by phosphorylation of its conserved C-terminal tyrosine residue (Cartwright, 1987; Ziegler *et al.*, 1989). The mouse *hck* gene, targeted in this way in undifferentiated ES cells, expressed increased Hck tyrosine kinase activity and needed less (≈15 times) LIF than the concentration normally required in the culture medium to maintain ES cells in an undifferentiated state. Hck was found to be physically associated with gp130, and participated in signal transduction from the LIF receptor (Ernst *et al.*, 1994).

Human recombinant LIFR is a 190 KDa glycoprotein that specifically binds human LIF with low affinity but does not bind mouse LIF (Gearing *et al.*, 1992). Mouse βg plasmacytoma cells transfected with the human LIF receptor display novel high affinity LIF receptors that are presumed to consist of transfected receptors in association with endogenous mouse high affinity-converting sub-units (Gearing *et al.*, 1992).

Although LIF receptors have a broad tissue distribution, only specific subsets of cells within these tissues are receptor positive (Nichols *et al.*, 1996). Receptor autoradiography with ^{125}I-LIF was carried out to determine which cells of newborn rat bone bound LIF specifically (Allan *et al.* 1990). Osteoclast preparations from new-born rat long bones were prepared in a way that ensured the cultures were heavily contaminated with osteoblasts, macrophages, and other cell types. Specific binding of ^{125}I-LIF was demonstrated to cells with the characteristics of osteoblasts and macrophages, but not to osteoclasts or their precursors (Allan *et al.*, 1990). However,

murine haematopoietic stem and progenitor cells express receptor for IL-1α, IL-3, IL-6 and G-CSF, not for M-CSF, GM-CSF or LIF (McKinstry *et al.*, 1997).

A protein that specifically binds LIF has been isolated from normal mouse serum (Layton *et al.*, 1992). This protein, which appeared to be a glycoprotein with an apparent molecular mass of 90 KDa, specifically bound murine LIF with an affinity comparable to that of the low-affinity cellular LIF receptor (Layton *et al.*, 1992). The protein has been identified as a soluble truncated form of the α-chain of the cellular LIF receptor (Layton *et al.*, 1992). Since normal serum concentrations of this protein can block the biological actions of LIF in culture, this protein may serve as an inhibitor of the systemic effects of locally produced LIF (Layton*et al.*, 1992).

The biological activity of LIF on murine ES cells, and on other cell types, is shared by different growth factors such as Oncostatin M (OSM) and ciliary neurotrophic factor (CNTF). These molecules exhibit related three-dimensional structures (Bazan, 1991), and share the requirement for the trans-membrane signal-transducing protein gp130 (with which they form a tertiary complex) for their effect on ES cells (Taga *et al.*, 1992).

The targeting of the LIFRβ sub-unit by gene mutation in the mouse causes perinatal death, placentation disruption by poor intra-uterine nutrition, decreased fetal bone volume and increased osteoclast numbers, neural defects and metabolic disorders (Ware *et al.*, 1995). Li *et al.* (1995) used homologous recombination to generate a 20 kb deletion of the mouse LIF receptor gene (*lifr*) in embryonic stem cells and derived transgenic mice homozygous for the mutation. The new-born animals were small, lacking in vigour, and invariably died within 24 h of birth. These mice were severely deficient in motor neurones of the face and spine and neurones of the nucleus ambiguous. The authors concluded that a ligand for LIF-R is probably required for the normal development of motor neurones in brain stem nuclei and spinal cord.

To investigate the biological role of the ubiquitously expressed signal transducing receptor gp130, mice deficient in gp130 were produced by gene targeting, and their phenotype examined (Yoshida *et al.*, 1996). Mouse embryos homozygous for this

deficiency died between 12.5 days postcoitum and term. The mutant embryos were significantly deficient in pluripotential and committed haematopoietic progenitors in the liver and T cells in the thymus. It was concluded that gp130 plays a crucial role in myocardial development and haematopoiesis during embryogenesis.

2.4 *LIF* Expression *in vivo*

The *LIF* gene is normally expressed at low basal levels and is difficult to detect its protein product in adult tissues and organs by Northern hybridisation (Yamamori, 1991; Robertson *et al.*, 1993; Brown *et al.*, 1994; Omori *et al.*, 1996). Application of the RNase protection assay, has shown that *LIF* is expressed in variety of adult cell types and tissues in the mouse, including yolk sac, brain, intestine, heart, thymus, liver, lung, spleen, ovary, oviduct, testes, femur and skin (Bhatt *et al.*, 1991; Robertson *et al.*, 1993). In contrast to these observations, Shen and Leader (1992), using RNase protection assays on adult mouse tissues, found that *LIF* transcripts were only detected in the uterus, where their level fluctuated with the estrous cycle, peaking soon after ovulation.

In the mouse, the D-LIF and M-LIF transcripts are found to occur with non-identical but overlapping patterns of expression in different tissues, suggesting that these two molecules have different biological functions (Robertson *et al.*, 1993). *LIF* transcripts are detectable in primary mouse embryo fibroblast cultures (Bhatt *et al.*, 1991).

High levels of mouse *LIF* expression are found in the endometrial glands of the uterus, particularly at the time of blastocyst implantation, and in extra-embryonic membranes (Bhatt *et al.*, 1991; Conquet and Brulet, 1990). Human *LIF* expression occurs in the endometrium during menstruation with maximal expression between days 19 and 25 of the menstrual cycle (Nachtigall *et al.*, 1996). On the basis of these observations, it has been suggested that LIF plays a role in human embryonic implantation (Nachtigall *et al.*, 1996). This important topic is further discussed later in this Chapter.

Several factors are known to induce the expression of *LIF*. The normally low levels of LIF expression in the adult mouse can be induced to higher levels by the administration of lipopolysaccharide (LPS), a bacterial cell wall product responsible for endotoxic shock (Brown *et al.*, 1994).

LIF transcripts are not detectable in normal rat liver cells, but in the AAF/PH model (combined administration of a non-carcinogenic dose of 2-acetylaminofluorene (AAF) and partial hepatectomy (PH)), *LIF* expression is detected at significant levels (Omori *et al.*, 1996). The induction of LIF by LPS and other treatments that could be considered as "stressful", is thought to reflect the possible role of LIF as a mediator of the acute phase response to tissue damage (Brown *et al.*, 1994).

LIF has shared actions with other cytokine regulators and the elevation of LIF levels in local inflammatory lesions is also accompanied with the elevation of other pro-inflammatory agents, such as IL-6, IL-1 and macrophage-active M-CSF and GM-CSF (Metcalf, 1992). It is difficult to determine the role played by LIF in such situations where there are multiple regulator perturbations. Experiments involving the deletion of LIF by antibody or gene knockout (Metcalf, 1992) are helpful in this contest.

Metcalf and Gearing (1989) multiply infected cells of a murine haematopoietic cell line with a retroviral construct containing *LIF* cDNA. The host cells secreted high levels of LIF. When these cells were injection into unirradiated or irradiated syngeneic mice, the recipients were engrafted with LIF-producing cells in their marrow, spleen, and lymph nodes and had elevated levels of LIF in their serum. Within several weeks, these mice developed fatal syndrome characterised by loss of weight, excess bone deposition, calcification of the heart and skeletal muscle etc. Mice injected with control cells developed none of these lesions. The results were interpreted as indicating that LIF effects calcium metabolism and osteoblast formation, and may induce weight loss.

LIF is known to be a causative factor for cachexia and thrombocytosis in nude mice bearing human cancer cells (Akiyama *et al.*, 1997). The effects of recombinant

human LIF (rhLIF) in a primate model, cynomolgus monkey, was investigated by administering various amount of rhLIF subcutaneously (Akiyama *et al.*, 1997). rhLIF was able to increase platelet level and reduce subcutaneous fatty tissue. These biological effects disappeared soon after the cessation of treatment. The results showed that rhLIF induces weight loss and thrombocytosis in primates.

In murine diseases, including pancreatitis, osteosIcerosis, gonadal failure and thrombocytopenia, LIF is always observed at abnormally high levels (Metcalf, 1992). Interestingly, the human counterparts of these diseases have not been associated with elevated LIF levels (Metcalf, 1992).

It is known that LIF enhances the proliferation of normal and leukemic haematopoietic progenitors in the combination with other cytokines (Kurzrock *et al.*, 1991; Wetzler *et al.*, 1991; Takanashi *et al.*, 1993; Wetzler 1994) and the receptors have been found on the cell surface of certain human solid tumour cell lines such as melanomas and neuroblastomas (Godard *et al.*, 1992).

Cytokines (including LIF) are found to occur at unusually high levels in the blood of individuals affected with the progressive disorder, postmenopausal osteoporosis (Zheng *et al.*, 1997). It has been shown that some of these cytokines (eg. IL-1β and TNF-α) can prevent bone loss associated with this disease, but that LIF has no such affect (Zheng *et al.*, 1997).

Recently, a role for LIF in the early phase of allergic contact dermatitis has been suggested (Szepietowski *et al.*, 1997). This proposal was based on the finding that *LIF* mRNA expression is significantly increased in nickel-tested skin compared with both vehicle-tested and non-tested skin (Szepietowski *et al.*, 1997).

It seems that LIF is produced at multiple local sites in response to certain human diseases. However, the factors that stimulate this LIF expression, and the possible roles of LIF in overcoming the disease conditions, remain to be determined.

2.5 *LIF* Expression in vitro

Northern hybridisation analysis indicates that *LIF* expression is detectable *in vitro* in a number of murine and human cell lines and primary cells, including Krebs II ascites cells from which LIF was first identified (Rathjen *et al.*, 1990).

The regulation of *LIF* expression in cultured human endometrial cells by cytokines, steroid hormones and growth factors was investigated and no evidence was found that steroid hormones stimulate LIF mRNA expression and protein synthesis (Arici *et al.*, 1995). Growth factors including IL-1, TNF-α, platelet-derived growth factor, epidermal growth factor and transforming growth factor-β, are potent inducers of LIF expression in cultured endometrial stromal cells (Rathjen *et al.*, 1990; Arici *et al.*, 1995).

LIF expression can also be induced *in vitro* in lung tissue by culturing in serum-free medium (Brown *et al.*, 1994).

The LIF receptor and LIF signal-transducing component gp130 are expressed in the human breast carcinoma cell line MCF-7, and the addition of LIF to the culture medium stimulates the proliferation of these cells (Estrov *et al.*, 1995). The proliferation of other oestrogen-dependent and independent breast tumour derived cell lines is also stimulated by LIF (Estrov *et al.*, 1995). These result suggest that the receptors are biologically active in these cultured cells, and that LIF and LIF receptors may play a role in human breast cancer cell division.

LIF and IL-11 mRNA were detected in seven independently derived human melanoma cell lines (Nichols *et al.*, 1996). Five of these cell lines secreted LIF as determined by enzyme-linked immunosorbent assay (ELISA) and only one cell line express IL-11 at a low level; there were no effects of LIF and IL-6 on the proliferation of any of the seven cell lines (Paglia *et al.*, 1995). It would appear that LIF, IL-6 and IL-11 are not coordinately expressed in these cells but they might have some paracrine or endocrine function in the course of melanoma progression. This hypothesis is supported by the

complementary, tissue-specific expression of LIF, its receptor, and other cytokines belonging to the same family (Paglia *et al.*, 1995).

Biologically active mLIF, hLIF and oLIF can be synthesised *in vitro* in yeast cells after transformation with the expression vector YepsecI containing *LIF* cDNA (Baldari *et al.*, 1987; Gearing *et al.*, 1987; Gough *et al.*, 1988; Willson *et al.*, 1992).

Gearing *et al.* (1989) have shown that the *E. coli* expression plasmid pGEX2T (Smith and Johnson, 1988) incorporating *LIF* cDNA inserted immediately after a glutathione S-transferase (GST) reading frame, can be used to produce LIF as a fusion protein (GST-LIF) and in pure form. The fusion protein can be purified by affinity chromatography using Glutathione Sepharose 4B beads, and digested with thrombin to release the LIF moiety. Mouse and human LIF synthesised in this way have a high purity and retain biological activity as judged by *in vitro* assays.

2.6 Biological Properties of LIF

In vitro and *in vivo* studies have shown that LIF, like other cytokines, has the ability to influence the function and differentiation of a variety of cell and tissue types (Table 2.3). The content of Table 2.3 is not all-embracing, but is presented to illustrate the wide range of properties that have been ascribed to LIF.

Table 2.3 A sample of the biological properties of LIF

Body element or system	Identified actions of LIF	Reference
Murine M1leukemic cell line	Suppresses proliferation and induces cell differentiation *in vitro*	Gearing *et al.*, 1987; Hilton *et al.*, 1988; Metcalf *et al.*, 1988; Tomida *et al.*, 1984; Lowe *et al.*, 1989; Abe *et al.*, 1989; Koopman & Cotton, 1984; Williams *et al.*, 1988; Lotem *et al.*, 1980.
Murine *myc*-transformed erythroleukemic cells	Stimulates proliferation *in vitro*	Corey *et al.*, 1991
Human DA1-1a leukemic cell line	Stimulates cell proliferation *in vitro*	Moreau *et al.*, 1987
Human HL60 & U937 myeloid leukemic cell lines	Suppresses proliferation *in vitro*	Maekawa and Metcalf, 1989; Maekawa *et al.*, 1990.
Murine spleen and bone marrow stem cells from LIF-deficient animals	Exogenous LIF stimulates cell proliferation *in vitro*	Escary *et al.*, 1993
Murine spleen and bone marrow	Induces thrombocytosis and haematopoietic progenitor cells *in vivo*	Pruijt *et al.*, 1997.
Murine and human liver cells	Induces differentiation and inhibition of proliferation *in vitro*	Gearing *et al.*, 1987; Moreau *et al.*, 1988
Rat liver cells	Stimulates acute phase protein synthesis in hepatocytes *in vitro*	Baumann and Wong, 1989; Fey and Gauldie, 1990; Baum *et al.*, 1989a, 1989b
Murine and human blood cells	Initiates clonal proliferation of haematopoietic stem cells, and facilitation of insertion of genetic material into such cells *in vitro*	Laery *et al.*, 1990; Fletcher *et al.*, 1990
Murine blood cells	Stimulates the growth of erythroid and megakaryocytic elements, and retards the growth of lymphocytes *in vivo*	Metcalf *et al.*, 1990; Metcalf *et al.*, 1991
Murine macrophage progenitor cells	Stimulates maturation of macrophages *in vitro*	Hilton *et al.*, 1988; Metcalf *et al.*, 1988; Lotem *et al.*, 1989
Murine embryonal cells	Inhibits differentiation of mouse ES cells *in vitro*, without affecting proliferation	Williams *et al.*, 1988; Smith *et al.*, 1988; Smith & Hooper, 1987
Murine bone	Stimulates bone re-modeling and resorption *in vitro*	Metcalf and Gearing, 1989; Abe *et al.*, 1986

Table 2.3 (Continued)

Murine bone	Stimulates changes to long bones *in vivo*, including the accumulation of elongated stellate cells.	Metcalf and Gearing, 1989a; Reid *et al.*, 1990
Murine bone	Stimulates trabecular bone mass *in vivo*	Metcalf and Gearing, 1989a; 1989b
Murine osteoclasts	Inhibits plasminogen activator activity *in vitro*	Martin *et al.*, 1992
Human lipid metabolism	Inhibits lipoprotein lipase *in vitro*	Mori *et al.*, 1989
Human nervous system	Directs the formation of neurotransmitter sympathetic neurones *in vitro*	Mori *et al.*, 1989
Rat nerve cells	Binds to distinct regions of the nervous system at defined developmental stages *in vivo*	Qiu *et al.*, 1994; 1997
Rat nervous system	Induces of neuronal differentiation *in vitro* & *in vivo*	Yamamori *et al.*, 1989; Cheng and Patterson, 1997
Rat neurones	Controls neuronal phenotypic decisions *in vitro*	Patterson and Chun, 1974; Patterson *et al.*, 1975; Patterson and Chun, 1971
Rat dorsal root ganglia cells	Induces sympathetic sprouting *in vivo*	Thompson and Majithia, 1998
Murine adult neuronal tissue	Affects the response to tissue injury *in vivo*	Kurek *et al.*, 1998
Cultured murine neural crest cells	Development of sensory neurones and dorsal root ganglia *in vitro*	Murphy *et al.*, 1993
Cultured murine dorsal root ganglia cells	Enhances survival *in vitro*	Murphy *et al.*, 1993
Cultured mouse embryos	Required along with Nerve Growth Factor (NGF) for differentiation of neuro-filament negative precursors into mature sensory neurones *in vitro*	Murphy *et al.*, 1993
Mouse adipocytes	Suppresses lipoprotein lipase (LPL) activity and prevents accumulation of lipids *in vitro* & *in vivo*	Mori *et al.*, 1989; Metcalf *et al.*, 1992; Hilton *et al.*, 1992
Human carcinoma cells	Induces apoptosis and proliferation *in vitro*	Kamohara *et al.*, 1997
Human breast cancer cells	Stimulates growth *in vitro*	Dhingra *et al.*, 1998
Murine teratocarcinoma cells	Inhibits differentiation of F9 cells *in vitro*	Hirayoshi *et al.*, 1991
Murine muscle cells	Stimulates proliferation of myoblasts *in vitro* and *in vivo*	Kurek *et al.*, 1996
Rat prospermatogonial stem cells	Stimulates proliferation *in vitro*	Nikolova *et al.*, 1998

Marsupial mammals have unique reproductive and developmental processes (Tyndale-Biscoe and Renfree, 1987). A major reason for initiating this study of marsupial LIF was to determine the extent to which these features could perhaps be attributed to differences between eutherian and marsupial LIF. The properties of LIF in relation to embryonic cells, embryonic development and blastocyst implantation are now reviewed briefly.

2.6.1 The effect of LIF on ES cells in culture

In the process of compaction during mouse embryogenesis, cell segregation starts at about 3 days after fertilisation and two populations of cells are generated, the epithelial cell layer trophectoderm and the inner cell mass (ICM), a pluripotential stem cell lineage from which some of the extra-embryonic membranes and all tissues of the embryo proper are developed. The pluripotency of cultured ICM cells can be maintained in culture by the participation of a feeder layer of mitotically inactivated mouse embryo fibroblasts, leading to the establishment of mouse embryonic stem (ES) cell lines (Martin *et al.*, 1975; Evans and Kaufman, 1981; Martin, 1981). The pluripotency of the ES cells was found to be retained indefinitely by the provision of a soluble factor, differentiation inhibitory activity (DIA) produced from a number of sources (Hooper *et al.*, 1987; Smith and Hooper 1987; Koopman *et al.*, 1984). Purified recombinant LIF was demonstrated to substitute for DIA, or for the presence of a feeder layer, in the maintenance of totipotent ES cell lines (Gearing *et al.*, 1987; Williams *et al.*, 1988) and it was subsequently shown that DIF and LIF were identical products of the same gene (Smith *et al.*, 1988). It has been confirmed that feeder layers of the type used to establish mouse ES cells in culture, secrete LIF (Rathjen *et al.*, 1990). The use of purified recombinant LIF as a direct additive to the culture media was an important advance in the culture and maintenance of ES cells.

Experiments to examine the biological effects of cytokines on murine ES cells have shown that LIF is not unique in its ability to suppress ES cell differentiation. LIF and

OSM, for example, maintain close to 100% of ES cells in an undifferentiated state, but CNTF is far less efficient in this respect (Piquet-Pellorce *et al.*, 1994).

In the presence of LIF, ES cells display the stem cell phenotype of compact colonies. The proportion of colonies with this phenotype is related to the concentration of LIF in the culture medium (Williams *et al.*, 1989). ES cells will differentiate and die over a 3-6 day period if LIF is removed from the culture medium (Williams *et al.*, 1989; Pease and Williams, 1990; Conquet and Brulet, 1990). Undifferentiated ES cells maintained in the presence of LIF, form germ-line chimeras when injected into blastocysts (Williams *et al.*, 1989; Pease and Williams, 1990).

LIF is also used as a substitution for feeder layers in attempts to isolate and culture stem cells from livestock species, including the pig (Evans *et al.*, 1990; Piedrahita *et al.*, 1990; Strojec *et al.*, 1990), sheep (Handyside *et al.*, 1987; Li and Trounson, 1990) and cow (Evans *et al.*, 1990; Hassan-Hauser *et al.*, 1990). Recently, conditions for culturing porcine embryonic stem cells using human LIF (hLIF) have been optimised (Moore and Piedrahita, 1997). However, to date, it has proved impossible to establish long term ES cell cultures from any species other than the mouse.

In their attempts to establish rat ES cell lines, Takahama *et al.* (1998) showed that the culture supernatant a rat *LIF* cDNA-transduced rat fibroblast cell line could maintain the stem cell phenotype of rat ES cells and that this effect was stronger than that of mouse LIF.

2.6.2 A Role for LIF in Embryogenesis and Blastocyst Implantation

While *LIF* is expressed at low levels in many foetal and adult mouse tissues, high level *LIF* expression is detected only in the endometrial glands of the uterus, specifically on the fourth day of pregnancy (Bhatt *et al.*, 1991; Shen and Leder, 1992), a time which coincides with the hatching of the blastocyst from the zona pellucida and its implantation in the uterine wall (Finn and McLaren, 1976), and with the presence of high circulating oestrogen levels (McCormack and Greenwald, 1974;

Shen and Leder, 1992). *LIF* expression also occurs in the endometrial glands of pseudo-pregnant female mice, reaching a peak when blastocysts would normally implant (Bhatt *et al.*, 1991; Shen and Leder, 1992).

In mice that have had their ovaries removed, and in unmated female rabbits, the uterine levels of LIF protein are very low but can be increased by treatment with progesterone alone or in combination with estradiol-17β (Yang *et al.*, 1995b). These findings suggest that *LIF* expression is differentially regulated during the oestrous cycle, possibly by progesterone and estrogen. *In vitro* studies, on the other hand, have shown that levels of *LIF* mRNA in mouse endometrial cell cultures are not enhanced significantly by treatment with oestradiol, progesterone, or a combination of the two (Lavranos *et al.*, 1996).

Further investigations in the laboratory mouse have shown that *LIF* mRNA is expressed in the differentiated trophectoderm, but not in the pluripotential inner cell mass, whereas *LIF*-receptor mRNA is found in the inner cell mass but not in the trophectoderm (Rathjen *et al.*, 1990; Nichols *et al.*, 1996). This complementary expression pattern suggests the paracrine coupling between stem cells and differentiated progeny at the earliest stage of mammalian development (Nichols *et al.*, 1996).

LIF expression in normal human endometrium during the human menstrual cycle, and in the embryo, has been studied using a polyclonal antiserum to detect LIF protein, and RNase protection assays to detect LIF transcripts (Charnock-Jones *et al.*, 1994). RNase protection assays showed that LIF mRNA was either absent or present at very low levels during the proliferative phase, but was present at high levels during the mid and late secretory phases. Immunocytochemistry demonstrated an absence of LIF protein from both the glandular epithelium and stromal tissue in the proliferative phase, but gave clear and reproducible staining in glandular epithelium in the mid to late luteal phase with faint staining in stromal tissue (Charnock-Jones *et al.*, 1994).

Northern hybridisation analysis has shown that *LIF* mRNA is present in human endometrial tissue of proliferative and secretory phases (and decidua) from the first

trimester of pregnancy (Kojima *et al.*, 1994). The application of quantitative RT-PCR to stroma-enriched (SF) and epithelium-enriched (EF) fractions of endometrial tissues showed that the levels of *LIF* mRNA are higher in the EF than in the SF (Kojima *et al.*, 1994), and that LIF mRNA is detected only during the mid-and late secretory phase of the cycle after day 20. Western blot analysis detected a 45 kDa LIF protein in an extract from late secretory tissue (Vogiagis *et al.*, 1996). These findings are compatible with a role for LIF in the human blastocyst implantation.

Using Northern analysis and immunolocalization, Vogiagis *et al.* (1997) examined the expression of *LIF* in endometrial tissues from cyclic and pregnant ewes. *LIF* expression remained constant throughout the oestrous cycle and was present during early pregnancy. LIF protein was detected in the cellular compartments of the endometrium and also in the trophoblast cells of day 17 blastocysts.

Ovariectomised steroid-treated ewes were used to study whether steroid hormones regulate endometrial *LIF* expression (Vogiagis *et al.*, 1998). Using antibodies to detect LIF, it was found that oestradiol had little effect on treated ewes in comparison to ovariectomised animals. However, treatment with oestradiol and progesterone had an inhibitory effect of LIF expression in ovariectomised ewes. This suggests that steroid hormones may be involved in the regulation of *LIF* expression (Vogiagis *et al.*, 1998). Oestradiol (not progesterone) was also showed to up-regulate *LIF* expression in bovine oviduct epithelial cells and fibroblasts (Reinhart *et al.*, 1998).

Secretion of LIF in women with extrauterine pregnancy was examined in supernatant taken from cultured decidual explants (Hambartsoumian, 1998b). High concentrations of progesterone in circulating plasma were associated with low levels of *LIF* expression.

Song *et al.* (1998) have show that LIF is expressed in the uterus of mink just prior to implantation and during the first two days after implantation, but not during diapause. *LIF* expression was localised in the uterine epithelial glands.

Using molecular markers to follow the differentiation of defined cell types, it has been demonstrated that LIF selectively inhibits the formation of primitive ectoderm while permitting the differentiation of primitive endoderm (Shen and Leder, 1992). Mouse blastocysts cultured in media containing LIF have a larger trophectoderm and a normal sized inner cell mass, indicating that the LIF-acting site is most likely the trophectoderm (Robertson *et al.*, 1990). *LIF* receptors are found on the trophectoderm of the expanded blastocysts (Nichols *et al.*, 1996).

The addition of human LIF to mouse ES cell culture medium has little influence on the development of 8-cell mouse embryos through to the blastocyst stage of development. However, the proportion of blastocysts hatched from the zona pellucida is increased (Lavranos and Seamark, 1989), ie, LIF has an embryotrophic effect. The addition of human LIF to the culture medium increases the viability *in vitro* of day 5 ovine embryos, and also increases the pregnancy rate when such embryos are re-implanted into recipient ewes (Fry *et al.*, 1992). Human LIF effects the development of cultured early stage sheep embryos in culture (Fry *et al.*, 1991), but mouse LIF has little effect. This was attributed, perhaps naively, to the closer degree of sequence identity between human and sheep LIF than between mouse and sheep LIF (Fry *et al.*, 1991). The presence of human LIF in the culture medium can also increase the success rates of cultured embryo implantation in cattle an pigs (Fry *et al.*, 1991).

Bovine embryo stem cells maintained in culture with LIF for 14 days show a phenotype that is not consistent with that occurring normally *in vivo* (Hassan-Hauser *et al.*, 1990). Unlike the mouse where hatching of the blastocyst and implantation are always coincidental (Orsini and McLaren, 1967, Finn and McLaren, 1967), these processes are about 10 days apart in livestock and the embryo is rapidly expanding prior to implantation (Bochier, 1969; Bindon, 1971). Therefore, LIF may play a somewhat different role in cattle, sheep and pigs from that in rodents. Investigations of the role of LIF during early pregnancy in livestock may lead to ways of using LIF i) to improve the rate of successful embryo manipulation, and ii) to establish viable ES cell lines.

Purified cultures of human cytotrophoblasts have been used to study the effects of LIF on several morphological and biochemical markers of trophoblastic differentiation. Investigation of LIF mRNA and protein found that LIF is expressed in the fallopian tubes during the menstrual cycle. The highest levels of LIF were found in the tubal mucosa and in the more distal segments of the fallopian tube, the area where fertilisation, early embryonic growth and ectopic implantation occur (Keltz *et al.*, 1996). Human placenta expressed the LIF receptor components LIFR and gp130 (Kojima *et al.*, 1995) suggesting a regulatory role for LIF in trophoblast growth and differentiation during human pregnancy.

In a recent study, LIF secretion was assayed (using enzyme-linked immunosorbent assay - ELISA) in explanted cultures of secretory and proliferative phase endometrium from fertile and infertile women. Fertile women had LIF levels that were significantly higher in the secretory phase tissue than in the proliferative phase tissue. In contrast, infertile women showed no such increase. It was concluded that deregulation of endometrial *LIF* expression could be a possible cause of infertility. However, like so many studies attempting to establish the roles of LIF *in vivo*, the authors appear to be confusing correlation with causation.

These and other studies indicate that *LIF* expression is under maternal control, precedes implantation and coincides with blastocyst formation (Bhatt *et al.*, 1991). The picture to emerge is that LIF is secreted by the endometrial glands, binds to specific receptors on the trophectoderm and probably other embryonic cell types, and triggers the expression of genes whose products affect blastocyst implantation and embryogenesis.

The observation referred to above suggests that LIF may play a role in regulating the growth and development of early mouse embryos. Furthermore, the coincidental timing of i) *LIF* expression in the endometrial glands and ii) implantation of the blastocyst, suggests a possible role for LIF in the process of blastocyst implantation. These putative roles for LIF were investigated, with surprising results, using the techniques of targeted gene replacement in ES cells and transgene expression in transgenic mice (Shellard *et al.*, 1996).

The mouse gene encoding LIF was mutated to disrupt the open reading frame, and one of six homologous recombinant clones was used to produce ES chimeras (Stewart *et al.*, 1992). Heterozygous offspring were selected and used to produce individuals homozygous for the mutant *LIF* allele; these mutant homozygotes were used to investigate the effect of LIF deficiency on reproduction and development.

Males homozygous for LIF deficiency were fertile and able to sire offspring from both wild-type and heterozygous females. Female homozygotes, when mated with either heterozygous or homozygous mutant males, produced morphologically normal blastocysts which failed to implant. If LIF was "secreted" into the uterine environment with small pumps, fertility was restored. When blastocysts recovered from mutant homozygous x homozygous matings were transferred to three-day pseudo-pregnant wild-type recipients, they implanted and developed to term. The analysis of female mutant homozygotes on day 7 of gestation revealed that their blastocysts were not surrounded by a zona pellucida and there was no overt indication that implantation had occurred or any evidence of a decidual response in the uterus which should have started on the fifth day of gestation.. **Thus the uterine expression of *LIF* in mice is essential for implantation.** It will be of interest to find out whether other mammals share this requirement. If so, it may be possible to develop methods for controlling pregnancy by manipulating the levels of LIF in females. This may be important in establishing successful pregnancies from embryos produced by *in vitro* fertilisation (Stewart *et al.*, 1992).

LIF-deficient mice derived by gene targeting have dramatically decreased numbers of stem cells in their spleen and marrow. When spleen and marrow cells from these mice were injected into lethally irradiated wild-type animals, the recipients survived long-term, showing that the LIF-negative stem cells from the LIF knock-out mice remain pluripotent (Escary *et al.*, 1993).

In a recent review article, Stewart and Cullinan (1997) summarise the evidence that LIF is required to promote embryo attachment and decidualization of the uterus. If LIF is not present, neither of these events occur.

Given the large number of instances where *LIF* expression appears to be correlated with various cellular phenotypic phenomena (Table 2.3) the surprising result from these knock-out experiments is that mice homozygous for the *LIF* mutation were viable and apparently developmentally "normal", as long as they developed in a maternal environment where *LIF* was expressed.

There are various possible conclusions to be drawn from these experiments. Perhaps the disrupted *LIF* transcript in the knock-out mice retained some degree of function. Perhaps LIF is absolutely required for implantation but is redundant in its role in other cellular processes, only being essential under rare and adverse environmental conditions. Perhaps LIF has no important role other than its involvement in implantation.

The further elucidation of the role of the LIF during the early stage of both maternal and embryonic and/or fetal development awaits a thorough analysis of the temporal and spatial patterns of expression of *LIF* and requires a great deal more research. Although a number of groups has been working on the *LIF* gene of different eutherian species, studies in a wider range of species will contribute to the understanding of *LIF* gene expression and evolution.

Chapter 3 Materials and Methods

3.1 Materials

3.1.1 Experimental Animals

Animals were obtained from the *Sminthopsis crassicaudata* colony, maintained by the Department of Genetics, University of Adelaide. DNA used to construct the genomic library was extracted from the liver of animal 1998.1a, using standard techniques.

Some tissues used for RNA extraction were obtained from Dr. W. G. Breed, Department of Anatomical Science, University of Adelaide.

3.1.2 Chemicals

Analytical Reagent grade (AR) chemicals were used in this project and were purchased from *BDH*, with the exception of the following:

Molecular biology grade, DNA grade, and low melting temperature (LMT) agarose were purchased from *PROGEN Industries Limited.*

Low melting point agarose (Ultra Pure) and agarose (Ultra Pure) were purchased from *BRL*, A Division of *Helena Laboratories (Australia) Pty Ltd.*

Acrylamide and bisacrylamide ("Electran" grade) were purchased from *BDH Chemicals Australia Pty Ltd.*

Ammonium persulphate (AR) was purchased from *May and Baker.*

The reduced form of glutathione was purchased from *Sigma Chemical Company.*

Agar, tryptone, kanamycin, N-lauryl sarcosine, IPTG (isopropyl -B-D-thiogalactoside) and X-gal (5'-Bromo-4-chloro-3-indolyl-B-D-galacto-pyranoside) were purchased from *Boehringer Mannheim GmbH, Biochemica.*

Polyethylene glycol (8,000) was purchased from *Sigma Chemical Company.*

Bio-gel P-60 resin was purchased from *Biorad Laboratories, Inc.*

Sepharose® CL-6B was purchased from *Pharmacia Biotech.*

Glutathione Sepharose® 4B was purchased from *Pharmacia Biotech.*

3.1.3 Isotopes

α-^{32}P-dATP was purchased from *Bresatec Pty Ltd* and *Amersham Australia Pty Ltd.*

3.1.4 Vectors

pBluescript II KS$^+$, a product of *Stratagene*, was a gift from Professor Peter Rathjen, Department of Biochemistry, University of Adelaide.

pGEM-11zf (+), pGEM-T, and λ GEM-11 *Bam*HI arms were purchased from *Promega.*

pGEX2T, a product of *Pharmacia*, was a gift from Professor Robert Saint, Department of Genetics, University of Adelaide.

3.1.5 Bacterial Strains

DH5α *E.coli* was a gift from Dr. R. M. Hope, Department of Genetics, University of Adelaide.

XL-1 Blue recA1, endA1, gyrA96, thi-1, hsdR17, SupE44, relA1, lac, [F' proAB, lacIq ZDM15] Tn10 (tet^r) was purchased from *Stratagene*.

JM109 endA1, recA1, gyrA96,thi-1, hsdR17, (rk^-,mk^+0, relA1, SupE44,l $^-$, D (las^- proAB) [F', traD36, proAB, $lacI^q$, ZDM15] was purchased from *Promega*.

KW251 ($mcrA^-$, $mcrB^-$), LE392 cells were purchased from *Promega*.

XL-1 Blue MRF'D (mcrA)183, D (mcrCB-hsdSMR-mrr)173, end A1, SupE44, thi-1, recA1, gyrA96, relA1, lac[F', proAB, $lacI^q$, ZDM15, Tn10 (tet^r)] was purchased from *Promega*.

VCSM13 helper phage (approximately 1 X 10^{11} pfu/ml) was purchased from *Stratagene*.

Packagene® Extracts packaging extract was purchased from *Stratagene*.

BL21 strains *E. coli*. BF- dcm, ompT, hsd (rB-mB-) gal was a gift from Dr. Joan Kelly, Department of Genetics, University of Adelaide.

3.1.6 Enzymes

Restriction enzymes were purchased from *Boehringer Mannheim*, *Promega* and *Progen*.

T4 DNA ligase, Bovine pancreas DNase I, RNase A, RNase T, RNase H, and Klenow fragment of DNA polymerase I were purchased from *Boehringer Mannheim*.

Taq DNA polymerase and sequencing grade *Taq* DNA polymerase were purchased from *Promega* and *Bresatec*.

3.1.7 DNAs

Lambda *Hind*III and *SPP-1 Eco*RI molecular weight markers were purchased from *Progen* and pUC19 *Hpa*II molecular weight standards were purchased from *Biotech*. High molecular weight markers were purchased from *BRL*.

Salmon sperm DNA was purchased from *Sigma*.

PBluescript II KS^+ T3 promoter primer (20mer) was a gift from Professor Peter Rathjen. T7 promoter primer (20mer) and primers for genomic sequencing were synthesised with a BECKMAN oligo 1000 DNA synthesiser.

Ultrapure dNTPs (2'-deoxynucleoside 5'-triphosphate minimal diphosphate) were purchased from *Pharmacia* and *Promega*.

Random hexamer primers for oligo labelling were purchased from *Bresatec* and *Promega*.

Random hexamer primers and poly $(dT)_{12-18}$ primers for first strand cDNA synthesis were purchased from *BRL*.

3.1.8 Kits

Wizard™ PCR Preps DNA purification system was purchased from *Promega*.

QIA Preps Spin plasmid kit was purchased from *QIAGEN*.

Sequenase® Version 2.0 DNA sequencing kit was purchased from *USB*.

Megaprime® DNA labelling System was purchased from *Amersham Life Science*.

SuperScript™ Pre-amplification System for First-strand cDNA System was purchased from *GIBCO-BRL*.

Trizol One-step RNA Purification System was purchased form *BRL Life Technologies Inc*.

3.1.9 Other Materials

X-ray film was purchased from *Fuji Photo film Co., Ltd.;* developer and fixer were purchased from *Kodak*.

Photographic film was purchased from *Polariod* and *Agfa*.

Hybond-N^+ nucleic acid transfer membrane was purchased from *Amersham Life Science*.

3.2 Methods

3.2.1 Construction of *Sminthopsis crassicaudata* genomic DNA library

3.2.1.1 Partial digestion of genomic DNA, separation on agarose gel and recovery of DNA fragments

A range of small scale reactions was performed, using the restriction enzyme *Sau*3A, to establish the optimum condition for generating DNA fragments of size 11-23 kb. *Sau*3A enzyme concentrations of 1 u/mg to 0.004 u/mg were used. The concentration found to be optimal (0.008 u/mg) was used for the large scale preparation of partially digested *S. crassicaudata* genomic DNA. High-molecular-weight genomic DNA (150 μg) was digested at 37°C for 1 hour. The reaction was stopped by heating to 70°C for 15 minutes, and the partially digested DNA sample was applied to 0.4 % w/v low melting temperature (LMT) agarose gel and electrophoresed at 4°C. The gel region containing DNA fragments ranging in size from 11-23 kb was excised under long

wavelength UV light (360 nm). The gel slice was equilibrated for 1 hour at room temperature in a solution of 5 mM EDTA, 100 mM NaCl. The gel slice was incubated in fresh buffer for a further 10 minutes at 68°C before re-equilibration to 37°C. To the re-equilibrating mixture, agarase was added to a final concentration of 2 u/100 µl gel and the mixture was incubated overnight at 37°C. The DNA was extracted once with an equal volume of phenol, and twice with chloroform. Two volumes of cold (-20°C) 100% v/v ethanol were added to the aqueous layer, and DNA precipitation was carried out at -20°C overnight. Precipitates were centrifuged at 13,000 X g in a microcentrifuge for 15 minutes, the DNA pellet was washed twice with 70% v/v ethanol, dried at room temperature and re-dissolved in an appropriate volume of double distilled water. The quality and concentration of recovered DNA fragments were assayed by UV examination on 0.8 % w/v agarose mini-gel.

3.2.1.2 Ligation of *S. crassicaudata* DNA fragments with λ arms

(1) Ligation

Lambda GEM-11 arms were pre-treated with *Bam*HI and calf intestinal alkaline phosphatase so that genomic DNA fragments with *Sau*3A ends could be ligated to the complementary termini of the arms.

To determine the optimal conditions for ligation, a small scale reaction was carried out using different molar ratios of vector:genomic DNA. The molar ratio of λ arms (43 kb) to genomic insert (average 17 kb) was varied between approximately 1:3.5 and 1:0.5. Ligation reaction containing no genomic DNA were used as controls to determine background levels of re-ligated arms. An optimal molar ratio of arms:inserts proved to be 2:1.

For the large-scale ligation, 200 ng of λGEM-11 *Bam*HI arms and 200 ng of *Sau*3A partially digested *S. crassicaudata* genomic DNA fragments were added to one Weiss unit of bacteriophage T4 DNA ligase in 1 X ligation buffer (supplied as 10 X buffer with ligase by the manufacturer). This mixture was incubated at room temperature for three hours (*Promega Technical Bulletin No.55*).

(2) Packaging the ligated DNA

Packagene® *In Vitro* Packaging System was used for the packaging of ligated phage DNA. The packaging extract was allowed to thaw on ice, and incubated with the ligation mix for 2 hours at room temperature. SM buffer (50 mM Tris-HCl pH 7.4, 100 mM sodium chloride, 10 mM magnesium sulphate, 0.1 % w/v gelatin) was then added to a final volume of 500 µl. The phage was then mixed with 25 µl of chloroform and stored at 4°C (*Promega Technical Bulletin No.5*).

3.2.2. Isolation of recombinant bacteriophage clones

3.2.2.1 Phage growth

(1) Preparation of host cells

E. coli strain KW251 (purchased as glycerol stocks) was used as the host bacteria for propagating λ phage. Bacteria were grown overnight on LB-agar plates in the presence of 0.2 % w/v maltose and 10 mM magnesium sulphate. A single colony of KW251 was inoculated in 50 ml of TB medium (1.0 % w/v Bacto-tryptone, 0.5 % w/v NaCl) in the presence of 2 % w/v maltose and 10 mM $MgSO_4$ and then incubated at 37°C overnight with vigorous shaking. The cell cultures were stored at 4°C before use.

(2) Titration

TB top agarose (0.7 % w/v) was made by adding 0.7 g agarose to100 ml of TB medium. The mixture was heated in a microwave oven to melt the agarose, and after cooling to about 60°C, 1 ml of 1 M $MgSO_4$ solution was added.

A series of dilutions (10^{-2}, 10^{-3} and 10^{-4}) of the packaging extracts in phage buffer was made. 100 ml of diluted phage solution was added to 100 ml of KW251 bacteria and the mixture was incubated at 37°C for 20-30 minutes to allow the phage to absorb. TB agarose (3 ml pre-heated to 45°C) was added to the phage/KW251 mix and immediately poured onto pre-warmed (37°C) LB-agar plates. The top agarose was

allowed to harden at room temperature and the plates were incubated overnight at 37°C. An average titter 1.55 X 10^8 pfu/ml, was obtained.

3.2.2.2 Plaque lifting onto Hybond-N^+ membrane

Colonies/plaques were blotted onto Hybond-N^+ membrane according to the procedures specified by the manufacturer (*Amersham Life Science, Protocol version 2.0*).

Prior to the blotting procedure, overnight plates were hardened at 4°C for three hours. Hybond-N^+ membrane was placed on the agarose surface. Marks were made on the membrane and top agarose for later orientation to ensure correct identity of plaques. The membrane was removed after one minute and placed, DNA side up, for seven minutes, on a pad of absorbent filter paper (*Whatman 3MM*) soaked in denaturing solution. The membrane was then placed, colony side up, on a pad of absorbent filter papers soaked in neutralising solution and left for 3 minutes. This process was repeated with a fresh pad soaked in the same solution. The membrane was washed in 2 X SSPE, and allowed to air dry.

The membrane was placed, DNA side up, on a pad of filter papers soaked in 0.4 N NaOH. Efficient DNA fixation was achieved by leaving for 20 minutes. The membrane was then rinsed in 5 X SSPE with gentle agitation for no more than 1 minute. The membrane was used either directly for hybridisation, or was store at 4°C until needed.

3.2.2.3 Hybridisation of colony/plaque lifts to radio-labelled nucleic acid probes

Membranes were rinsed in 5 X SSPE and pre-hybridised at 65°C for one hour in hybridisation solution (5 X SSPE, 5 X Denhardt's solution, 0.5 % w/v SDS) with sheared single-stranded salmon sperm DNA added to a final concentration of 20 µg/ml. Membranes in the same hybridisation bottle were separated by nylon filter (*Polymon*). Labelled probe was denatured by heating at 95°C for 5 minutes and added

to the pre-hybridisation solution. Incubation was continued at 65°C for 16-20 hours. Membranes were then washed twice (10 minutes per wash) at room temperature in 2 X SSPE, 0.1% w/v SDS, followed by one wash at 65°C in 0.1 X SSPE, 0.1 % w/v SDS for 10 minutes. Membranes were wrapped in plastic sheets, and autoradiographed by exposure to X-ray film at -70°C for from one to seven days in a photographic cassette, with and without intensifying screen.

3.2.2.4 Isolation of plaques

Plaques that showed hybridisation were picked by stabbing through the top agarose and agar using a micro-pipette tip with cut off end. The plug of agarose was expelled into a 1.5 ml microcentrifuge tube containing 100 µl of SM buffer and incubated at 4°C overnight. λ phage diffused through the agarose into solution. Next morning, SM buffer (50 mM Tris-HCl pH 7.4, 100 mM sodium chloride, 10 mM magnesium sulphate, 0.1 % w/v gelatin) was added to a total volume of 1 ml, one drop of chloroform was added, and the mixture left at room temperature for 2 hours. The phage diffused into the SM buffer which was stored at 4°C (Sambrook *et al.*, 1989).

3.2.2.5 Isolation of recombinant λ phage

Single phage plaques were recovered from L-agar plates and stored in 100 µl of SM buffer with chloroform at 4°C. This solution was added to 300 µl of overnight cultured KW251 and incubated at 37°C with shaking for 20 minutes. This culture was then used to inoculate 100 ml of pre-warmed (37°C) LB medium containing 10 mM $MgSO_4$ in a flask. The incubation was continued at 37°C with agitation for another 7-8 hours. Usually, cell lysis had occurred during this time. If it had not, 500 µl of chloroform was added and incubation was continued for a further 15 minutes at 37°C. To precipitate cellular debris, the lysate was centrifuged at 8000 X g for 10 minutes and the supernatant was transferred to a sterile tube and stored at 4°C.

RNase A and DNase I were added to the supernatant, each to a final concentration of 1 µg/ml. The supernatant was incubated at 37°C for 1 hour. PEG 8,000 (polyethylene

glycol, molecular weight = 8,000) was added to a final concentration of 10 % w/v and NaCl to a final concentration of 1 molar. The mixture was kept on ice for 3-4 hours before being centrifuged at 11,000 X g for 10 minutes at 4°C. The supernatant was removed by aspiration and the tube containing the pellet was kept in an inverted position on a piece of paper towel to allow all of the fluid to drain away. The pelleted phage particles were resuspended in SM buffer by gentle vortexing. The suspension was centrifuged at 8,000 X g at 4°C for 2 minutes to remove debris and then extracted once with equal volume of phenol saturated with TE buffer (pH 8.0) and once with an equal volume of phenol/chloroform (1:1) saturated with TE buffer. The upper aqueous phase was transferred to a fresh tube and after an equal volume of chloroform/isoamyl alcohol (24:1) had been added, the extraction procedure was repeated. An equal volume of 100 % v/v isopropanol was added to the upper aqueous phase in a fresh tube, the tube was mixed and left at -70°C for 4 hours before being centrifuged at 12,000 X g for 20 minutes at 4°C to recover the phage DNA. The pellet was washed with 70 % v/v ethanol, vacuum dried at room temperature, resuspended in 1 X TE buffer pH 8.0 and stored at -20°C.

3.2.3 Mapping of λ bacteriophage DNA

3.2.3.1 Purification of λ bacteriophage DNA

Phage DNA was purified as follows: 100 µl of phage DNA in a microcentrifuge tube was incubated at 37°C for 45 minutes with 0.08 mg/ml of proteinase K and 0.05 mg/ml of DNase-free RNase A. An equal volume of phenol was added to the tube which was then shaken for 1 minute, and centrifuged at top speed in a microcentrifuge. The upper aqueous phase was transferred to a fresh tube and the purified bacteriophage DNA was precipitated with 1/10 volume of 3 M NaAC pH 4.6 and 2 volumes of 100 % v/v ethanol at -70°C for 30 minutes. The DNA was recovered by centrifugation for 10 minutes at 4°C. The pellet was washed with 70 % v/v ethanol, vacuum dried at room temperature and re-dissolved in double distilled water.

3.2.3.2 Restriction mapping of λ bacteriophage DNA

Purified DNA samples were digested with different restriction endonuclease in 1 X restriction buffer at temperatures and times specified by the manufacturer. Following agarose gel electrophoresis, the DNA fragments were stained with ethidium bromide (*EtBr*) solution, and the gels photographed under UV light (254 nm). DNA fragment sizes were determined by comparing their mobilities with those of DNA molecular weight markers, using the computing program "FRAGRAP" (Duggleby *et al.*, 1981).

3.2.4 Sub-cloning λ bacteriophage DNA fragments in bacterial plasmid

3.2.4.1 Ligation into the vector pBluescript II KS^+

The λ phage DNA and pBluescript II KS^+ DNA were each cleaved (Section 3.2.3.2) with *Xho*I and *Eco*RI. The digested DNAs were electrophoresed in 0.8 % w/v agarose gels and bands containing DNA fragments were excised under a short wavelength UV light (302 nm), and then squashed in plastic bags. The squashed gel was put through a Sepharose CL-6B column and the purified DNA fragments were collected in a sterile microcentrifuge tube. DNA samples were quantified either spectrophotometrically (260 nm) or by comparison with known concentrations of molecular weight markers after treatment with *EtBr* and visualisation under UV light.

Ligations were carried out by mixing 100 ng of the digested pBluescript II KS^+ DNA with 300 ng of recovered DNA fragments and incubating overnight at 13°C in a 15 µl total volume of 1 X ligation buffer (0.05 M Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1mM ATP, 100 µg/ml DNase-free BSA) containing 2.5 units of T4 DNA ligase.

3.2.4.2 Preparation of competent bacterial cells

(1) Calcium chloride method

A single colony of XL-1 Blue bacteria isolated from a tetracycline (12.5 µg/ml) L-agar plate was incubated with vigorous agitation for 2 hours at 37°C in 10 ml of L-broth. L-broth medium (48 ml), containing 12.5 µg/ml tetracycline, was inoculated with 2 ml of the above culture and incubated overnight at 37°C with vigorous agitation until the $OD_{600} \approx 0.6$. The cells were pelleted by centrifugation at 4,000 X g for 2 minutes at 4°C and resuspended in ice-cold 0.1 M $MgCl_2$ solution. The cell suspension was re-pelleted by centrifugation at 4,000 X g for 10 minutes at 4°C and re-suspended in ice-cold 0.1 M $CaCl_2$ solution. The re-suspended cells were then incubated on ice for 20 minutes and pelleted by centrifugation at 4,000 X g for 10 minutes at 4°C. The cell pellet was re-suspended in ice-cold 0.1 M $CaCl_2$ solution containing 15 % v/v autoclaved glycerol. Aliquots (200 µl) of the cell suspension were stored in microcentrifuge tubes at -70°C.

JM109 competent cells were prepared as described above accept that cells from this strain were grown and kept on minimal plates (M-9 plates: 0.6 % w/v Na_2HPO_4, 0.3 % w/v KH_2PO_4, 0.05 % w/v NaCl, 0.01 % w/v NH_4Cl, 1.5 % w/v agarose pH 7.4 supplemented at 50°C with 1 mM thiamine-HCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.02 % w/v glucose). No tetracycline was added to the incubation mixture.

(2) Acidic method

A single colony of XL-1 Blue or JM109 bacterial cells was added to in 10 ml of L-broth medium and incubated overnight at 37°C with vigorous agitation. L-broth medium (48 ml) was inoculated with 2 ml of overnight culture and incubated at 37°C with vigorous shaking until the $OD \approx 0.6$. The cells were then chilled on ice for 2 hours and centrifuged in pre-chilled tubes at 2,500 X g for 20 minutes. The cells were re-suspended by pipetting in 2 ml of ice-cold Trituration buffer (100 mM $CaCl_2$, 70 mM $MgCl_2$, 40 mM NaAC pH 5.5) (*Protocols and Applications Guide, Second Edition, Promega, 1991*) and this suspension was made up to a final volume of 50 ml with Trituration buffer. The cells were incubated on ice for 45 minutes, and then pelleted at 1,800 X g for 10 minutes. The pelleted cells were resuspended in 5 ml of ice-cold trituration buffer by gentle pipetting. Sterile glycerol was added to the cell

suspension to a final concentration of 15 % v/v and the suspension was stored at -70°C as 200 µl aliquots in *Eppendorf* tubes.

3.2.4.3 Transformation of *E. coli* XL-1 Blue cells with plasmid DNA

The ligated plasmid DNA was diluted to the final concentration of 50 ng/µl with TE buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0). Ligated plasmid DNA (50 ng in 1 µl) was added to 200 µl of competent cells that had been pre-thawed on ice. The mixture was incubated on ice for 30 minutes. It was found that a heat shock at 42°C for two minutes after incubation on ice increased transformation efficiency for this bacterial stain. Following the heat shock, the tube was cooled on ice for 2 minutes. The transformants were diluted in 0.5 ml of L-broth and incubated at 37°C for 1 hour with agitation, and plated on LB-agar plates containing 100 µg/ml ampicillin, 0.1 M IPTG and 40 µg/ml X-gal. Plates were incubated overnight at 37°C.

3.2.4.4 Selection for positive transformants

To help simplify cloning and sequencing procedures, pBluescript II phagemid (a cloning vector derived from pUC19) was used in this project. In this plasmid, nucleotides encoding a region of the *lacZ* gene are interrupted by DNA fragments that may be inserted into the multiple cloning site (MCS). Phagemids containing inserts are therefore deficient in β-galactosidase activity. This deficiency can be complemented by addition of a functional α-peptide when plated on selective medium containing X-gal and IPTG. Host/phagemid combinations without inserts generate blue colonies while the bacterial colonies having recombinant phagemids remain white.

A single white transformant colony was transferred to L-broth medium containing 100 µl/ml ampicillin, and cultured with agitation, overnight at 37°C.

3.2.4.5 Mini-preps of plasmid DNA

(1) Plasmid Mini-Boiling Prep

Cells from a single colony of recombinant pBluescript II KS^+ transformed XL-1 Blue bacteria were incubated at 37°C overnight in 3 ml of L-broth supplemented with ampicillin to a final concentration of 50 µg/ml. The overnight culture (1.5 ml) was pelleted by centrifugation at 4°C for 3 minutes. The pellet was resuspended in 110 µl of STETL buffer (8 % w/v sucrose, 0.5 % v/v Triton X-100, 50 mM Tris-HCl at pH 8.0, 50 mM EDTA and 0.5 µg/ml lysozyme) and the tube was placed in a boiling water bath for 30 seconds. The cell suspension was then centrifuged immediately at 4°C for 15 minutes. The pellet was removed with a sterile toothpick and discarded. The supernatant was further treated for 30 minutes at 37°C with RNase A at a concentration of 10 µg/ml. Isopropanol (110 µl) was added to the supernatant which was immediately centrifuged for 15 minutes. The DNA pellet was re-suspended in 2.5 µl of 1 X TE buffer. The amount of DNA recovered was sufficient and suitable for restriction digestion and gel electrophoresis.

Further purification, required for DNA sequencing, was carried out as follows. The DNA obtained as described above, was extracted twice with phenol/chloroform, and once with chloroform. DNA was precipitated by adding equal volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol. This mixture was incubated on ice for 15 minutes and centrifuged at top speed in a microcentrifuge at 4°C for 20 minutes. The DNA pellet was rinsed with 1 ml of 80 % v/v ethanol, centrifuged for 1 minute, vacuum dried, and resuspended in 15 µl of 1 X TE buffer pH 7.5.

(2) Lithium Mini-preps

The procedure described below, which involves the treatment of the bacterial cells with Triton X-100/LiCl and phenol/chloroform, was used for small scale isolation of plasmid DNA. This procedure solubilises plasmid DNA while precipitating chromosomal DNA along with cellular debris. A bacterial colony (from two to five mm in diameter) was picked using a micro-spatula and cells were transferred to a 1.5 ml microcentrifuge tube containing 100 µl TELT buffer (2.5 M LiCl, 50 mM Tris-HCl

pH 8.0, 62.5 mM Na_2-EDTA, 4 % w/v Triton X-100). The mixture was thoroughly vortexed and 100 µl of phenol/chloroform (1:1) was added. After further vortexing, the solution was centrifuged at maximum speed (13,000 X g), and the upper aqueous phase was transferred to a clean microcentrifuge tube. To precipitate the plasmid DNA, chilled (-20°C) 100 % v/v ethanol (150 µl) was added to the supernatant. DNA was pelleted by micro-centrifugation for 5 minutes at maximum speed. The supernatant was drained by inverting the tube, and the DNA pellet was washed with 1ml of 100 % v/v ethanol, dried under vacuum, and redissolved in 30 µl 1 X TE buffer.

(3) Alkaline lysis (based on Sambrook *et al.* (1989) with the modification suggested by *Applied Biosystems Inc.*)

L-both containing 100 µl/ml ampicillin was inoculated with a single colony of XL-1 Blue transformed with recombinant pBluescript II KS^+ and the cells were grown overnight at 37°C. The overnight culture was pelleted by centrifugation, the supernatant removed, and the pellet re-suspended in 200 µl of GTE (50 mM glucose, 55 mM Tris-HCl pH 8.0, 10 mM EDTA) buffer (Solution I). The contents was kept at room temperature for 5 minutes and 300 µl of freshly made 0.2 N NaOH/1 % w/v SDS solution (Solution II) was added and mixed gently. The mixture was incubated at room temperature for 5 minutes, and then 300 µl of 3 M NaAC pH 4.6 (Solution III) was added with mixing. Cell debris was separated from the supernatant by centrifugation at maximum speed for 10 minutes at 4°C. The supernatant was removed and transferred into a clean *Eppendorf* tube. RNase A was added to a final concentration of 20 µg/ml and the solution was incubated at 37°C for 45 minutes, and then extracted with an equal volume of chloroform. The upper aqueous phase was transferred to a clean tube and incubated at room temperature for 10 minute before being centrifuged at top speed for 10 minutes at 4°C in a microcentrifuge. The pellet was washed with 70 % v/v ethanol, dried in a SpeedVac concentrator (*Savant*) for 5-10 minutes and redissolved in 30 µl of double distilled water.

(4) QIAGEN® plasmid mini-preps

QIAGEN® Plasmid Mini Kit was purchased from *Bresatec* and used for high quality plasmid mini-preps. The 4.5 ml overnight culture was pelleted by centrifugation at 12,000 X g for 2 minutes and the pellet resuspended by pipetting up and down several times in 250 µl of P1 solution (50 mM Tris-HCl pH 8.0, 10 mM EDTA) containing 100 µg/ml RNase A. P2 solution (250 µl) (200 mM NaOH, 1 % w/v SDS) was added and the cell suspension mixed by inverting 6 times. The suspension was then incubated at room temperature for 5 minutes and after the addition of 350 µl of N3 buffer (3.0 M KAC pH 5.5), the contents was mixed by immediately inverting the tube six times. The suspension was centrifuged at 12,000 X g for 10 minutes at room temperature and the supernatant placed in a clean tube to which was added 48 µl of 2.0 M NaOH containing 2 mM EDTA. After incubation at room temperature for 5 minutes, 48 µl of 2.0 M NaAC pH 4.6 was added and mixed by vortexing. The supernatant was applied to a column (placed in a 2 ml collection tube) and the column was centrifuged at 12,000 X g for 1 minute. After the addition of 750 µl of Buffer *PE*, the column was again centrifuged at 12,000 X g for 1 minute. The column was transferred to a clean 1.5 ml *Eppendorf* tube and 50 µl of double distilled water was added to the column which was then incubated at room temperature for 1 minute. The purified DNA sample was collected after by spinning the column at 12,000 X g for 1 minute, and was stored at -20°C.

(5) Sepharose CL-6B column

A 0.5 ml *Eppendorf* tube was pierced once at the bottom with a 26 gauge, 1/2 inch needle and one drop of a suspension of glass beads (glass powder suspended in double distilled water) was added to the tube. Sepharose® CL-6B (600 µl) was added and the tube placed inside a 1.5 ml of *Eppendorf* tube. The column was then centrifuged at 3,000 X g for 4-5 minutes. Centrifugation could be repeated several times with extra Sepharose CL-6B beads added. The CL-6B column was moved to a clean 1.5 ml *Eppendorf* tube and the DNA sample was applied. After centrifugation at 3,000 X g for 4-5 minutes, purified DNA was collected in the 1.5 ml *Eppendorf* tube.

3.2.4.6 Large scale preparation of recombinant plasmid DNA

LB medium (10 ml) containing 50 µg/ml of ampicillin was inoculated with bacteria from a single colony of recombinant pBluescript II KS^+ and the culture was grown at 37°C overnight with agitation. A volume of 5 ml of the culture was added to 500 ml of LB medium supplemented with 50 µg/ml ampicillin, and the culture incubated overnight with shaking at 37°C.

The culture was centrifuged at 7,000 X g for 5 minutes and the pelleted cells were thoroughly resuspended in 4 ml of 1 X GTE solution (solution I). A total volume of 5 ml of 0.2 N NaOH/ 1.0 % w/v SDS (solution II) was added, mixed by inversion several times, and the cells were lysed at room temperature for 5 minutes before the addition of 5 ml of 3 M NaAC pH 4.6 (solution III). The contents was mixed by inversion several times and incubation at room temperature was continued for another 5 minutes to permit the separation of the cell debris and the chromosomal DNA from plasmid DNA in the supernatant. The precipitated cell debris and chromosomal DNA was removed by centrifugation at 15,000 X g for 10 minutes at 4°C. The solution was treated with DNase-free RNase A at a final concentration of 10 µg/ml, for 3 hours at 37°C, and then extracted twice with an equal volume of phenol/chloroform, and once with chloroform. After precipitation with an equal volume of 100 % v/v isopropanol at room temperature for 10 minutes, the plasmid DNA was pelleted by centrifugation at 15,000 X g for 15 minutes, washed with 70 % v/v ethanol, dried in a *SpeedVac* and redissolved in 0.5 ml of double distilled water.

3.2.5 General Recombinant DNA methods

3.2.5.1 Preparation of high molecular weight genomic DNA from animal tissues

Liver tissue from *S. crassicaudata* was used to prepare high molecular weight genomic DNA. About 100 mg of freshly frozen tissue was ground into a fine powder in liquid nitrogen and suspended in 2 ml of a solution containing 10 mM Tris-HCl pH 8.0/ 0.1 M EDTA pH 8.0. N-laurylsarcosine was added to a final concentration of 1.0

% w/v and RNase A was added to a final concentration of 40 µg/ml. The suspension was incubated at 37°C for 1 hour and proteinase K was added to a concentration of 100 µg/ml, followed by incubation overnight at 37°C. The solution was then extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol. The upper aqueous phase was transferred to a clean tube and the DNA was precipitated by adding NaAC pH 5.2 to a final concentration of 0.3 M, and 2 volumes of 100 % v/v ethanol. After mixing, the sample was incubated at -20°C for 1 hour and the DNA was spooled out and washed with 75 % v/v ethanol, dried in a *SpeedVac*, and dissolved in 1 X TE buffer for storage at -20°C (Biln and Stafford, 1976).

3.2.5.2 Restriction digestions

Digestions were performed in a total volume of 10-15 µl, using the optimal conditions specified by the manufacturers of the particular restriction enzymes. Five units of enzyme were used to digest 1 µg of DNA at the recommended temperature. Incubation was carried out in 2-3 hours for small scale digestions and overnight for large scale digestions.

3.2.5.3 Electrophoresis of restricted DNA

Electrophoretic separation of DNA fragments was usually carried out on 0.8 % w/v or 1.0 % w/v agarose gels with 1 X TAE buffer (50 X TAE = 2.0 M Tris 5 mM EDTA pH 8.0, 5.70 % v/v glacial acetic acid). Samples were mixed with gel-loading buffer and centrifuged briefly before being added to the wells. The electrophoresis was performed at 5 volts per cm width of the gel slab. The gel was stained by immersing it in a solution of 0.5 µg/ml ethidium bromide for 10 minutes, and de-stained in H_2O for 10-15 minutes before being viewed and photographed under UV light.

3.2.5.4 Purification of DNA fragment

(1) Freeze-squeeze spinning

Restricted DNA was electrophoresed on agarose gel and the gel region containing the DNA fragment of interest was excised under long wave length (360 nm) UV light, sealed in a small plastic bag and frozen at –20°C for 30 minutes. The bag was then squeezed to expel solution from the agarose gel. This freeze-squeeze procedure was repeated two more times. Agarose gel was removed by centrifugation at 13000 X g for 5 minutes and the supernatant was transferred to a clean tube where 1/5 volume 7.5 M NH_4AC solution and 2 volumes of 100 % v/v ethanol were used to precipitate the DNA at -70°C for 2 hours or more. The DNA precipitate was collected by centrifugation at 13000 X g at room temperature for 10 minutes, washed with 70 % v/v ethanol, dried under vacuum at room temperature for 10-15 minutes, and resuspended in double distilled water.

(2) LMT agarose gel

DNA samples were electrophoresed on a horizontal slab gel of low melting temperature (LMT) agarose (usually 0.8 % w/v) at 4°C for 3 hours at 7.5 v/cm. The gel was stained with EtBr (1 µg/ml) for 10 minutes and the DNA fragment of interest was localised by viewing under long wave length UV light (360 nm). The DNA band of interest was sliced from the agarose gel and placed in a clean *Eppendorf* tube. After the addition of an equal volume of 1 X TE buffer (10 mM Tris-HCl pH 7.4, 1mM EDTA pH 8.0), the tube was incubated at 65°C until the agarose had melted, and the mixture was extracted once with an equal volume of pre-warmed (at 37°C) phenol, once with equal volume of phenol/chloroform (1:1), and finally with an equal volume of chloroform. DNA was precipitated by incubation at -20°C for 2 hours in 2 volumes of 100 % v/v ethanol, and pelleted by centrifugation at 13,000 X g for 20 minutes at 4°C. The pellet was washed with in 70 % v/v ethanol, dried under vacuum and redissolved in double distilled water.

3.2.5.5 Quantification of nucleic acids

Spectrophotometric measurement was used to quantify the DNA in large, relatively pure samples. Absorbance was read at a wavelength of 260 nm in a spectrophotometer (*DMS 100 UV Visible Spectrophotometer, Beckman*). An OD_{260} of one was taken to correspond to 50 µg/ml (ds-DNA), 40 µg/ml (ss-DNA and RNA), and 20 µg/ml

(oligonucleotides). The ratio of OD_{260}/OD_{280} was used to estimate nucleic acid purity. Pure preparations have an OD_{260}/OD_{280} of 1.8-2.0.

When only small amounts of nucleic acid were available or when the purity of the sample was sub-optimal, nucleic acid concentration were estimated on an agarose gel stained with EtBr by comparison under UV light with known concentrations of nucleic acid molecular weight markers.

3.2.5.6 Random hexamer method for α-^{32}P-dATP-labelling of DNA probes

The procedure developed by Hodgeson and Fisk (1987), was modified as follows. The DNA to be labelled (100 ng) was mixed with 60 ng of hexamer primer in a total volume of 12 µl, and incubated at 100°C for 5 minutes, and then on ice for 5 minutes. The following were then added: 30 µ*Ci* of α-^{32}P-dATP, 5 units of Klenow enzyme mix (Large fragment of DNA polymerase I) and labelling solution to 1 X concentration (10 X buffer = 0.5 M Tris-HCl pH 6.9, 0.1 M $MgSO_4$, 1 mM DTT, 0.6 M each of dATP, dGTP and dTTP). The solution was mixed thoroughly, centrifuged briefly, and incubated at room temperature for 1.5-2 hours. The reaction was terminated by the addition of 32 µl of stop solution (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 % w/v SDS).

3.2.5.7 Column purification of unincorporated nucleotides

A 0.5 ml *Eppendorf* tube was pierced once at the bottom and five times on the cap with a 26 gauge needle. The column was prepared by placing 150 µl of Bio-Gel 50-100 resin at the base of the tube, overlayed by 450 µl of Bio-Gel 100-200. The tube was placed within a 1.5 ml *Eppendorf* tube and the resin compacted by centrifugation at 1000 X g for 2 minutes. The resin was then washed twice with 100 µl of stop solution. The oligo labelling reaction mix (50 µl total) was loaded onto the column and centrifuged through at low speed (1000 X g) for 2 minutes. The column was washed with 50 µl of stop solution. α-^{32}P-dATP-labelled probe was collected in the outer tube (ie the column flow-through) while the unincorporated nucleotides

remained in the resin. The probe was either used immediately for the hybridisation, or stored at -20°C for later use.

Scintillation counting was used to measure the radioactivity of the probe. Labelled probe (1 µl) was diluted in 1 ml of scintillation fluid, and the number of counts per minute were read by a Liquid Scintillation System (*Beckman scintillation counter, LS 3801 series*). A probe was considered suitable for use if it contained greater than 5 X 10^8 cpm/µl.

3.2.5.8 Alkaline Southern transfer of DNA

Hybond-N^+ (*Amersham*) and the alkaline blotting protocol (*Version 2.0*) provided by the manufacturer were used for Southern transfers. The method is an adaptation of that originally developed by Southern (1975).

Restricted DNA samples were electrophoresed in a 0.8 % w/v agarose gel, stained in EtBr and photographed under UV light with a Polariod camera. The gel was soaked in 0.25 M HCl solution for 7.5 minutes to denature the ds-DNA and then soaked in a fresh solution of 0.25 M HCl for a further 7.5 minutes. The gel slab was rinsed several times in double distilled water and placed on top of a "bridge" of Whatman 3MM paper wicks saturated with transferring solution (0.4 N NaOH) over a stainless-steel tray filled with the same solution. The pre-cut gel-sized piece of Hybond-N^+ membrane was laid on top of the gel and the edges were sealed with parafilm. Two pieces of Whatman 3MM paper of the same size, pre-soaked in transfer solution, were added on top of the membrane. At all stages, air bubbles were carefully avoided. A stack of absorbent papers was then placed on top, followed by a glass plate and an appropriate weight. Transfer took place overnight at room temperature after which the filter was rinsed in 5 X SSPE or 5 X SSC solution, dried in air, sealed in a Whatman 3MM paper pocket in a plastic bag and stored at -20°C.

3.2.5.9 Stripping probe from Hybond-N^+ filters

For stripping filters, a modification of the manufacturer's (*Amersham Life Science*) recommendation was used. Filter were boiled for at least 10 minutes in an aqueous solution of 0.5 % w/v SDS. The stripped filters were rinsed in 2 X SSC, briefly air dried, sealed within a Whatman 3MM paper pocket in a plastic bag, and stored at -20°C for re-use.

3.2.5.10 Oligo-nucleotide synthesis

DNA oligonucleotides for use as primers were synthesised with an Oligo 1000 DNA Synthesiser (*Beckman*) in the Department of Genetics. On completion of synthesis, the column was removed and was firmly attached to a syringe. The syringe/column assembly was screwed onto a 1.5 ml screw-capped *Eppendorf* tube that contained 0.5 ml of AMA reagent (Ammonia and Methylamine, DNA UltraFast Cleavage and De-protection Kit, *Beckman*). Then, the *Eppendorf*/column/syringe assembly was inverted, the AMA was washed through the column and air bubbles were removed. This assembly was kept in the reverse position and incubated at room temperature for 5 minutes before the liquid was pushed into the *Eppendorf* tube. Detached from the assembly, the tube was incubated at 65°C for 10 minutes and cooled at room temperature for more than 30 minutes.

"Raw" oligo solution (120 µl) was added to a clean tube and mixed with 10 volumes of butan-1-ol by vigorous vortexing for 15 seconds. The precipitated oligonucleotide was collected by centrifugation at 13,000 X g for 5 minutes at room temperature in a microcentrifuge. The pelleted DNA was dried under vacuum and resuspended in 120 µl double distilled water, to give a DNA concentration of about 250 µg/ml.

3.2.5.11 Polymerase Chain Reaction (PCR) and cloning PCR products

(1) Standard PCR reactions

PCR amplification was based on a standard protocol and the conditions were adjusted according to need. PCRs were carried out in a volume of 60 µl with 1 X *Taq* polymerase reaction buffer ($MgCl_2$-free), 100 ng of each of the reverse and forward primers, 0.5 mM of each dNTP, 3.5 mM $MgCl_2$, 100 ng of plasmid DNA template or 0.5 µg of genomic mammalian target DNA template, and 2 units of *Taq* polymerase. Reactions were conducted in an "A.R.N" Programmable Temperature Controller (*A.R.N. Electronics*). The reaction mix was incubated at 95°C for 15 minutes without the addition of *Taq* polymerase and then the polymerase was added, mixed and centrifuged briefly. The reaction solution was overlayed with 50 µl of mineral oil and after 2 minutes of denaturation at 95°C, amplification was carried out for 35 cycles (1 minute at 95°C for denaturation, 1 minute at 55°C for annealing and 2 minutes at 72°C for elongation). A final incubation at 72°C for 10 minutes was performed and the PCR product was stored at 4°C. Conditions that were varied included the concentration of $MgCl_2$, the concentration of target DNA and the cycling parameters. PCR products were analysed by electrophoresis on agarose gel.

(2) Wizard™ PCR Preps column purification of PCR products

The Wizard PCR Preps System (*Promega*) provided a reliable and efficient way to purify PCR amplified ds-DNA away from contaminants. The method involves two steps - first, the use of electrophoresis to separate non-specific amplified fragments from the ds-DNA and second, purification of the ds-DNA from other impurities by binding the DNA to, and then eluting from the PCR Preps Resin using double distilled water or TE buffer. PCR products were purified directly without the electrophoretic step if the amplification was specific. An aliquot of 100 µl Direct Purification Buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$ and 0.1 % v/v Triton X-100) was mixed with an equal volume of PCR products and vortexed briefly before the addition of 1 ml of Resin. The content was then vortexed briefly 3 times over a one-minute period before column purification. The PCR products were fractioned on a LMT agarose gel to separate them from non-specific amplification products, stained with EtBr and visualised under UV light (302 nm) where the desired DNA band was excised using a clean, sterile razor blade. The agarose section was transferred to a clean 1.5 ml *Eppendorf* tube, incubated at 65°C until the gel was complete melted and

mixed with 1 ml of Resin. DNA fragments were purified through a Wizard™ PCR Preps column as described above.

(3) Direct cloning of PCR amplified fragments

The pGEM®-T Vector System (*Promega*) was used for cloning PCR products, and protocol provided by the manufacturer was followed with minor modifications. The single overhanging 3' terminal thymidine at the insertion site gives high efficiency ligation of PCR products by providing a compatible insertion site yet preventing re-circulation of the vector.

The PCR amplified DNA fragments were electrophoresed on agarose gel and purified using a Wizard PCR Preps mini-column. Ligation reactions were performed in a total volume of 10 µl in 1 X ligation buffer. The reaction mix contained 2.5 units of T4 DNA ligase, 300 mM Tris-HCl pH7.8, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, 50 ng (1 µl) pGEM-T vector and 25 ng PCR product (about 0.5 kb). The ligation reaction was incubated at 4°C overnight. Recombinant pGEM-T plasmids were transformed as described in Section 3.2.4.3, except that high efficiency JM109 competent cells were used.

3.2.5.12 DNA fragment de-phosphorylation

Both fragment and vector DNA should have compatible ends for cloning ligations. When single restriction enzymes are used to prepare vectors, the 5' phosphate groups must be removed to prevent re-circularisation of the vector during the ligation. To do this, calf intestine alkaline phosphatase (CIAP) was used with accordance of the protocol by Sambrook *et al.* (1989) and referred to the Technical Bulletin No. 87, *Promega* (1994).

Endonuclease digestion of an insert and/or vector DNA sample was carried out for 1 hour in a final volume of 50 µl in restriction digestion buffer (Section 3.2.5.2) containing 10 µl DNA with 2-3 fold excess of restriction enzyme. A mini-gel was run

to ensure complete digestion and CIAP buffer was added to give a final concentration of single strength (10 X buffer = 10 mM $ZnCl_2$, 10 mM $MgCl_2$ and 100 mM Tris-HCl pH 8.3). CIAP was added to a concentration of 0.01 u/pmol ends, and the final reaction volume was made up to100 µl with double distilled water. The reaction components were incubated at 37°C for 1 hour. Another 0.01 unit CIAP/pmol of ends was added and incubation was carried out at 37°C for an additional 30 minutes. CIAP was removed from the mix either by incubation at 56°C for 30 minutes in the presence of proteinase K (100 µg/ml), 0.5% w/v SDS and 5 mM of EDTA (pH 8.0), or extraction with TE-saturated phenol/chloroform, followed by extraction with chloroform:isoamyl alcohol (24:1). The phosophorylated DNA fragments were precipitated by adding either 1/10 volume of 3 M NaAC pH 7.0 or 0.5 volume of 7.5 M NH_4AC, and then adding 2 volumes of ethanol and incubating at -70°C for 30 minutes. The DNA precipitate was collected by centrifugation at 12,000 X g for 10 minutes. The pellet was washed with 70% v/v ethanol, dried under vacuum, redissolved in double distilled water and quantified absorption spectroscopy.

3.2.6 DNA sequence determinations

3.2.6.1 Preparation of template DNA for sequencing

(1) Single-stranded plasmid DNA

A singe, well-isolated colony of bacteria was used to inoculate 5 ml of 2 X YT medium (1.0 % w/v NaCl, 1.0 % w/v Yeast extracts and 1.6 % w/v Bactotryptone) containing 100 µg/ml antibiotics and VCM13 helper phage at 10^7-10^8 pfu/ml (m.o.i ~10). The culture was incubated overnight at 37°C with agitation. Cultured cells (1.2 ml) were pelleted by centrifugation and the supernatant was transferred to a clean *Eppendorf* tube followed by the addition of 150 µl 20 % w/v PEG 8,000/2.5 mM NaCl solution. This mixture was inverted several times, the phage particles were allowed to precipitate on ice for 15 minutes and were pelleted by centrifugation at 13,000 rpm for 5 minutes. The supernatant was drained off and the tubes were centrifuged for a few more seconds to remove residual liquid. The pellet was resuspended in 400 µl of 0.3 M NaAC pH 5.5/1 mM EDTA solution by vortexing

vigorously and extracted with an equal volume of phenol/chloroform. After centrifugation in a microcentrifuge for 2 minutes, the upper aqueous phase was transferred to a clean *Eppendorf* tube and mixed with 1 ml of ethanol by inverting. The ss-DNA pellet was collected by centrifugation at 13,000 rpm for 5 minutes, dried under vacuum and re-dissolved in 25 µl of double distilled water.

(2) Denaturation of double-stranded DNA

Double-stranded plasmid DNA samples in aqueous solution, prepared as described in Section 3.2.4.5, were further purified for sequencing using the following procedure. ds-DNA (40 µl) was digested with 1 µl of 10 mg/ml RNase A at 37°C for 45 minutes and then 10 µl of 1.0 M NaOH/1.0 mM EDTA solution was added to the solution, mixed, briefly centrifuged and incubated for a further 1 hour at 37°C. The sample was purified using a Sepharose CL-6B column (see Section 3.2.4.5).

3.2.6.2 Sequencing reactions

DNA sequencing of ss-DNA and/or alkaline denatured super-coiled DNA (Chen and Seeburg, 1985) was performed by the di-deoxy chain termination method (Sanger *et al.*, 1977) using both the *fmol*™ DNA Sequencing System from *Promega* and Sequenase® Version 2.0 from *United States Biochemicals*. The sequence of both strands of the DNA insert was determined progressively by genomic walking and sub-cloning.

(1) Sequencing reactions using the *fmol*™ sequencing system

The protocol provided by the manufacturer was followed with slight modifications. For each set of sequencing reactions, four thin walled 0.5 ml of microcentrifuge tubes were labelled A, C, G and T respectively. The appropriate d/ddNTP mix (2 µl) was added to each tube and the tubes retained on ice until used. A 16 µl cocktail was made which contained 0.5 µg DNA sample, 50 ng of sequencing primer, 15 µ*Ci* α-^{32}P-dATP, and 5 µl of *fmol*™ Sequencing 5 X Buffer. The cocktail was mixed by flicking the tubes and then 1 µl of sequencing grade *Taq* DNA polymerase (5 u/µl)

was added and mixed by gentle pipetting. An aliquot of 4 µl of the above mix was added to each labelled tube and after mixing and a brief centrifugation, the contents of each tube was overlaid with two drops of mineral oil. Reactions were carried out in an A.N.R. Programmable Temperature Controller, initially pre-heated to 95°C. Reactants were incubated at 95°C for 2 minutes and then thermal cycling was performed for 30 rounds (0.5 minute at 95°C for denaturation, 0.5 minute at 62°C for annealing and 1 minutes at 72°C for elongation). A final incubation at 72°C for 10 minutes was used before the reactions tubes were stored at -20°C overnight.

(2) Sequencing with Sequenase® Version 2.0

Sequenase® Version 2.0 T7 DNA Polymerase Sequencing Protocol from the manufacturer, *USB*, was used with some modifications. Template DNA (7 µl ≈1.5 µg) prepared as described in Section 3.2.6.1, was mixed with 50ng (1 µl) of sequencing primer, 2 µl of 5 X Sequenase Reaction Buffer (200 mM Tris-HCl, 100 mM $MgCl_2$, 250 mM NaCl), and the final reaction volume was made up to 10 µl with double distilled water. This reactants were incubated at 65°C for 2 minutes, allowed to cool to room temperature over a period of about 30 minutes, and then kept on ice. Sequenase Version 2.0 was diluted 1:8 in dilution buffer (10 mM Tris-HCl pH 7.5, 5 mM DTT, 0.5 mg/ml BSA) and 2 µl (3.25 units) of this diluted solution was added to the annealing mix with 1 µl of 0.1 M DTT, 2 µl of 10 X labelling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP), 5 µ*Ci* of α-^{32}P-dATP (0.5 µl of 10 µ*Ci*/µl stock, *Amersham*). The mixture was incubated at room temperature for 4 minutes and at the same time, 2.5 µl of the appropriate di-deoxy termination mixture was added to each of 4 labelled tubes ("G", "A", "T", "C") which had been pre-warded at 37°C for 4 minutes. The termination mixes used were as follows. ddG – 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 8 µM ddGTP, 50 mM NaCl; ddA – 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 8 µM ddATP, 50 mM NaCl; ddC – 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 8 µM ddCTP, 50 mM NaCl; ddT – 80 µM dGTP, 80 µM dATP, 80 µM dCTP, 80 µM dTTP, 8 µM ddTTP, 50 mM NaCl. The labelling mix (4 µl) was added to each of the termination tubes, mixed by pipetting and incubated at 37°C for 4 minutes. Stop

solution (4 µl) (95 % v/v formamide, 20 mM EDTA, 0.05 % w/v bromophenol blue, 0.05 % w/v xylene cyanol FF) was added to each termination tube, mixed and the tubes either kept on ice for direct use, or stored at -20°C overnight.

3.2.6.3 DNA sequencing PAGE (Sanger and Coulson, 1978)

Polyacrylamide gel electrophoresis (PAGE) was used to separate terminated nucleotide fragment which were visualised autoradiographically after exposure to RX X-ray film (*Fuji*). The gel was made of 6.0 % w/v polyacrylamide containing 7.0 M urea in 1 X TBE buffer diluted from 10 X TBE stock solution (0.89 M Tris-base pH 8.0, 0.89 M boric acid and 20 mM EDTA). The gel solution was filtered and de-gassed for 15 minutes under vacuum before being poured between two 40 cm X 40 cm glass plates separated by 0.25 mm spacers. Polymerisation was catalysed by 0.05 % TEMED and 0.04 % ammonium persulphate at room temperature for at least 2 hours. The gels were pre-run in a sequencing apparatus (Model S2, *BRL Life Technologies Inc.*) for 30 minutes at 1700 Volts with maxima set at 50 mA and 100 W. Sequence reactants (3 µl) were denatured for 2 minutes at 80°C and then applied to the wells. Electrophoresis was continued until 10 minutes after the xylene cyanol dye reached the bottom of the gel. If necessary, a second or/and a third loading was made and after electrophoresis was completed, the DNA was fixed to the gel by soaking in fixation solution (20 % v/v methanol, 10 % v/v glacial acetic acid in double distilled water) with agitation on a rotating shaker for 20 minutes. The gel was oven dried at 65°C for at least 2 hours and exposed to X-ray film (*Fuji*) overnight at room temperature.

3.2.6.4 DNA sequence analysis

(1) Nomenclature

Throughout this thesis, the following abbreviations are used to describe DNA and protein sequences (Table 3.1).

Table 3.1 Abbreviations used to describe various *LIF* sequences

Abbreviation	Species	Sequence	GenBank Locus Identification	References
hLIF	*Homo sapiens* (Human)	Mature LIF coding region	HUMALIFA	Stahl *et al.*, 1990
bLIF	*Bos taurus* (Cattle)	Mature LIF coding region	BOVLIFA	Kato *et al.*, 1996
mLIF	*Mus musculus* (Home mouse)	Mature LIF coding region	MUSALIFA	Stahl *et al.*, 1990
oLIF	*Ovis aries* (Ovine)	Mature LIF coding region	A05963 A05964 A05965	Willson *et al.*, 1992
pLIF	*Sus scrofo* (Porcine)	Mature LIF coding region	A05961 A05962	Willson *et al.*, 1992
rLIF	*Rattus norvegicus* (Rat)	Mature LIF coding region	RATLIF	Yamamori *et al.*, 1989
sLIF	*S. crassicaudata* (Fat-tailed dunnart)	Mature LIF coding region		This thesis

(2) DNA Sequence comparisons

The computer programs FASTA and BLAST were used to search DNA sequence data bases for sequences that showed significant identity to *sLIF*. DNA sequences were edited by the program SEQFIX. The program MAP was used to identify restriction sites in DNA sequences. Comparisons between pairs of DNA or protein sequence were carried out using SEQH and SEQA. Multiple sequence alignments made use of the program CLUSTAL-W (Higgins and Sharp, 1989). For some comparisons (eg intron sequences) DOTPLOT was used (Maizel and Tenk, 1981). Aligned sequences were edited and displayed using GENEDOC (Nicholas, 1997).

(3) Rates of nucleotide substitution

The minimum numbers of nucleotide substitutions that have occurred during the separate evolution of pairs of *LIF* sequences (ie sequences taken from different taxa) since they last shared a common ancestor, were calculated using the method of Li (1993).

(4) Phylogenetic analyses

LIF DNA sequences were extracted from GenBank (Table 3.1) and aligned using CLUSTAL-W. Maximum parsimony analyses of the aligned sequences (coding regions only) were carried out using DNAPARS. The *sLIF* sequence was used as an outgroup in these tests as it can reasonably be assumed that the separation of the stem marsupials from the stem eutherians pre-dated the separation of the various eutherian taxa. Robustness of phylogenetic trees was tested by bootstrapping (Felsenstein, 1985) and was based on 1,000 pseudo-replicates of the original data set obtained using SEQBOOT and CONSENSE. DNAPARS, SEQBOOT and CONSENSE are part of the PHYLIP group of programs (Felsenstein, 1993). All computer algorithms were accessed on-line through ANGIS (Australian National Genomic Information Services).

(5) Prediction and analysis of biological properties of sLIF

The GCG program TRANSLATE carried by ANGIS was used to convert *sLIF* cDNA sequence into its coded amino acid sequence. Mature LIF polypeptide from human, mouse, cow and *S. crassicaudata* were analysed by PEPTIDESTRUCTURE and PLOTSTRUCTURE (Chou and Fasman, 1978; Nishikawa, 1983). PEPTIDESTRUCTURE makes secondary structure predictions for a peptide sequence. The predictions include (in addition to alpha, beta, coil, and turn) measures for antigenicity (Jameson and Wolf, 1988), glycosylation site (Wolf *et al.*, 1988; Jameson and Wolf, 1988), hydrophobicity (Kyte and Doolittle, 1982), and surface probability (Emini *et al.*, 1985). PLOTSTRUCTURE displays the predictions graphically. The polypeptide pI and molecular weight of LIF proteins were calculated using Compute pI/Mw at ExPasy (Switzerland). Compute pI/Mw is a tool which allows the computation of the theoretical pI (isoelectric point) and Mw (molecular weight) for a list of SWISS-PROT and/or TREMBL entries or for a user entered sequence (Bjellqvis *et al.*, 1993; 1994). Prediction of transmembrane regions and their orientation of LIF proteins were made using the program TMpred at ISREC (*Institut Suisse de Recherche Experimentale Sur le Cancer*, Swiss Institute for Experimental Cancer Research). The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins. The prediction is

made using a combination of several weight-matrices for scoring (Hofmann and Stoffel, 1993). 3 dimensional structure of sLIF was predicted using computing program SWISS-MODEL (ProModII) that is based on one or more template structures. SWISS-MODEL is an automated protein-modelling server that makes protein modelling accessible to all biochemists and molecular biologists world wide. This program searches for suitable templates, checks sequence identity with target, creates ProModII jobs, generate models with ProModII and energy minimisation with Gromos 96 to give the final result in PDB files (Peitsch and Jongeneel, 1993; Peitsch, 1995a; 1995b; 1996; 1997). LIF PDB files were viewed using the 3D viewer RASMOL, software for visualisation of molecular structures (Christopher *et al.*, 1996; Sayle *et al.*, 1995; Sanchez-Ferrer *et al.*, 1995).

3.2.7 **Northern analysis of RNA prepared from adult tissues**

3.2.7.1 Isolation of adult tissues from adult *S. crassicaudata*

The desired tissue was collected by normal surgical procedures after the animal had been sacrificed. Tissue was rinsed in PBS, dried using clean lint-free tissue, and either used directly for extraction of total RNA or snap-frozen in liquid nitrogen and stored at -70°C for later use.

3.2.7.2 Extraction of total RNA from adult tissues and organs

Successful cDNA synthesis is largely dependant on the availability of good quality RNA. It is important to optimise the condition of RNA isolation and to prevent degradation by RNases. A single-step RNA isolation method was used, employing the TRIZOL™ reagent (which contains guanidine isothiocyanate) (Simms *et al.*, 1994). Tissue (100 mg) was homogenised in 1ml of TRIZOL™ reagent using a motor-driven homogeniser (Lab. Stirrer RS21, *Chiltern Scientific*) and centrifuged for 10 minutes at 13,000 rpm in a microcentrifuge at 4°C. The clear supernatant was transferred to a clean sterile tube and incubated at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes. Chloroform (200 µl per ml of TRIZOL reagent) was added, the components mixed vigorously, stood at room temperature for

3 minutes and centrifuged at 13,000 X g in a microcentrifuge for 15 minutes at 4°C. The upper colourless aqueous phase was transferred to a clean tube, and the RNA was precipitated by mixing with 0.5 ml of isopropanol, incubating at room temperature for 10 minutes, and pelleting by centrifugation at 13,000 X g in a microcentrifuge for 10 minutes at 4°C. The RNA pellet was washed once with 75 % v/v ethanol and dried briefly (< 3 minutes) under vacuum before being dissolved in DEPC-treated double distilled water.

3.2.7.3 Formaldehyde agarose gel electrophoresis of RNA

RNA samples were electrophoresed on 1.4 % w/v agarose gels containing formamide and made in MOPS buffer (20 mM MOPS, 5 mM NaAC, 1 mM EDTA pH 7.0). Buffer plus agarose was heated in a microwave oven until the agarose melted. The solution was allowed to cool at room temperature to 50-60°C and made up to volume with double distilled water. Formaldehyde was added slowly to give a final concentration of 12.5 % v/v, and the gel was cast in a fume hood. RNA samples were precipitated in ethanol, pelleted by centrifuged, dried under vacuum for 2-3 minutes and dissolved in a solution of 3.5 µl double distilled water, 10 µl deionised formamide, 2 µl 10 X MOPS buffer, 1 µl EtBr, made up with double distilled water to a total volume of 20 µl. This solution was incubated at 65°C for 5 minutes, transferred immediately to ice and mixed with 5 ml of 5 X RNA tracking dye (8.0 % w/v Ficoll, 0.02 % w/v bromophenol blue, 0.04 % w/v xylene cyanol). Samples were loaded onto the gel in a fume hood and after electrophoresis at 70 mA for 4-5 hours in 1 X MOPS buffer, the gel was photographed under UV light.

3.2.7.4 Northern blotting of RNA to Hybond-N^+ nylon membrane

RNA was transferred from formamide - agarose gels to Hybond-N^+ membrane using Northern blotting. The gel was rinsed several times in double distilled water and soaked in 50 mM NaOH made in 1X SSC solution for 20 minutes. The gel was then soaked in 20 X SSC for 45 minutes to remove formamide. The capillary blotting procedures described in Section 3.2.5.8 was used for RNA blottings but 20 X SSPE

solution rather than 0.4 N NaOH was used as the transferring solution. The transfer was carried out overnight at room temperature. It was routinely confirmed that the RNA had transferred to the filter. The membrane was then rinsed in 2 X SSPE solution and the RNA fixed to the membrane by baking at 80°C for 2 hours without vacuum.

3.2.7.5 Hybridisation of Northern membranes with isotopic DNA probes

The conditions for the hybridisation and post-hybridisation washes of Northern membrane were the same as those used for Southern hybridisation (Section 3.2.2.3). Filters were wrapped in plastic sheeting and exposed to X-film at -70°C for about 1 week.

3.2.8 cDNA synthesis

3.2.8.1 Use of AMV reverse transcriptase

The first strand cDNA synthesis reaction was carried out in a total volume of 20 µl containing up to 2 µg of poly A^+ RNA. This volume was scaled up if more RNA was used. In a sterile RNase-free microcentrifuge tube, about 2 µg of total RNA sample was combined with 1 µl of hexamer (500 ng/µl) or the same amount of oligo $(dT)_{12-18}$ primer, heated at 65°C for 5 minutes and cooled at room temperature for at least 30 minutes. The tube was centrifuged briefly to collect the solution at the bottom and the following components were then added to the annealed template/primer mix: 4 µl of 5 X Buffer (250 mM Tris-HCl pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine, 50 mM DTT), 1 µl of 10 mM dNTPs, 1 µl of 100 mM DTT, 2 µl of RNasin® ribonuclease inhibitor (40 u/µl), 1 µl of AMV reverse transcriptase (10 u/µl) and double distilled water to 20 µl. After mixing, the solution was incubated at 42°C for 1 hour. The reaction was stopped by heating at 95°C for 5 minutes, and the reactants were stored at -20°C.

3.2.8.2 Use of SuperScript™ Pre-amplification System

SuperScript™ Pre-amplification System for First Strand cDNA Synthesis kit was purchased from *GIBCOBRL Life Technologies* and the protocol they supplied was followed for first strand cDNA synthesis.

Both oligo $(dT)_{12-18}$ and random hexamer primers were used. Before use, all components were thawed on ice, mixed and briefly centrifuged. The synthesis reaction was started with 10 µg of total RNA sample, mixed with 1 µl of random hexamer (50 ng/µl) or oligo $(dT)_{12-18}$ primer (500 ng/µl) in a total volume of 12 µl in double distilled water. This mixture was incubated at 70°C for 10 minutes and then kept on ice. To the tube was added 2 µl of 10 X PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 2 µl of 25 mM $MgCl_2$, 1 µl of 10mM dNTP mix and 2 µl of 100 mM DTT. Annealing took place at 42°C for 5 minutes. One µl of SuperScript II RT (200 u/µl) was added and the reaction components were incubated at 42°C for 50 minutes to allow cDNA synthesis. The reaction was terminated by heating to 70°C for 15 minutes. Alternatively, RNA templates were removed by incubation at 37°C for 20 minutes in the presence of 1 µl of RNase H (2 u/µl). The cDNA sample was used for PCR amplification immediately or stored at -20°C.

3.2.9 RT-PCR amplification of *sLIF* gene fragments

3.2.9.1 Isolation of intact RNA from formalin-fixed paraffin-embedded tissues

Formalin-fixed, paraffin-embedded tissue sections, cut from the uterus/embryo complex of *S. crassicaudata* during early stages of embryonic development, were used for the preparation of RNA samples. The procedure was adopted from Jiang *et al.* (1995) with minor modifications.

Paraffin was removed from the tissue sections by soaking the slides overnight in xylene, after which the cover-slips could be removed easily. The tissue sections were

re-hydrated by washing in serial dilutions of ethanol, and air-dried for several minutes at room temperature. The sections were scraped off the slide using a pipette tip, transferred to a 0.5 ml microcentrifuge tube, and incubated at 37°C for 1 hour to allow for DNA and protein digestion. The incubation solution (40 µl) contained 1.25 X PCR buffer (200 mM Tris-HCl, 500 mM KCl), 6.25 mM $MgCl_2$, 5 units of RNasin (*Promega*), 2 mM DTT, 1 unit of RNase-free DNase I (*Pharmacia*) and 0.1 mg/ml proteinase K. After incubation, the solution was then heated at 95° C for 15 minutes to inactivate the DNase and proteinase K. Following centrifugation at 13,000 X g for 7 minutes, the supernatant was transferred to a clean tube and stored at -70°C. This material was used for the cDNA synthesis as described above, and for the RT-PCR reactions described below.

3.2.9.2 Primer design for amplification of cDNA fragment encoding mature sLIF

Primers were designed to amplify *sLIF* cDNA encoding the mature sLIF protein (555 bp). At both the 5' and 3' end of the cDNA, a *Bam*HI restriction site was introduced in order to facilitate in-frame, unidirectional cloning. The amplified fragment included a stop codon from *sLIF*. When cloned into pGEX2T expression vector, the stop codon included in this amplified fragment will be followed by three stop codons originally in the open reading frame (GST Gene Fusion System, Second Edition 1994, *Pharmacia Biotech*).

The 5' primer, designated *Exp1*, was a 22mer with the following sequence:
5' TAGGATCCCCCCTACCCATCAC 3'.
The 3' primer, named *Exp2*, was a 27mer with the following sequence:
5' ATGGATCCTAGAAGGCCTGGGCCACCA 3'. *Bam*HI sites are underlined.
These primers were designed from the *sLIF* genomic sequence that had been obtained previously in this study.

3.2.9.3 RT-PCR amplification

First strand cDNA was synthesised as described in Section 3.2.8. The PCR reactions were initially carried out using the standard protocol (Section 3.2.5.11) with

modification to gain optimal PCR amplification. The first strand cDNA (2 µl) was mixed with 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 100 ng of each of *Exp1* and *Exp2*, in a final volume of 50 µl in 1 X PCR buffer. DNA was denatured prior to the addition of 1µl diluted (2 u/µl) *Taq* polymerase (*Bresatec*) by incubating the reaction mixture at 95°C for 15 minutes. The reaction mixture was overlaid with mineral oil (50 µl) and heated to 95°C for 2 minutes. Thermal cycling involved 35 repeats. Each cycle consisted of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 2 minutes. After a final 10 minute elongation step at 72°C, samples were stored at 4°C.

3.2.9.4 Analysis of RT-PCR products

Products of RT-PCR were electrophoresed on 2 % w/v agarose gels, stained with EtBr, photographed under UV light (Section 3.2.5.11) and analysed by Southern blot hybridisation using probes derived from λ clones for exon 2 and exon 3 of the *sLIF* gene as described in Section 3.2.4.

3.2.10 *In vitro* expression of *sLIF*

3.2.10.1 Modification of *sLIF* cDNA fragment

An aim of this project was to express *sLIF* cDNA *in vitro* after inserting this cDNA into the expression vector pGEX2T. Incorporation of *Bam*HI sites in the primers used for RT-PCR in the derivation of *sLIF* cDNA, permitted the cDNA to be inserted into the vector, in-frame, so as to retain the appropriate signal peptidase cleavage site (thrombin). However, the cDNA possessed an internal *Bam*HI site that had to be modified and this was carried out using oligonucleotide-directed PCR (see Results Section 4.3.1).

3.2.10.2 Growing the bacterial strain BL-21 and making competent cells

No specific hosts are required for propagating pGEX2T or for expressing its fusion proteins, since the vectors possesses a functional *lacI*^{q} gene. The proteinase-deficient bacterial strain BL-21 was used as host for the vector. BL-21 cells from a glycerol stock stored at -70°C, were streaked onto a LB-agar plate, grown overnight at 37°C, and a single colony was used to inoculate 50 ml of L-broth medium. After incubation at 37°C with agitation until the OD_{600} = 0.4-0.5, the cells were pelleted by centrifugation (3000 X g for 15 minutes at 4°C) and then gently re-suspended in 1/10 volume (5 ml) of ice cold TSS buffer (Transformation and Storage Solution: 1 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 0.8 g PEG 3350, 5.0 ml DMSO, 5.0 ml $MgCl_2$, dissolved in 100 ml sterile double distilled water, adjusted pH 6.5 and stored at 4°C). The suspended cells were stored on ice and used within 2-3 hours for transformation (Chung *et al.*, 1989).

3.2.10.3 Preparation of pGEX2T expression vector

pGEX2T plasmid (*Pharmacia*) was used for cloning and expressing *sLIF*. Plasmid DNA was diluted with double distilled water to a concentration of 200 ng/µl and 1 µl of this solution was used to transform bacterial cells of the strain DH5α that had been grown at 37°C overnight in the presence of ampicillin. LB medium (20 ml) was inoculated with cells from a single colony and the culture incubated overnight at 37°C with shaking. Plasmid DNA (30 µl), prepared as described in Section 3.2.4.5, was digested with *Bam*HI. After checking that digestion was complete, 7 units of calf intestinal alkaline phosphatase (CIAP) was added with CIAP 10 X reaction buffer to a final concentration of 1 X and the solution was incubated at 37°C for 30 minutes for complete dephosphorylation of phosphate groups from 5' termini. The reaction was terminated by adding 2 µl EDTA, and heating at 85°C for 15 minutes. The reaction mix was extracted with an equal volume of phenol and the upper aqueous phase was extracted with equal volume chloroform/isoamyl alcohol and purified through the Wizard PCR Preps System column (Section 3.2.5.11).

3.2.10.4 Ligation of *sLIF* to pGEX2T

For ligations, a ratio of 1:5 linearised pGEX DNA : *sLIF* DNA was recommended by the commercial supplier. The molars of linear DNA ends was calculated by the following formula,

Molar ends = 2 X (g of DNA) ÷ [(No. of bp) X (649 Daltons/bp)]

100 ng pGEX DNA (0.06 pmol ends) needs 50 ng of *sLIF* (≈ 0.55 kb) (0.3 pmol ends) according to the recommended ratio.

The ligation to form *pGEX2T-sLIF* was carried out at 4°C for 4 hours in a total volume of 20 µl of 1 X ligation buffer, containing 1 mM ATP with 2.5 units of T4 DNA ligase and terminated by heating at 65°C for 10 minutes. Part of the ligated DNA was used immediately for transforming BL-21 competent cells; the remainder was stored at -20°C.

3.2.10.5 Transformation

The procedure recommended by *Pharmacia* for the pGEX2T system was followed with some modifications. Freshly prepared BL-21 competent cells (1 ml) were added to a pre-chilled tube and mixed with 10 µl of each ligation reaction mix or 1 ng of un-cut vector by gently swirling. The mixture was incubated on ice for 45 minutes, in a 42°C water bath for exactly 2 minutes, and then chilled on ice for at least 2 minutes. Transformed cells (200 µl) were diluted in LB medium to a final volume of 1ml and incubated at 37°C for 1 hour with shaking. Cells (200 µl) transformed with plasmid carried insert and cells (20 µl) transformed with uncut plasmid vector without insert were plated onto LB-agar plates supplemented with 100 µg/ml ampicillin and incubated overnight at 37°C. This cell culture was diluted 1:10 in LB medium and incubated at 37°C for 30 minutes with shaking. The cells were stored at -70°C in the presence of 200 µl of sterile 80% v/v glycerol.

3.2.10.6 Analytical scale preparation of GST-sLIF fusion protein

Cells from a single colony of the transformed bacteria were used to inoculate 25 ml of ampicillin supplemented LB medium. The incubation was carried out overnight at 37°C with agitation. Overnight cultured cells (2.5 ml) were diluted (1:10) in 50 ml of LB medium containing 100 µg/ml of ampicillin, and incubated at optimal temperature until $OD_{600} \approx 0.6$. IPTG was added from a 0.1 M stock solution to a final concentration of 0.1 mM and incubation was continued for 1 hour with shaking. The culture was transferred to a clean tube and the cells were pelleted by centrifugation at 7000 X g for 7 minutes at 4°C. The pelleted cells were resuspended in 10 ml of 1 X TTBS buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 % v/v Triton X-100) and incubated on ice for 15 minutes with 100 µg/ml lysozyme. PMSF was added to the suspension to a final concentration of 0.1 mM and the cells were lysed by sonication using an MSE sonicator (*TOSCO Thomas Optical & Scientific Co. Pty Ltd*, serial no. PG-471). The crude sonicate was centrifuged at 12,000 X g for 10 minutes at 4°C to remove the insoluble cell debris and the supernatant was passed through a sterile 0.45 µm filter. Triton X-100 was then added to a final concentration of 1.5 % v/v and the solution was stored at -70°C.

3.2.10.7 Preparative scale preparation of GST-sLIF fusion protein

After optimal conditions for over-expressing the GST-sLIF fusion protein had been established from pilot experiments, these conditions were scaled up for use in the large scale preparation. Cells were treated identically to those used in the analytical scale preparation (Section 3.2.10.6) except that 5 ml of overnight cultured cells were used to inoculate 150 ml of ampicillin-supplemented LB medium and then over-expression of the fusion protein was induced as described in Section 3.2.10.6. Bacteria were harvested by centrifuging at 7000 X g for 7 minutes at 4°C in an SS-34 rotor (*Sorvall*), washed with 30 ml of 1 X TTBS buffer, drained and placed on ice. Cells were completely re-suspended in 30 ml of ice-cold TTBS buffer and were disrupted

using the condition described earlier (Section 3.2.10.6) in the presence of 0.1 mM PMSF. Triton X-100 was added to a final concentration of 1 % v/v and the crude extracts were centrifuged at 12,000 X g for 10 minutes at 4°C. Supernatants were transferred to clean tubes and stored at -70°C.

3.2.10.8 Analysis of proteins on SDS-PAGE

The Laemmli standard protocol for discontinuous polyacrylamide gel electrophoresis under denaturing condition was used in this project (Laemmli, 1970). The separating gel monomer solution was made by combining the following reagents: 2.5 ml of 1.5 M Tris-HCl pH 8.8 solution, 100 µl of 10 % w/v SDS stock solution, 4.0 ml of de-gassed 30 % w/v stock solution of acrylamide/bisacrylamide and double distilled water (to a total volume of 10 ml). Mini-PROTEIN® II Cell gelling system (*Bio-Rad*, serial no. 125BR) was used for gel casting. A comb was inserted into the assembled gel apparatus and the above gel mixture, together with 50 µl of fresh-made 10 % w/v ammonium persulfate and 5 µl of TEMED, was poured into the assembly. The top of monomer solution was slowly overlaid with water-saturated isobutanol. After polymerisation (45 minutes at room temperature), the overlay solution was washed off with distilled water and the space for the stacking gel was dried with Whatman 3MM paper. A clean comb was inserted and 10 ml of stacking solution (0.5 M Tris-HCl pH 6.8, 100 µl of 10 % w/v SDS, 1.3 ml of degassed 30 % w/v stock solution of acrylamide/bisacrylamide, 50 µl ammonium persulfate, 10 µl TEMED in double distilled water) was added until all the teeth had been covered. The gel was allowed to polymerise at room temperature for 30-45 minutes. The comb was removed by pulling it straight up slowly and gently. The wells were flushed with double distilled water before use.

Cell extracts (10 µl) were mixed with an equal volume of 2 X SDS sample solution (304 mg Tris-base, 4.0 ml glycerol, 0.4 g SDS, 0.2 mg bromophenol blue, 0.4 ml β-mercaptoethanol, made up to 15 ml with double distilled water, and adjusted pH 6.8) and heated at 100°C for 5 minutes in a sealed screw-cap microcentrifuge tube. Samples were added to the gel wells, the latter having first been filled with SDS

electrophoresis buffer (5 X buffer = 9.0 g Tris-base, 43.2 g glycine, 3.0 g SDS dissolved in 600 ml double distilled water). The gel was run at 200 V for about 45 minutes at room temperature. When the bromophenol blue dye had reached the bottom of the gel slab, electrophoresis was stopped and the gel was stained for 1 hour with 0.1 % w/v Coomassie brilliant blue R-250 in fixative (40 % v/v methanol, 10 % v/v glacial acetic acid), de-stained with fixative, and dried between two sheets of Cellophane, at 80°C for 2 hours under vacuum.

3.2.10.9 Releasing GST-sLIF fusion protein from inclusion bodies

Over-expressed protein was obtained as described in Section 3.2.10.7. The crude cell extract, supernatant, pellet, cell debris and inclusion bodies were analysed by SDS-PAGE. It was found that about 80 % of the fusion protein was insoluble and precipitated in the pellet. Lowing the IPTG concentration used for induction, altering induction time, inducing for shorter times, inducing at a higher cell density and lowing the growth temperature (Smith and Johnson, 1988; Schein, 1989) were used in attempts to solubilise the fusion protein. Optimal conditions were found to be: bacterial cells grown at 30°C until $OD_{600} \approx 0.6$ and induced with 0.1 mM IPTG for 3 hours. Under these conditions 70-80 % of the fusion protein was recovered in a soluble form.

3.2.10.10 Affinity chromatographic purification of GST-sLIF

(1) Preparation of Glutathione Sepharose 4B slurry

Glutathione Sepharose® 4B (Pharmacia) (GS4B) was used to purify the recombinant protein. An aliquot of 1.33 ml of a thoroughly suspended 75 % w/v slurry of GS4B was transferred to a centrifuge tube (to give 1 ml of bed volume) and sedimented by centrifugation at 500 X g for 5 minutes. The supernatant was discarded and the GS4B washed three times with 10 volumes of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.3). PBS (1 ml) was added to the washed GS4B resulting in a 50 % w/v slurry that could be stored at 4°C for up to one month.

Used GS4B was re-generated for re-use by washing with 2-3 bed volumes of alternating high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M NaAC, 0.5 M NaCl, pH 4.5) buffers. This cycle was repeated 3 times followed by re-equilibration with 3-5 bed volume of PBS.

(2) Batch purification of GST-sLIF fusion protein

Crude cell extract (10 ml) was thawed on ice and added to1 ml of GS4B slurry that had been washed three times in 10 ml of TTBS (0.1 % v/v Triton X-100 in TBS) and three times in 10 ml of TBS (25 mM Tris-HCl pH 8.0, 150 mM NaCl). After incubation with agitation at room temperature for 30-45 minutes, and centrifugation at 500 X g for 5 minutes, the supernatant was removed. The pelleted GS4B with the bound GST-sLIF was washed three times in 10ml of 1 X TTBS and three times in 10ml of 1 X TBS at room temperature. After each wash the Sepharose was sedimented by centrifugation for 5 minutes at 500 X g. The Sepharose slurry was gently re-suspended in 1 ml of elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl pH 8.0) and incubated at room temperature for 15 minutes to elute the bound fusion protein. The protein in the supernatant was recovered by centrifugation at 500 X g for 5 minutes and saved in a fresh tube. The elution and centrifugation steps were repeated twice more and the purified protein samples stored at -70°C.

(3) Column purification of GST-sLIF fusion protein

A 5 ml syringe plugged with silicolnised glass wool was used to form a column of GS4B beads. 1 ml of GS4B slurry, prepared as described in Section 3.2.10.10, was placed in the cylinder and washed 3 times with 5 ml of TTBS buffer and 3 times in 5 ml of TBS buffer. Cell extract (10 ml) was applied to the column and allowed to flow through. The matrix was washed by the addition of 10 ml of TTBS buffer and allowed to drain: this process was repeated twice. Three more washes in TBS buffer were carried out and the syringe barrel was capped when the column had been drained. Elution buffer (1 ml) was slowly added to the column and the column eluate containing the fusion protein was collected. This procedure was repeated twice. The purified protein samples were stored at -70°C.

3.2.10.11 Separation of sLIF from the fusion protein

Thrombin digestion was used to separate sLIF from the fusion protein obtained using either the batch or column procedures described above. 10 µl of thrombin (1 digestion unit/µl) was added per mg fusion protein, and the solution was incubated overnight at room temperature. The glutathione was removed by extensive dialysis against 1 X TBS and purified by batch or column preparation as described above. The purified sLIF protein was in the flow-through.

Purified sLIF protein was also obtained by direct thrombin digestion of the fusion protein while bound to the GS4B Sepharose (Gearing *et al.*, 1989). Ten digestion units of thrombin in cleavage/storage buffer (TBS supplemented with 2.5 mM $CaCl_2$, 0.1 mM DTT and 0.01 % w/v sodium azide) were applied to the protein-bound matrix in a tube or on the column, and incubated at room temperature overnight. The purified sLIF protein in batch preparation was collected in the supernatant after centrifuging the suspension at 500 X g for 5minutes. The sLIF protein prepared by the column method was released when the cap was removed and the flow-through was collected in a new tube.

3.2.10.12 Determination of protein concentrations

The method for determining sLIF protein concentration was adapted from Bradford (1976). Initially, known protein standards of 2, 4, 6, 8,10, 12 and 14 µg BSA prepared in 0.15 M NaCl solution, were added to separate wells in a microtitre tray and made up to 160 µl with the buffer of choice. 1/10, 1/25 and 1/50 dilutions of the protein sample to be tested were made up to 160 µl before the addition of 40 µl of Bradford reagent (100 mg Coomassie brilliant blue G-25 dissolved in 95 % v/v ethanol, 100 ml 85 % v/v phosphoric acid, made up to 1 litre with double distilled water). Following thorough mixing, the absorbence was read on a microplate reader at 650 nm, calibrated against the sample buffer plus Bradford reagent alone. A standard curve

was plotted for absorbence at 650 nm and the concentration of the protein sample determined.

3.2.10.13 Bio-assay of sLIF using mouse Embryonic Stem (ES) cell cultures

(1) ES Cell culture

The murine ES cell line E14 was used for this investigation. This is a growth factor-dependent cell line which requires the presence of LIF in the culture medium, for proliferation. The cell line came originally from Anna Michelska (Murdoch Institute, Melbourne) and has been maintained in the laboratory of Professor Peter Rathjen (Department of Biochemistry, University of Adelaide).

DMEM medium [Dulbecco's modified Eagle's medium (DMEM, *GIBCO* Cat. No. 430-2100EB, high glucose with L-glutamine, without sodium pyruvate and sodium bicarbonate)] was used to make ESDMEM medium (incomplete medium) by adding 0.044 mM $NaHCO_3$, 0.125 % w/v Gentamycin and 0.1 % v/v β-mercaptoethanol and adjusting to the pH to 7.3. ESDMEM medium was sterilised by filtration (0.4 μm pore size) and supplemented with 10 % v/v fetal calf serum (FCS), 1 % w/v glutamine and 1000 u/ml LIF (ARMRAD, Melbourne, Australia) in the final solution (complete medium). ES cells were cultured in the complete ESDMEM medium by Dr. Joy Rathjen, Department of Biochemistry. Since ES cells are not tolerant to frequent changes in the pH of the culture medium, the correct pH was maintained by the presence of sodium bicarbonate in the medium and 10 % v/v CO_2 in the incubator. To avoid frequent opening and shutting of the door the incubator was used only for ES cell culture. The culture vessels were pre-coated with 0.1 % w/v gelatin (*Sigma Porcine skin* 300 *bloom*, St Louis, MO, USA) in PBS (stocks: 0.2 % w/v gelatin in PBS, sterilised by autoclaving) for at least 30 minutes at room temperature prior to seeding with ES cells. Gelatin-PBS was removed by rinsing with culture medium prior to use. The maintenance of freshly prepared cell culture vessels at 37°C for 3 days was found to obtain a high proportion of undifferentiated ES cells (Smith, 1991).

To passage ES cells, cell clumps were first dispersed into a single cell suspension by treatment with trypsin. The culture medium was removed and the cells washed with PBS. Five mls of EGTA (*Sigma*) in 1 X PBS was added and the cells were left at room temperature for about 5 minutes to disperse the clumps of cells. Under a microscope, cell boundaries within the clumps become obvious after this treatment. After removal of EGTA, the cell clumps were further broken up in 1 ml of trypsin solution (0.1 % v/v trypsin in 1 X Versene solution) per 10 mm dish by pipetting cells vigorously for about 30 seconds. The Versene solution was diluted from 10 X stock solution (2 g EDTA, 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, 11.5 g Na_2HPO_4 in 1 L of MQ H_2O, filter sterilised and stored at 4°C) (*Sigma*). The single cell suspension was transferred into 4 ml complete ESDMEM medium and collected by centrifugation at 1,200 rpm for 5 minutes. Cells were gently re-suspended in 10 ml complete ESDMEM medium and a flask seeded at a density ranging from 1.5 X 10^3 to 3.0 X 10^3 cells/cm^2 (ie. a dilution of 1:20-1:40). ES cells were passaged every 3-4 days and maintained for a maximum of 34 passages. The trypsinised cells could be washed and resuspended in 90 % v/v FCS, 10 % v/v DMSO, and frozen at -80°C (>10^6/ml), and transferred for long term storage to liquid nitrogen. To establish a fresh culture, frozen cells were thawed rapidly at 37°C.

(2) ES cell differentiation assay

Passage 21 E14 cells were plated at a density of 500 cells per well (growth area 0.785 cm^2) in 48-well multi-well plates (Sigma) in 0.5 ml ESDMEM complete culture medium. To aid cell adhesion, the wells had first been pre-treated with 0.2 % w/v gelatin in PBS for 30 minutes at room temperature. Complete ESDMEM culture medium with 1000 units of mLIF per ml was used to maintain undifferentiated ES cells as a positive control. The same medium, but without added LIF, was used to allow cells to differentiate, ie as a negative control. ES cells were cultured for 5 days followed by microscopic examination. Cells were further checked for differentiation using an alkaline phosphatase expression test (see below).

Recombinant mouse LIF (mLIF) and *S. crassicaudata* LIF (sLIF) proteins were recovered using the GST fusion system (Section 3.2.10.11). The recombinant protein samples were concentrated using Centricon-3 column units (*Amicon*, Beverly, MA,

USA). The samples were diluted to their original volume in 1X PBS to reduce the effect of detergent in the elution buffer on their biological function. The concentration of mLIF and sLIF was adjusted in small aliquots to give approximate equivalent concentration assessed as gel electrophoresis. A concentration of 0.08 ng/ml in both mLIF and sLIF was obtained and mLIF protein at this concentration had been estimated as 1000 units. A concentration of 100 ng/ml was used in the initial well. This initial concentration was 50 % serially diluted to 0.1 ng/ml over 9 wells. Assays with mLIF and sLIF were incubated under identical condition at 37°C, 10 % v/v CO_2 in air for 5 days.

(3) Identification of differentiated cells

Alkaline phosphatase (APase) (E.C. 3.1.3.1) was used as an indicator of ES cells differentiation. Activity of this enzyme varies in most adult tissues (Dixon and Webb, 1964), but constantly high activities are widely distributed in embryos. Tissues in the embryo are rich in alkaline phosphatase at early stages of development, particularly primordial germ cells (Mintz, 1959; Berstine *et al.*, 1975). Alkaline Phosphatase, Leucocyte Diagnostics System (*Sigma*, No. 86) was used to detect APase by following the protocol supplied by the manufacturer with some minor modifications. Two mls of a diazonium salt solution [1 ml of 0.4 M sodium nitrite solution added to 1 ml of FRV-alkaline solution (fast red violet based solution in 0.4 M hydrochloric acid with stabiliser), mixed by gentle inversion and allowed to stand for 2 minutes] was diluted in 45 ml of deionised H_2O and then further diluted by the addition of 1 ml of naphthol AS-BI alkaline solution. The components were mixed thoroughly and poured into a *Coplin* jar. A citrate-methanol-formaldehyde fixative solution (5 ml of citrate solution plus 13 ml methanol and 16 ml of formaldehyde, placed in a glass bottle, capped tightly and stored at 4°C for not more than 4 weeks) was pre-warmed to room temperature (18-26°C) and used for 5 seconds to fix the ES cells. The fixed cells were rinsed gently in deionised water for 45 seconds and incubated with the alkaline-dye solution from the *Coplin* jar at room temperature for 15 minutes in the dark. The stained cells were rinsed with deionised water for 2 minutes, counter-stained for 2 minutes with Hematoxylin solution (Gill No.3), rinsed thoroughly in tap water and air dried for microscopic examinations.

Chapter 4 Results

4.1 Identification and Characterization of *LIF* from a *S. crassicaudata* Genomic DNA Library

4.1.1 Cloning vector and probes

The Lambda GEM®-11 vector (Fig. 4.1) was used for the construction of the genomic DNA library. λ-GEM-11 is a multi-functional replacement type genomic cloning vector that will accept DNA fragments ranging from 9 kb to 23 kb. T7 and T3 promoters, which flank the multiple cloning site (MCS), can be used as universal sequencing primers. A high recombinant efficiency, up to 3 X 10^7 pfu/μg of input DNA, can be archived with low non-recombinant backgrounds. Being a replacement vector, λ-GEM-11 provides maximum space for the insertion of exogenous DNA fragments, thereby reducing the number of individual recombinants necessary for complete library representation of a whole genome. The λ GEM-11 half-site arms obtained commercially had already been digested with *Xho*I, dephosphorylated, and the first two over hanging nucleotides of the *Xho*I site had been filled in. This allows for the rapid cloning of *Mbo*I or *Sau*3A digested genomic DNA. Non-productive ligation of genomic DNA with the central stuffer fragments is extremely rare.

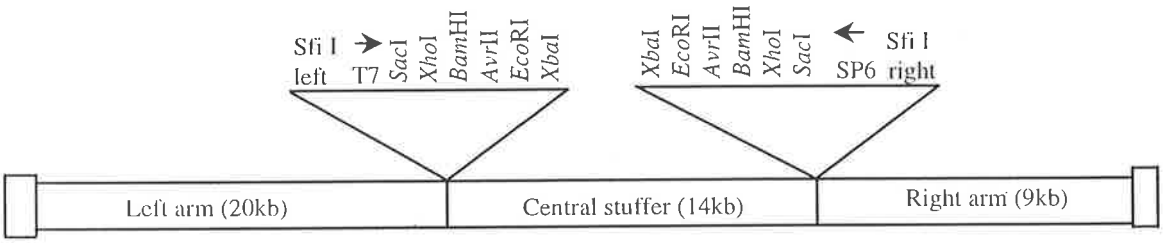


Fig. 4.1 Lambda GEM-11 Vector

The probes used for screening the genomic library for clones containing the *S. crassicaudata LIF* gene were obtained from the mouse clone, pDR1 (Rathjen *et al.*, 1990) and the human clone, HpGEM1 (Smith *et al.*, 1988).

pDR1 is a cDNA containing the entire murine *LIF* coding region. The clone was obtained following amplification by RT-PCR of Ehrlich ascites RNA. cDNA was inserted into and cloned into the *Eco*RI restriction site of pBluescript II KS$^+$. The insert can be excised by *Eco*RI digestion (Fig. 4.2a). HpGEM1 contains a ≈700 bp *Pst*I fragment incorporating the entire *hLIF ORF* (Smith *et al.*, 1988) in pBluescript II KS$^+$ (Fig. 4.2b).

The most conserved coding region of the *LIF* gene in both mouse and human corresponds to the N-terminus of the LIF protein. *SmaI* digestion of pDR1 and HpGEM1 releases a fragment that contains this conserved region, together with a small amount of vector sequence (Rathjen, 1993, personal communication).

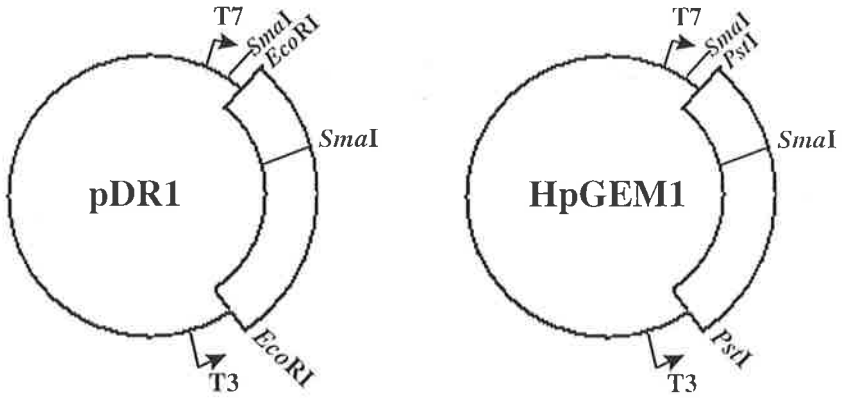


Fig. 4.2 Diagrams of mouse (pDR1) and human (HpGEM1) *LIF* clones used as probes in this project. (a) Mouse *LIF* cDNA ORF cloned in the EcoRI site of pBluescript II KS$^+$ (Rathjen *et al.*, 1990). (b) Human *LIF* cDNA ORF (about 700 bp) cloned in the *Pst*I site of site of pBluescript. II KS$^+$ (Smith *et al.*, 1988).

4.1.2 Construction of genomic DNA library

4.1.2.1 Partial digestion and size fraction of genomic DNA

High molecular weight genomic DNA prepared from the liver of an adult female (I.D.= 1 998.1a) was partially digested with *Sau*3A. This enzyme recognizes the four

base sequence 5'-GATC-3' and generates ends suitable for *Bam*HI restricted vector ligation. The digestion was conducted at 37°C for 1 hour and the conditions for partial digestion were optimized, by varying the concentration of *Sau*3A, to give fragments of average size 10kb - 23kb. The optimal concentration of *Sau*3A was found to be 1/128 units/μg of DNA (Fig. 4.3, track 8). This concentration was employed in the large scale preparation of DNA fragments.

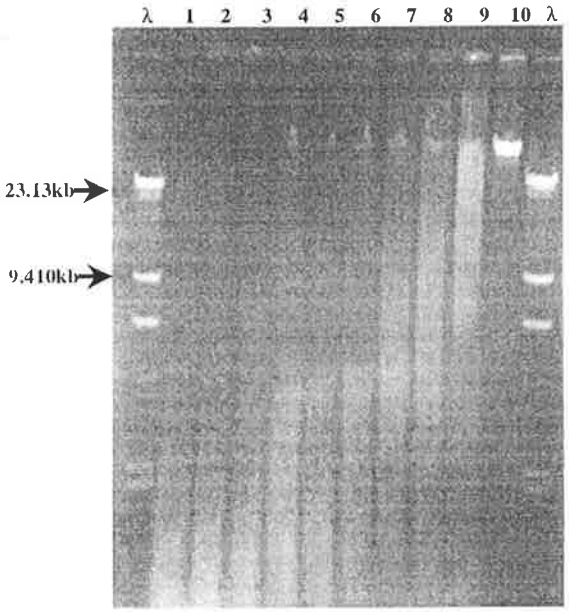


Fig. 4.3 Agarose gel electrophoresis of *Sau*3A partially digested *S. crassicaudata* DNA.

Tracks λ- λDNA/*Hind*III marker
1- 1unit of *Sau*3A/mg of DNA
2- 1/2 unit of *Sau*3A/mg of DNA
3- 1/4 unit of *Sau*3A/mg of DNA
4- 1/8 unit of *Sau*3A/mg of DNA
5- 1/16 unit of *Sau*3A/mg of DNA
6- 1/32 unit of *Sau*3A/mg of DNA
7- 1/64 unit of *Sau*3A/mg of DNA
8- 1/128 unit of *Sau*3A/mg of DNA
9- 1/256 unit of *Sau*3A/mg of DNA
10- 0 unit of *Sau*3A/mg of DNA

The *Sau*3A partial digested genomic DNA was fractioned on a 0.5 % w/v LMT agarose gel and stained with an ethidium bromide solution. The agarose gel slice containing DNA fragments within the desired 10-23 kb size range was excised under long-wave UV light, equilibrated in a solution of 5 mM EDTA/100 mM NaCl and then digested with agarase. The purified DNA sample was examined by electrophoresis on a 0.8 % w/v agarose gel (Fig. 4.4) to ensure that the recovered DNA was within the anticipated size range. It can be seen that the DNA fragments were of the expected size range, ie. about 17 kb on average.

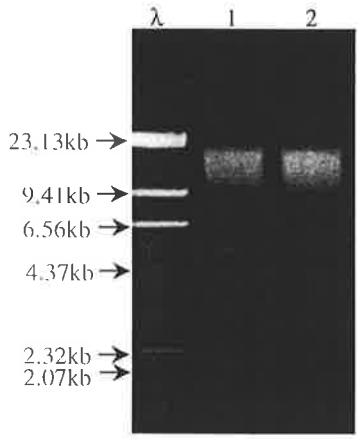


Fig. 4.4 An agarose gel showing the purified 10 - 23kb DNA fragments.
Track λ - λ DNA/*Hind*III size markers
Tracks 1 & 2 - purified, size selected *S. crassicaudata* genomic DNA.

4.1.2.2 Determination of optimal ligation condition

Test ligations were conducted with varied insert/vector arm ratios. A "no inserts" ligation control of λGEM-11 arms only was carried out in parallel with the test ligation to check for re-ligated vector arms. The insert/vector molar ratios were 0:1 (A), 3.5:1 (B), 2:1 (C), 1:1 (D) and 1:2 (E). Ligates were packaged (Packagene® Lambda DNA Packaging System) and the titres determined (Table 4.1).

Table 4.1 Titres of the phage from genomic DNA library. The bacterial strain KW251 was used for the titration. Cells were incubated overnight at 30°C with vigorous agitation and temporally kept at 4°C immediately after the incubation. Titrations were conducted on LB plates.

Ligation	No. of plaques 10^{-2} dilution	No. of plaques 10^{-3} dilution	No. of plaques 10^{-4} dilution	Average titre (pfu/ml)
A	0	0	0	0
B	confluent	76	8	7.6×10^7
C	confluent	155	18	1.55×10^8
D	confluent	46	13	4.6×10^7
E	confluent	67	12	6.7×10^7

Assuming a total haploid genome of 1×10^9 bp, it can be estimated that a library of 7.6×10^5 plaques is required to contain at least one copy of a unique 17 kb fragment with 99% probability (Kaiser and Murray, 1986). The highest titre obtained (>1.55 X

10^8) came from the ligation using a 2:1 vector/insert ratio. All four test ligations (B-E) gave titres of > 7.6 X 10^5.

4.1.3 Isolation of the phage clone λ-sLIF from the *S. crassicaudata* genomic library

Ligated λ DNA from the ligation C was diluted by 10^3 and plated on 140 mm petri dishes to give isolated plaques. Plaques were allowed to develop for approximately 16 hours until they were almost touching one another and DNA from the plaques was transferred to nylon membrane Hybond N^+ using the plaque lifting technique (Benton and Davis, 1977).

To isolate λ DNA clones containing the *S. crassicaudata LIF* gene (see Section 3.2.2.5), the entire ORFs encoding LIF from mouse (*mLIF ORF*) and human (*hLIF ORF*) were used as probes. *mLIF ORF* cDNA was removed from pDRI by *Eco*RI digestion, and *hLIF ORF* cDNA was excised from HpGEMI using *Pst*I. These fragments were purified using agarose gel electrophoresis (Fig. 4.5), labelled with α-^{32}P-dATP (to an activity of 10^5 – 10^7 cpm/ml) by random extension, and used to probe the *S. crassicaudata* genomic library.

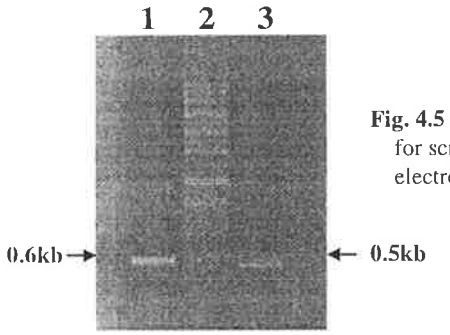
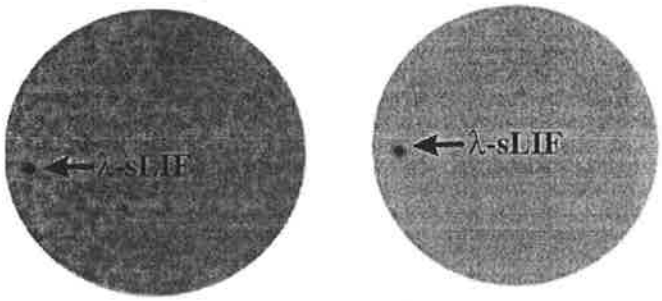


Fig. 4.5 Preparation of *mLIF* and *hLIF* cDNA fragments for use as probes for screening the *S. crassicaudata* genomic library. The samples were electrophoresed on 0.8% w/v agarose gel and stained with EtBr.
Track 1 - *hLIF ORF*
2 - SPP-1 DNA molecular weight maker
3 - *mLIF ORF*

One clone from ligation C showed significant hybridization to the *mLIF ORF* after a low stringency wash (30 minutes at room temperature with agitation in 2 X SSPE, 0.1 % w/v SDS) (Fig.4.6a). Further high stringency washes (30 minutes at room temperature with agitation in 1 X SSPE, 0.1 % w/v SDS followed by a wash at 68°C

with agitation for 15 minutes in a fresh change of the same solution) significantly reduced background hybridization (Fig. 4.6b).



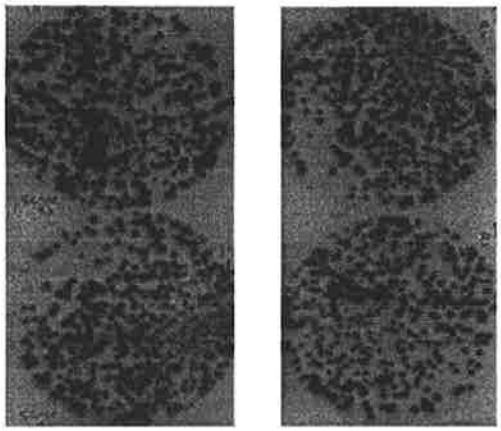
(a) (b)

Fig. 4.6 Autoradiograph of a plaque lift from plating C (2:1 vector/insert ratio ligation). The arrow indicates the clone that showed specific hybridization to *mLIF ORF*.

(a) low stringency post-hybridization washes.

(b) The same filter after high stringency washes.

A plug containing the positive plaque was incubated overnight at 4°C in SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8.0 mM $MgSO_4$, 0.01 % w/v gelatin) to allow the plaque particles to diffuse into solution. For a second round of screening, bacteriophage suspensions obtained from the plug were replated at an adjusted density and plaques were transferred to nylon membrane and probed with *mLIF ORF* or *hLIF ORF* using the procedures and conditions described above. All plaques from this second screening showed strong hybridization to both *mLIF ORF* and *hLIF ORF* probes. Non-hybridizing plaques were not observed (Fig. 4.7). This positive λ DNA clone was purified by two rounds of screening. Recombinant bacteriophage DNA (named λ-*sLIF*) was isolated from a single plaque retrieved from the second screening.



(a) (b)

Fig. 4.7 Second round screening. The plaque lifts were hybridized
(a) with the *Eco*RI cDNA insert of pDRI (*mLIF ORF*).
(b) with *Pst*I cDNA insert of HpGEM1 (*hLIF ORF*).
The upper filter in (a) is the same as the lower filter in (b). All the plaques showed positive hybridization with both probes.

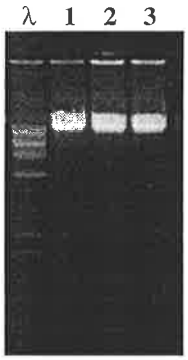


Fig. 4.8 λ-*sLIF* bacteriophage DNA isolated from lambda lysates
Track λ - λHindIII DNA marker
Track 1- 3 phage DNA prep samples

4.1.4 Preliminary characterization of λ-*sLIF*

λ-*sLIF* bacteriophage DNA was analysed by restriction endonuclease digestion. Since the insert was cloned into a *Bam*HI site of the λGEM-11 vector, restriction enzymes with recognition sequences located outside of *Bam*HI (ie. *Sac*I and *Xho*I) were chosen first for use in digestions. Other commonly used restriction enzymes were used in attempts to completely excise the inserts from the recombinant phage DNA. DNA restriction fragments were separated by electrophoresis on 1.0 % w/v agarose gel, stained with EtBr and photographed. DNA fragment sizes were estimated by

comparison with DNA molecular weight markers. Figure 4.9 shows λ-*sLIF* DNA fragments digested with various endonucleases.

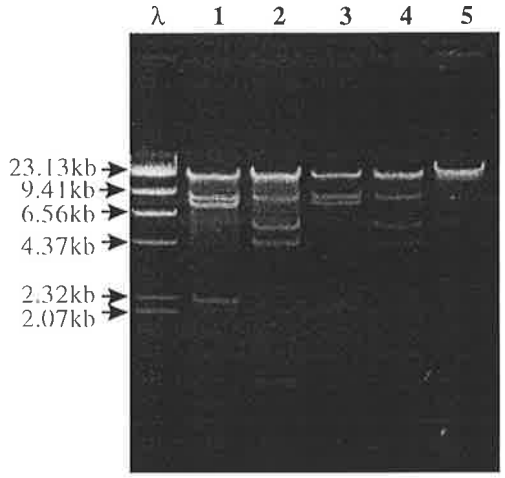


Fig. 4.9 Restriction analysis of λ-*sLIF*. Restriction fragments were electrophoresed on 1.0% w/v agarose gel, stained with EtBr solution and photographed under UV light.

λ - λDNA/*Hind*III molecular weight marker
1 - Reprecipitated λ-*sLIF* digested with *Xho*I
2 - Reprecipitated λ-*sLIF* digested with *Sfi*I
3 - Column purified λ-*sLIF* digested with *Xho*I
4 - Column purified λ-*sLIF* digested with *Sfi*I
5 - λ–*sLIF* undigested

The λ-*sLIF* DNA restriction fragments (from a different gel to that shown in Fig. 4.9) were transferred to Hybond-N^+ nylon membrane by alkali blotting (Reed and Mann, 1985) and hybridized with the *mLIF ORF* probe. Washes of the membrane were carried out in 2 X SSPE/0.1 % w/v SDS at room temperature for 30 minutes and then in 1 X SSPE/0.1 % w/v SDS at 65°C for 15 minutes. Autoradiography showed that *mLIF ORF* hybridized to a 8.75 kb *Xho*I fragment and to a 14 kb *Eco*RI fragment (Fig. 4.10). Since no *Eco*RI sites remain in the λGEM–11 arms used for library construction, the hybridization result suggest the presence of two *Eco*RI sites within the insert, with the hybridizing 5 kb fragment being linked to the right arm of the vector to form a 14 kb fragment. The DNA fragment between the *Eco*RI sites is about 4.5 kb. A more extensive restriction analysis of λ-*sLIF* was carried out (Fig. 4.11), and the result used to construct the restriction map shown in Fig. 4.12.

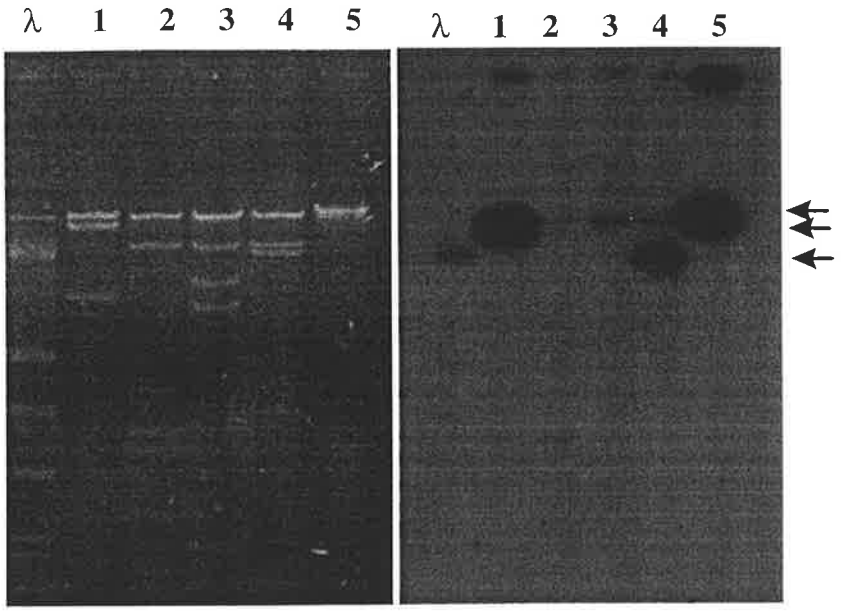


Fig. 4.10 Southern hybridization of λ-sLIF fragments. DNA fragments separated on agarose gel were alkali blotted to Hybond-N^+ membrane, hybridized with α-^{32}P-dATP labelled *mLIF ORF* probe and autoradiographed at -70°C overnight. Strong hybridization signals are located to a 8.5 kb *Xho*I fragment and a 14 kb *Eco*RI fragment linked with the right arm of λGEM-11 vector.

λ - λDNA/HindIII molecular weight marker

1 - λ-*sLIF* digested with *Eco*RI

2 - λ-*sLIF* digested with *Sac*I

3 - λ-*sLIF* digested with *Sfi*I

4 - λ-*sLIF* digested with *Xho*I

5 - λ–*sLIF* undigested

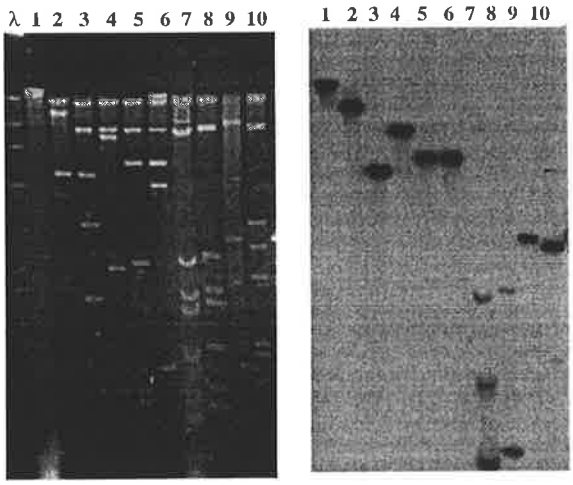


Fig. 4.11 Restriction and Southern analysis of λ-*sLIF*. (a) 1.0% w/v agarose gel containing a series of restriction digests of λ-*sLIF*. (b) An autoradiograph of the gel, using *mLIF* as probe. Tracks are:

λ – λDNA/*Hind*III molecular weight marker

1- λ-*sLIF* undigested

2- λ-*sLIF* digested with *Eco*RI

3- λ-*sLIF* digested with *Eco*RI and *Xho*I

4- λ-*sLIF* digested with *Xho*I

5- λ-*sLIF* digested with *Xho*I and *Sfi*I

6- λ-*sLIF* digested with *Sfi*I

7- λ-*sLIF* digested with *Sfi*I and *Sac*I

8- λ-*sLIF* digested with *Sac*I

9- λ-*sLIF* digested with *Xba*I

10- λ-*sLIF* digested with *Bam*HI

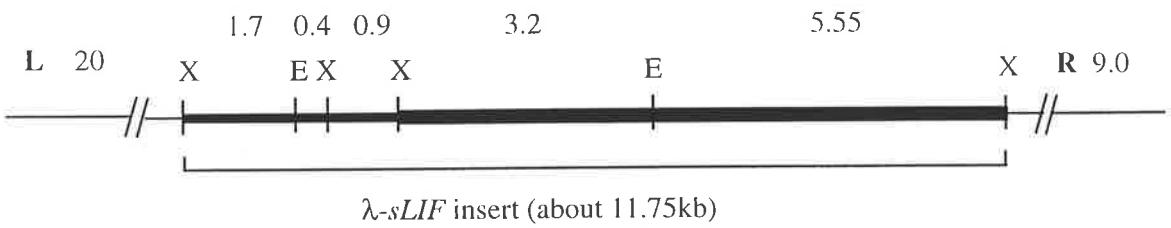


Fig. 4.12 A restriction map of λ-*sLIF*. The thick line shows the 11.75 kb insert of λ-*sLIF* phage DNA and the 8.75 kb *mLIF* positive fragment is displayed by a thicker line. The numbers indicate the fragment size in kb. The enzymes used are X: *Xho*I; E: *Eco*RI.

To further characterize the 8.75 kb *Xho*I fragment, which was expected to contain at least part of the *S. crassicaudata LIF* gene, the fragment was sub-cloned in the vector pBluescript II KS$^+$. The sub-clone was named *psLIF-1* (Fig. 4.13). Figure 4.14 shows the restriction pattern of *psLIF-1*, indicating that two fragments of the expected sizes (3.2 kb and 5.55 kb) were generated by *Xho*I and *Eco*RI double digestion.

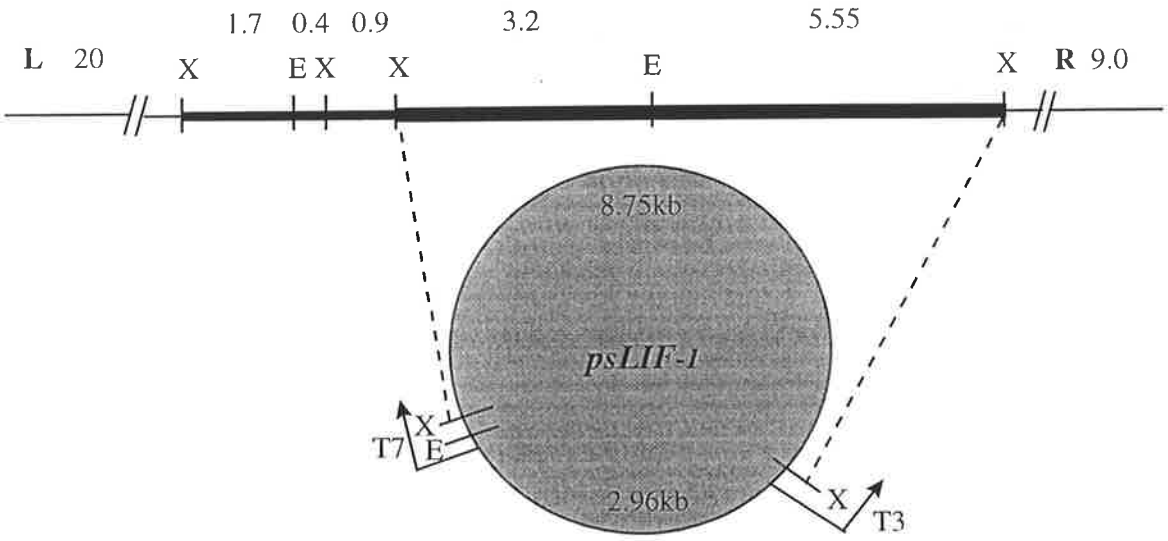


Fig. 4.13 Construction of sub-clone *psLIF-1*. The 8.75 kb *Xho*I fragment that showed hybridization *mLIF ORF* was cloned in the *Xho*I site of pBluescript II KS$^+$.

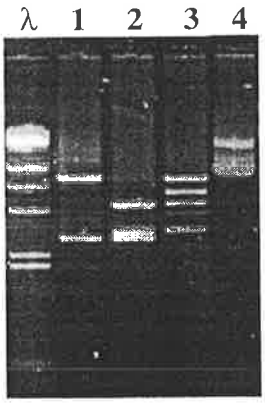


Fig. 4.14 Restriction analysis of *psLIF-1*. Fragments were separated by electrophoresis on a 0.8% w/v agarose gel, stained in ethidium bromide solution and photographed under UV light (360nm).

Track λ - λDNA/*Hind*III molecular weight marker
1 - *psLIF-1* digested with *Xho*I
2 - *psLIF-1* digested with *Xho*I and *Eco*RI
3 - *psLIF-1* digested with *Eco*RI
4 - *psLIF-1* undigested

4.1.5 Authentication of *psLIF*-1

Endonuclease digestions were conducted on *psLIF*-1 plasmid DNA and the results are shown in Fig. 4.15.

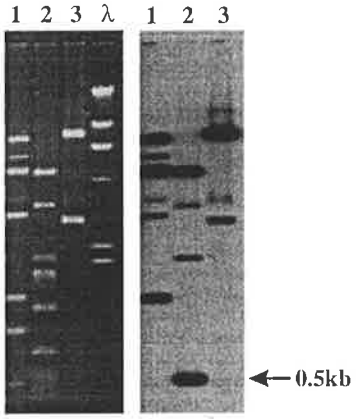


Fig. 4.15 Identification of a 0.5 kb positive fragment from *psLIF*-1.

(a) *psLIF-1* was digested with different restriction enzymes, fractioned on 0.8 % w/v agarose gel, sained with EtBr solution and photographed under UV light.

(b) DNA fragments from the gel (a) were transferred to Nylon membrane Hybond- N^+ and hybridizied with *hLIF ORF* (*Pst*I insert of HpGEM1) labelled with α-^{32}P- dATP and autoradiographed on X-ray film. Note the strong hybridization of the *hLIF* probe (*hLIF ORF*) to the 0.5 kb *Sac*I fragment of *psLIF-1*.

Tracks are:

1 - *psLIF*-1 digested with *Bst*XI (at 55°C for 2 hours)

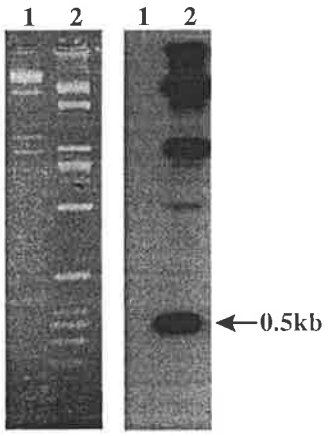
2 - *psLIF*-1 digested with *Sac*I (at 37°C for 2 hours)

3 - *psLIF*-1 digested with *Xho*I (at 37°C for 2 hours)

4 - λDNA digested with *Hind*III DNA size marker

It can be seen that a 500 bp *Sac*I fragment hybridized strongly to the *hLIF ORF* probe. This result is consistent with the Southern hybridization results described in Section 4.1.4 (see track 9, Figure 4.11). The 500 bp *Sac*I fragment is likely to contain a region of DNA that has high sequence identity to a coding region of the human *LIF* gene. This *Sac*I fragment could correspond to any one of about 5 fragments of approximately this size seen on the ethidium bromide stained gel (Fig. 4.15a). Further restriction digestions and Southern analysis were carried out to help resolve

this uncertainty. *Sac*I digested *psLIF*-1 DNA fragments, when electrophoresed on a 2.0 % w/v agarose gel, revealed four clearly separated fragments in the region of the hybridizing band (Fig. 4.16a). Southern analysis showed hybridization of *mLIF ORF* to the second largest fragment in this group of four (Fig.4.16). *psLIF*-1 plasmid DNA was digested with *Sac*I and fractioned on 2.0 % w/v agarose gel. The gel slice containing the hybridizing fragment was excised under a long wavelength UV light and the DNA was purified by the freeze-squeeze method (Section 3.2.5.4). This DNA fragment was ligated into *Sac*I linearized pBluescript II KS^+ and the clone so obtained was designated *pXSa*10. After infection with VCM13 helper phage, single-stranded *pXSa10* plasmid DNA was isolated, purified as described in Section 3.6.6.1 (Fig. 4.17) and used directly as a template for DNA sequencing. Sequencing was carried out using the *fmol*™ system and Sequenase® Version 2.0 (Section 3.2.6).



(a) (b)

Fig. 4.16 Southern analysis of *psLIF-1*.

(a) *psLIF-1* was digested with *Sac*I and fractioned on 2.0% w/v agarose gel;

(b) autoradiography of Gel (a) using *hLIF ORF*.

Tracks are:

1 - λDNA/*Hind*III marker

2 - *psLIF*-1 digested with *Sac*I

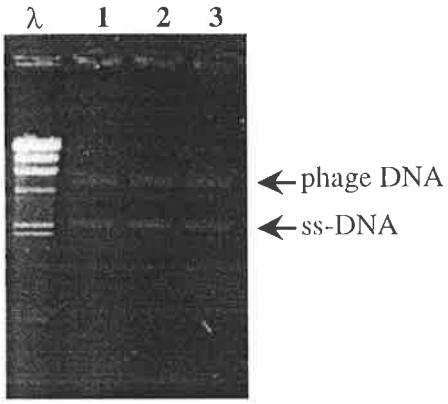


Fig. 4.17 Generation of ss-DNA from pXSa10 by phagemid pBluescript II KS$^+$. The purified ss-DNA was electrophoresed on an agarose gel, stained with EtBr solution and photographed under UV light. The larger of the 2 fragments in lanes 1 - 3 is VCSM phage DNA (6 kb), the smaller fragment is the ss-DNA.
Tracks are:
λ - λDNA/*Hind*III marker
1 - ss-DNA preparation 1
2 - ss-DNA preparation 2
3 - ss-DNA preparation 3

A search of GENBANK revealed that the nucleotide sequence of the *Sac*I fragment of *psLIF*-1 (552 bp) showed a high level of identity to *LIF* genes from various species, and based on sequence homology, the region from nucleotide 278 to 457 was identified as exon 2 of *S. crassicaudata LIF*. The alignment of *pXSa10* sequence and part of the human *LIF* gene is shown in Figure 4.18. *pXSa10* has extensive sequence identity to *LIF* genes from other mammals (Table 4.2A). This confirms that *psLIF*-1 is a clone from the LIF gene of *S. crassicaudata*.

```
           210        220        230        240        250        260
pXSa10 CTAGACCTAGAAGTATAAGCTCCTCCGGTTCCTTCCAAGCCCCTCCACTCATAGACCCCT
                                          :::: :  ::: ::  ::::: :   : :
HUMLIF CCCATCCGGTGTGCCATGACCCCAGGCCACCCTTTCCTGCCTTTCTACTCATGGCTTCTT
        2400       2410       2420       2430       2440       2450

           270        280        290        300        310        320
pXSa10 CCCCACTGTTTCCAGGAGTCATGCCTCTGCTGCTGGTTCTGCACTGGAAACATGGGGCCG
       ::  ::::: :::::::: :::: :::::: :::::::::::::::::::::::::: :
HUMALI CCTGACTGTCCCCAGGAGTTGTGCCCCTGCTGTTGGTTCTGCACTGGAAACATGGGGCGG
        2460       2470       2480       2490       2500       2510

           330        340        350        360        370        380
pXSa10 GGAGCCCCCTACCCATCACCCCCGATGATCCCAAATGCGAAATGCGCCACCAGTGCCCAG-
       :::::::::: :::::::::::: :   :  ::: :: :  :: ::::::: :: : :
HUMLIF GGAGCCCCCTCCCCATCACCCCTGTCAACGCCACCTGTGCCATACGCCACCCATGTCACA
        2520       2530       2540       2550       2560       2570

           390        400        410        420        430        440
pXSa10 GCAACCTCACGTTCCAGATCCGGAACCAGCTGAATCAGCTCAATAGCAGCGCCCCGGAAC
       :::::::: :  :::::::: ::: ::: :::   :::::::: :::: :::   :  :
HUMLIF ACAACCTCATGAACCAGATCAGGAGCCAACTGGCACAGCTCAATGGCAGTGCCAATGCCC
        2580       2590       2600       2610       2620       2630

           450        460        470        480        490        500
pXSa10 TCTTCACCTATTATGTGAGTGACCCCGTCCGCCCTTCCTGCCCCCAGCCCCCAGCCGGGG
       :::: :     ::::: ::: :::::
HUMLIF TCTTTATTCTCTATGTAAGTTACCCCTGGGATACTGACAGGAGATGGCAGGGAGGGGGCT
        2640       2650       2660       2670       2680       2690
```

Fig. 4.18 Alignment of the *pXSa10* sequence from *S. crassicaudata* and a region of the human *LIF* gene. Identical nucleotides are indicated by ":". The exon 2 region of human *LIF* and the corresponding region of *pXSa10* are shown in bold type.

Table 4.2A DNA sequence comparisons of *pXSa10* with other sequences in the GENBANK database.

LIF gene source	nt overlap	identity (%)
Mouse *LIF*	235	71.5
Human D-factor	252	71.8
hLIF cDNA	195	75.4
Human *LIF*	236	70.3
Bovine *LIF*	264	68.2
Porcine *LIF*	252	69.0
Rat CNDF (*LIF*)	184	74.5

4.1.6 Generation of *psLIF1.1* and *psLIF1.2*

The two restriction fragments generated by double digestion of *psLIF*-1 with *Xho*I and *Eco*RI, were purified using low melting temperature (LMT) agarose gel electrophoresis and CL-6B Sepharose column gel filtration, and cloned into *Xho*I/*Eco*RI double digested pBluescript II KS$^+$ plasmid vector (Fig. 4.19). The ligated plasmid DNAs were transformed to XL-1 Blue competent bacteria. The transformed cells were plated onto L-agar selective plates (containing 100 μg/ml of ampicillin, 12.5 μg/ml tetracycline, 0.1M IPTG, and 40 μg/ml of X-gal) and grown overnight at 37°C. Few colonies (<10) were observed on the control plates (inoculated with XL-1 Blue cells transformed with religated pBluescript II KS$^+$ only), indicating a low background with native pBluescript II KS$^+$ and implying some incompleteness of the vector double digestion during the preparation of pBluescript II KS$^+$ for the ligation. The bacterial cells harbouring pBluescript II KS$^+$ phagemid with insert grow as white colonies on XL-1 Blue bacteria strain (containing the *lacZΔM15* on an F′ episome) while the non-insert transformants grow as blue colonies on the plates. The cells from these white colonies are expected to be both tetracycline resistant (tet^R) and ampicillin resistant (amp^R) on the selective plates. Ampicillin selects for cells that harbour the plasmid and tetracycline selects for cells containing the F′ episome.

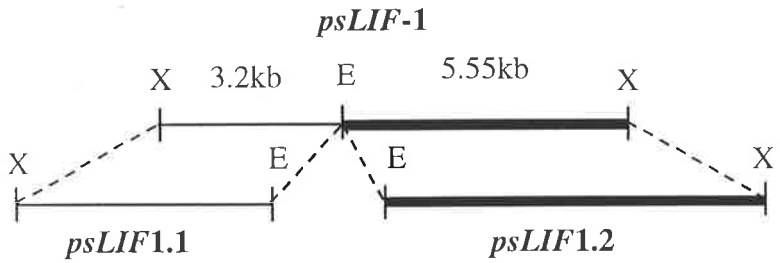
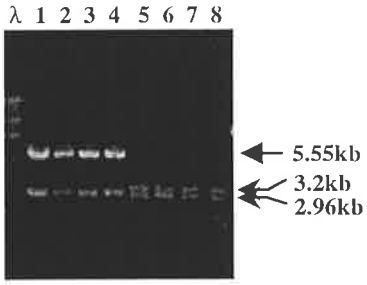


Fig. 4.19 Sub-cloning *psLIF-1* to generate the clones *psLIF1.1* and *psLIF 1.2*

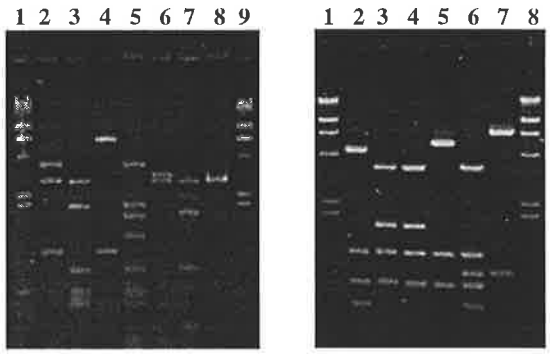
4.1.7 Sub-cloning and mapping of *psLIF*1.1 and *psLIF* 1.2

Well-isolated single colonies were picked and used to prepare plasmid DNA using the procedures described in Section 3.2.4.5. The sub-cloned DNA fragments were found to be of the expected size (Fig. 4.20).

Fig. 4.20 Analysis of the subclones *psLIF*1.1 and *psLIF*1.2. Plasmid DNA isolated from single colonies was digested with *Xho*I and *Eco*RI, separated on 0.8% w/v agarose gel, stained with EtBr solution and photographed under UV light.
Track λ -λDNA/*Hind*III marker
1-4 - *psLIF*1.2 clones
5-6 - *psLIF*1.1 clones



Plasmid DNA samples were prepared from *psLIF*1.1 and *psLIF* 1.2 (Section 3.2.4.6), digested singly or doubly with various restriction enzymes, and analysed on 1.0 % w/v agarose gels (Figure 4.21).



(a) (b)

Fig. 4.21 Restriction analysis of sub-clones *psLIF*1.1 and *psLIF*1.2. Digested DNA samples were separated on 1.0% w/v agarose gels, stained with EtBr solution and photographed under UV light.

Tracks are: (a) 1 & 9 - λDNA/HindIII DNA markers
2 - *psLIF1.2* digested with *Xho*I, *Eco*RI and *Sac*I
3 - *psLIF1.2* digested with *Xho*I, *Eco*RI, *pst*I and *Sac*I
4 - *psLIF1.2* digested with *Pst*I
5 - *psLIF1.2* digested with *Sac*I
6 - *psLIF1.1* digested with *Pst*I
7 - *psLIF1.1* digested with *Xho*I, *Eco*RI, *Pst*I and *Sac*I
8 - *psLIF1.1* digested with *Xho*I, *Eco*RI and *Pst*I
(b) 1 & 8 -λDNA/HindIII DNA markers
2 - *psLIF1.2* digested with *Xho*I and *Hind*III
3 - *psLIF1.2* digested with *Xho*I, *Eco*RI and *Hind*III
4 - *psLIF1.2* digested with *Eco*RI and *Hind*III
5 - *psLIF1.2* digested with *Hind*III
6 - *psLIF1.2* digested with *Pst*I and *Hind*III
7 - *psLIF1.2* digested with *Pst*I

The DNA on these gels (and on other gels containing restricted *psLIF*1.1 and *psLIF*1.2) was Southern transferred to nylon membrane and hybridized with *mLIF ORF* and *hLIF ORF* probes. The hybridization results (not shown) were used to construct a restriction map (Fig. 4.22).

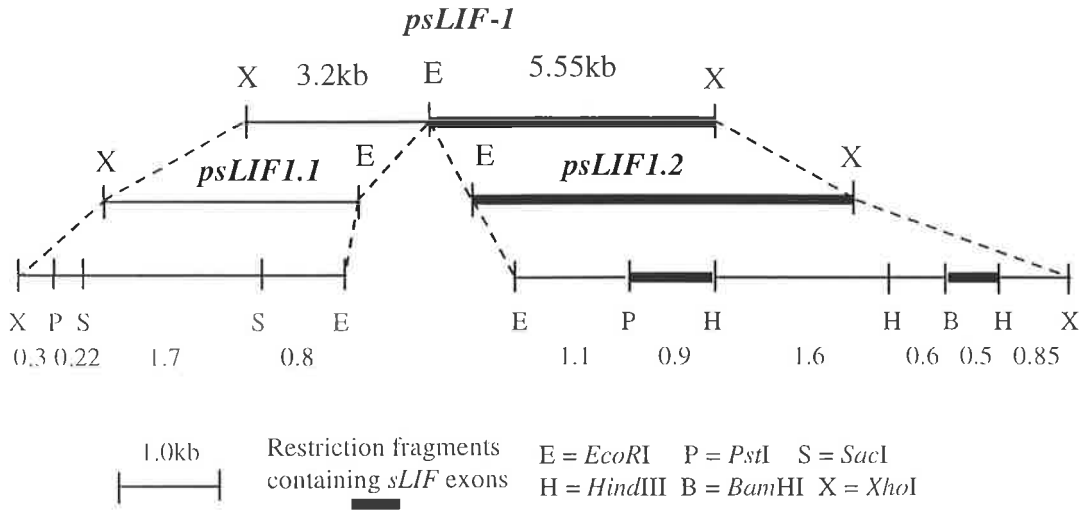


Fig. 4.22 Restriction map of the genomic inserts of *psLIF*1.1 and *psLIF*1.2

Since the 5.55kb fragment of *psLIF*-1 sub-cloned into *psLIF*1.2 had been shown to hybridize to m*LIF ORF* and *hLIF ORF* (see Fig. 4.19), further investigations of this sub-clone were commenced. The *psLIF1.2* was sub-cloned in pBluescript II KS^+ vector (Fig. 4.23).

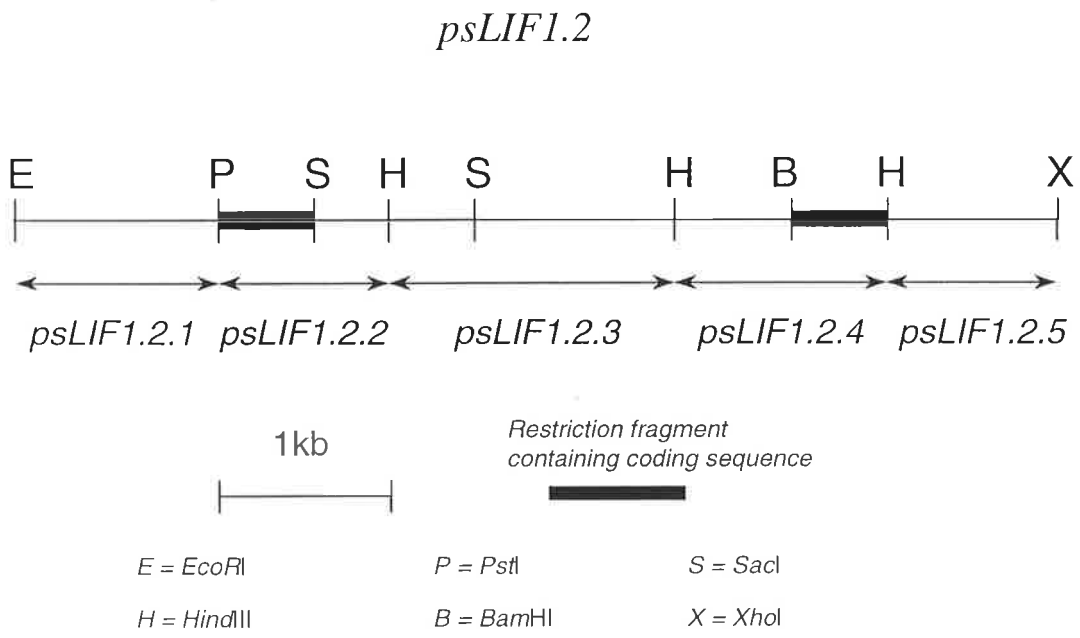


Fig. 4.23 Five sub-clones generated from *psLIF*1.2.

The plasmid DNA from each sub-clone was isolated, purified, and restriction digested to excise the inserts; these were separated on 0.8 % w/v agarose gel (Fig. 4.24a), Southern transferred to nylon membrane and hybridized with α-^{32}P-dATP labelled *mLIF ORF* probe. Hybridizing bands were detected in *psLIF1.2.2* and *psLIF1.2.4* (Fig. 4.24b). However, the background levels were very high and the bands of specific hybridization do not show clearly on the scanned image (Fig. 4.24b). The filter was stripped (Section 3.2.5.9) and re-hybridized with *Sac*I insert of *pXSa10* (which had been shown to contain the exon 2 region of *LIF*). The resultant autoradiograph showed hybridization of *pXSa10* probe to the insert (Fig. 4.24c), indicating that exon 2 of *sLIF* is located in the sub-clone. It was predicted that the other fragment from *psLIF1.2*, which showed hybridization to *mLIF ORF* and *hLIF ORF* (Fig. 4.22), must be from the downstream exon 3 region of *sLIF* gene. This deduction was proved by DNA sequencing analysis of *psLIF1.2.4*. The DNA fragment cloned in *psLIF1.2.4* (Fig. 4.23) was isolated, radioactively labelled and hybridized to the same membrane, after it had first been stripped of probe. No hybridization was detected between this fragment and other fragments except *psLIF1.2.4* (Fig. 4.24d).

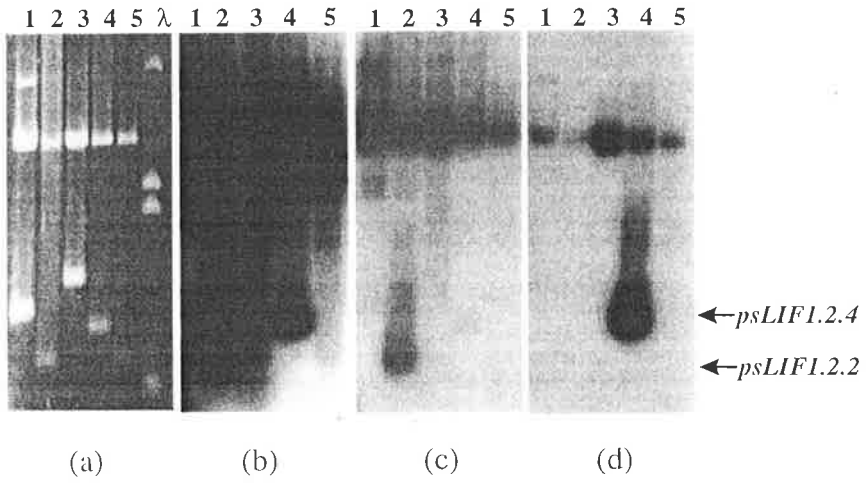


Fig. 4.24 Southern blot analysis of the sLIF clone *psLIF1.2* using different *LIF* probes. Plasmid DNA from the 5 sub-clones was digested with different restriction enzymes, fractioned on 0.8 % w/v agarose gel (a), Southern transferred to nylon membrane and hybridized *with mLIF ORF* (b), exon 2-containing *pXSa10* insert (c), and exon 3 coding region fragment from *psLIF1.2.4* (d). Both exons are present within *psLIF1.2*.

4.2 Genomic Sequence of *sLIF*

4.2.1 Sequencing strategy

The clone *psLIF1.2* and its five sub-clones, *psLIF1.2.1*, *psLIF1.2.2*, *psLIF1.2.3*, *psLIF1.2.4* and *psLIF1.2.5* were used as the sequencing templates. For each sub-clone, the universal primer pairs located within the cloning vectors (T7 promoter region and T3 promoter region), were used for sequencing. Sequencing primers within the cloned fragments were designed and used for genomic "walking". DNA sequences were determined from both strands. Plasmid DNA samples were prepared as described in Section 3.2.4.6, and were digested with RNase A (at a final concentration of 50 µg/ml) to ensure against RNA contamination. The RNA degraded DNA samples were denatured in a freshly made solution of 1 N NaOH/1 mM EDTA at 37°C for 1 hour before the samples were "cleaned" using CL-6B columns. In order to obtain good quality DNA template for sequencing reactions, it proved to be important that the final concentration of sodium hydroxide in the DNA sample did not exceed 0.25 N. It was also found that the use of high temperatures (45°C and 95°C) in the termination and denaturation steps improved the quality of sequencing profiles (The manufacturer of Sequenase recommends 37°C and 75°C to 80°C respectively). ss-DNA samples were also generated for sequencing as described in Section 3.2.6.1. The three sequenced regions are shown in Figure 4.25A, and the sequences are given in Fig. 4.25B.

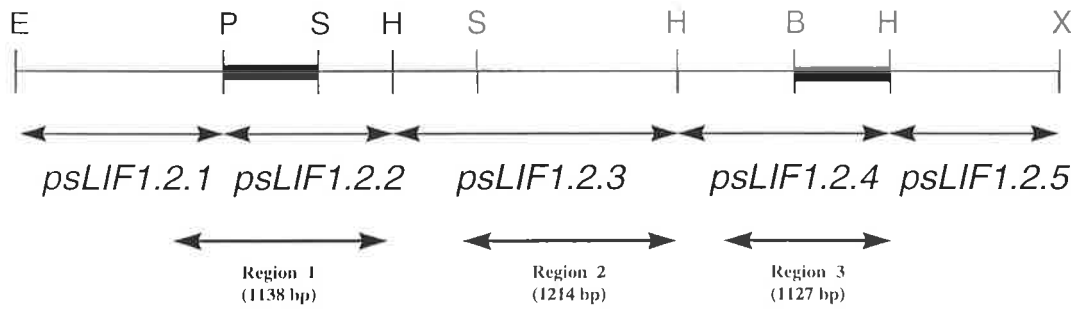


Fig. 4.25A Restriction map of *psLIF1.2*, showing sub-clones and sequenced regions.

Region 1 (1138 bp)

CTGGCAGCCC GGGGGATCCA CTAGTTCTAG AGCGGCCGCC ACCGCGGTGG
AGCTCTGGGA CCCTGATGGA CGGGCCGCTG CCTTCCTCGT CCGAGCCTCT
GTGCTCCCCT CTGCAGATGA GGATGAGGAT TCTGGTCCGG CTGCCTGTCC
TGGCGTCTAG CCTTCACCTC CTTCTAGAAG AGAGAGTGGT AGGTTGTTAC
TAGTGATAGT GACCAGGCCG GGAGGCCACA GTCTAGGCCC TCTCTTCCCC
GGCCAAGCTA GACCTAGAAG TATAAGCTCC TCCGGTTCCT TCCAAGCCCC
TCCACTCATA GACCCCTCCC CACTGTTTCC **AGGAGTCATG CCTCTGCTGC**
TGGTTCTGCA CTGGAAACAT GGGGCCGGGA GCCCCCTACC CATCACCCCC
GATGATCCCA AATGCGAAAT GCGCCACCAG TGCCCAGGCA ACCTCACGTT
CCAGATCCGG AACCAGCTGA ATCAGCTCAA TAGCAGCGCC CCGGAACTCT
TCACCTATTA TGTGACCCCG TACCGCCCCT TCCTGCCCCC AGCCCCAAGC
CGCGTTCCTC CACTTGGCCG GGCAGGAAGT CTCTCTGTCT GCCAGCCAGG
CCCCACTGAG GGTGTGACCT AGATACCTGA TGGGGGGGGA GCTCAAGGGA
AAGCAGAAAG TCTGCCTCGG TCTGGGCCAG GCCATTGCAG CTCCCCCACC
CCCAAGTTTC TCATGTACGT TGCCTTGCGA CAAGACTGGT ATGTGGGTAG
GAAAGGGATC AACATGGCGG CTGGAGAGGA TGTTAGAGGG ACTGCCCCGA
GCTGAAGAGG CTGGAGTCAG GCTGAAGGCA TGGGGGATTC TGCCGAGGGG
CCAGCTTATC GATACCGTAT CGACCCTCAT CGTACGTACG GGGGTGCCGG
TACCGGCTGT GTGTGTGTCG CCGTTTGGTG CAGGGTTAAT TCTCTACGAG
CTTGGCGTAA TCAGTGGCTC ATAGCTCGTT TCGCCTGTAT CGTCGAGTTG
TTATCCGCTC ACAAGTTCCA CACAACACTA CGGCGGAAGC ATAAAGCCTG
GGGTAAGCCT AATCGAAGTG AGCTAACTCA CATTATTGCG TTGCGCTCAC
TGCCCGCTTT CAGTCGGAAC TTGGCAGCTG CTTATACG

Region 2 (1214 bp)

AGGAGAGGCT GGAGTCAGGC GAAGGCTGGG GATTCTGGCG AGGGGAAGCT
TCTCATGGAG GCCGAGCTTG GCGAGAAGGG ACAGAGAGGG AAAGGGCGGG
AGACAGAGAC GAGGACAGGC AGAAAGCAAC AGACCCAGAA TCCTCCATCC
TTCCAGCTGG TGCTGTTTTA GAACTTTAGA TCCAGGTCAT TTCACGGATG
GAAAAAATGA GACCCAGACC GAAGAAGCAC TTGCCCCAAA TCATATAGGA
GGTAGCTCAT AAAATCACAG GCTGACAGAG AGAGCTCATG GCTACGAGTC
CCGGCAATTA ACCCAAAAGG ACTCCTCCAG CCTCTGCTTG AAGACTTCCA
GTGACAGGGA GCTCACTACC TTCCCCAGCA GCCCATCCGA CTTTGAGATA
GCTCTCATTA CTGGGAAGGA TTCCTGGCAT GGAATTGTAC CTTCCTCCTC
TTGCTCCTGC CTCATGGCAA GACAAGTTGG TGCTCAGACC AGGTATGGCA
TCTCCTAAGC CTGGGCAACT CTCCACTGAA GCCAGCTGAC TCCCTGGGGA
TCCTCCAAGT AGAGGCTTGC ATGACCAGTG GATCGGGCAC TGTTGGACTC
ACTGGACTCT CTGAGACCCC TTTGAGGGCT AAGATTCTGT GATCCTGGGA
TCCCTAGTAG ACTTCCTCTC TGCCAAGAGA GATGGAGAAT AACAGAGATA
ACTGAGAGAC CAAAGACGAG CTAGAGACAC TGGACTGTGG AGATGCATGG
AGAGAGACTG GAGGAGAAGG AACTGATGAT GTAAAGGGAA AGATGAGTCG
TGCATGAGGC CTCCAAGAGA CTCGCTGTGG CCATCTAGCT GGTCCCAGGC
CTTCACATGA TTGATGGGGA AACAGGCCCA GAGGTGAGAG GTGACACACT
GAAGAGCTGG GCCTCGAAGA CCGATGGCCT TTTATTCCCA GGCCAGAGGT
ATGGCTCCTG ATGTTTCCAA ATCCCTGCCT TCCTCTGCAT CCTCCCCAGA
TTTTTAGCTT AGGGTTAGGG ACTGGGCACA AAGGCTTTAC TGACCAGGCC
CTCACATCTG GGGGCATGTC TAGGCCTTTC CCCTTTTCCC AGTCAGTAGC
CGTTGACGTG ATTCCTCCAA AACTGCCCCT TCGGAGCACC TGGGACGGTA
TACGGGAGGC AAGGGCCCAG AGCTCCCCTA GGCAGTCCCC GGCTGATGAA
AGCACCTCGT CTTG

Region 3 (1127 bp)

AATTCTCAGC TTTGAATCTA TGTCTAAGCA ACTCTCATTC TCTCTTTTAA
AGTCTCTCCC AGTTCTAACA TTGTGTTCTA AGCTCCAGTC TTTTCTCTGA
TGTTCTCTCT TCTAAAGTTC CTCCCAGCTC TGACATTCTG TGTTCTAAGC
TCCTCTCCTC CAGACGTTCT CTCTCCTCCT AAGTGTCCCC CTGCTCTGAG
GTTCTCTGAG ACTAGGAATA GCTGACAACG GTCATATCGA CTGCGGTCCA
TTGTCCTGCC TCCTGGGGTA CCCTGGTCTC TGCCCTTGTT CCAGATTCAG
TGTCCGGGGT CAGTCAAAGA CCTCCTGCCC CAGCCTCGCG CCGCGTCCCC
TTGTTGCCCT GAACCAGTTA GTTAATCCTT GTCAGTTCTT TTCTGTGTTC
TAG**TACAAGG CTCAGGGGGA GCCGTTCCCC AACAACCTGG ACAAGCTGTG**
CAACCCCAAC GTGACGGACT TCCCGCCCTT CCACGCCAAC GGGTCAAACA
AGGAAAAGCT GGTGGAGCTA TACCGGATCA TAGCCTACCT CAACGCCTCA
CTGGGCAACA TCACGCGGGA CCAGAAGGCC CTCAACCCCA GTCGGCTCTA
CCTCCTCAGC CGGCATAACA TGACCACGGC CATAATGAGG GGCCTGCTCA
ACAACCTCAC CTGTCGCCTG TGCAGGAAGT ACCACGTGGC TCACGTCTCG
GTCGCCTACG GCCCGGACAC GGCCAACAAG GACACTTTCC AGAAGAAGAA
GCTGGGCTGC CATTTGTTGG GGAAATACAA GCAGGTCATT TCTCTGGTGG
CCCAGGCCTT CTAGACAGCC GCAGCTCAAG GGGTCGTCCT CGACTGGTGG
GAGAGTCCCG ACCATCTCAG CCGGACTTGG GAGTGGCCTC CAGCATGGGG
CACTAGCCCA AGGAGGCAAG AGTGGCTCAG ACCGGGGAGA GCGGGACTTG
AAATCGCAAG CTTGGTCTGA GAGTCCACTC TCAGTCATCG GGCCTCAGGG
TCCCCGGGTA CCAGTCCAGT CCAGTAGTCA GTCATCGACC AGTCCACTGG
CTAAGCCTGG GCTGATGGGC AGGGGTGAGA CCATCATCTG GAGGGTGGTG
AGGTGAGAGT CAACCCCATC TGATCAG

Fig. 4.25B Sequences of the 3 regions of *psLIF1.2* shown in Fig. 4.25A. (Coding DNA in bold).

4.2.2 Sequence analysis

Comparison of *S. crassicaudata LIF* and the *LIF* genes from other species, revealed high levels of conservation of nucleotide sequence (sequence identity greater than 75 %) in segments (presumed to be coding regions) bonded by consensus splice donor and acceptor sites, embedded in less well conserved non-coding regions. The aligned *LIF* genes from human, mouse and *S. crassicaudata* and the locations of the exons are shown in Figure 4.26. It can be seen that *sLIF* has a relatively long intron 2 (>2.6 kb) compared to the intron 2 regions of *mLIF* and *hLIF*.

Fig. 4.26 Structural comparison of *sLIF*, *mLIF* and *hLIF* genes. The three *LIF* genes are aligned on the basis of the 5' end of exon 2. Thick lines indicate the exons and thicker lines represent the coding region for LIF proteins. The dashed line shows the uncharacterized 3' untranslated region of *sLIF* predicted from the *mLIF* and *hLIF* genes.

psLIF1.2.2 showed a high degree of sequence identity to homologous regions of *mLIF* and *hLIF*. Of 180 bases in exon 2, 134 bases are identical between *sLIF* and *hLIF* (Fig. 4.27). The level of sequence identity of exon 2 in these two *LIF* genes is about 75 %. The exon 2 area of *sLIF* is more similar to *hLIF* that bovine *LIF* (*bLIF*, 73 %) and *mLIF* (73.3 %) although it is unlikely that these small differences are significant. DNA in the region of the intron splice sites is highly conserved across species.

```
          260        270        280        290        300        310
psLIF122  CTAGACCTAGAAGTATAAGCTCCTCCGGTTCCTTCCAAGCCCCTCCACTCATAGACCCCT
                                          :::: :  :::  :: :::::: :    : :
HUMALIF   CCCATCCGGTGTGCCATGACCCCAGGCCACCCTTTCCTGCCTTTCTACTCATGGCTTCTT
          2400       2410       2420       2430       2440       2450

          320        330        340        350        360        370
psLIF122  CCCCACTGTTTCCAGGAGTCATGCCTCTGCTGCTGGTTCTGCACTGGAAACATGGGGCCG
          ::  :::::  ::::::::  :::: :::::: :::::::::::::::::::::::::::
HUMALIF   CCTGACTGTCCCCAGGAGTTGTGCCCCTGCTGTTGGTTCTGCACTGGAAACATGGGGCGG
           2460       2470       2480       2490       2500       2510

          380        390        400        410        420        430
psLIF122  GGAGCCCCCTACCCATCACCCCCGATGATCCCAAATGCGAAATGCGCCACCAGTGCCCAG
          :::::::::: :::::::::::  :   :  :::  :: :  :: ::::::::  :: :
HUMALIF   GGAGCCCCCTCCCCATCACCCCTGTCAACGCCACCTGTGCCATACGCCACCCATGTCACA
           2520       2530       2540       2550       2560       2570

          440        450        460        470        480        490
psLIF122  GCAACCTCACGTTCCAGATCCGGAACCAGCTGAATCAGCTCAATAGCAGCGCCCCGGAAC
           :::::::: :  ::::::: ::: ::: :::   :::::::  :::: :::   :  :
HUMALIF   ACAACCTCATGAACCAGATCAGGAGCCAACTGGCACAGCTCAATGGCAGTGCCAATGCCC
           2580       2590       2600       2610       2620       2630

          500            510        520        530        540        550
psLIF122  TCTTCACCTATTATGTGAGTGACCCCGTACCGCCCCTTCCTGCCCCCAGCCCCAAGCCG
          :::: :    ::::::  ::: :::::        :
HUMALIF   TCTTTATTCTCTATGTAAGTTACCCCTGGGATACTGACAGGAGATGGCAGGGAGGGGGC
           2640       2650       2660       2670       2680       2690
```

Fig. 4.27 Sequence alignment of *psLIF1.2.2* and *hLIF*. The identical nucleotides are indicated by ":". Exon 2 regions are shown in bold. The two *LIF* exon 2 regions are 75 % identical in sequence with the splicing sites conserved.

The DNA sequence of a 411bp segment determined from sub-clone *psLIF1.2.4* that contains exon 3 coding region, was found to be a homologue of the exon 3 coding region of other *LIF* genes. These segments encode the N-terminal part of the mature LIF protein. Figure 4.28 shows the region of high sequence identity (78 %) between *psLIF1.2.4* and the bovine *LIF* gene.

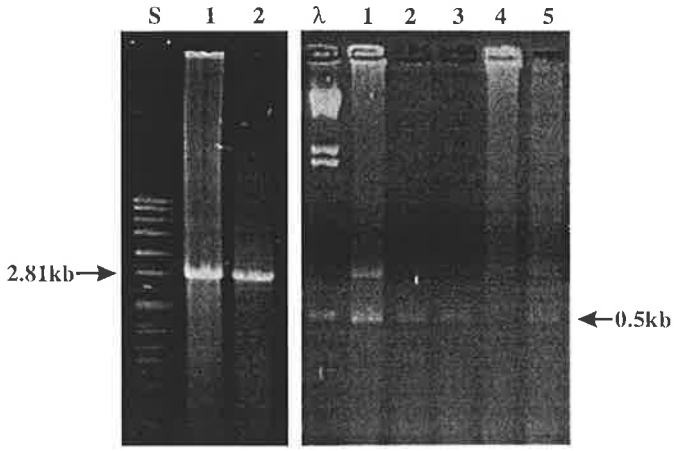
Fig. 4.28 Sequence alignment of *psLIF1.2.4* and a region of the bovine *LIF* gene, showing a highly conserved 411 bp segment which corresponds to exon 3 (in bold type). A *Bam*HI restriction site is underlined.

As deduced from the comparison with *hLIF* and *mLIF* genes, *psLIF1.2* contains two exon regions, exon 2 and part of exon 3 including the entire coding region for the mature LIF protein. The two exon regions in *psLIF1.2* are separated by a 2.6 kb intron. This intron is about 4 time greater in size than the intron 2 present in *hLIF* and about 5 times greater than that present in *mLIF*. A putative exon 1 of *sLIF* could not be identified by Southern hybridization or by DNA sequence comparisons with other *LIF* gene, since exon 1 in *LIF*, which contains a short coding region (18 bp) and 5' UTR, is not well conserved amongst species (Stahl *et al.*, 1990). The mature LIF protein coding regions in *S. crassicaudata*, deduced by comparison with *LIF* genes of other species, is 411 bp in length.

4.3 Isolation and Characterization of *S. crassicaudata* LIF cDNA

4.3.1 cDNA cloning

In order to clone the region of *sLIF* that encodes the mature LIF protein, the reverse transcription-polymerase chain reaction (RT-PCR) technique was employed using RNA isolated from adult brain and heart tissues (Section 3.8.1). The forward primer was designed from the region of *psLIF1.2.2* that encodes the first 6 amino acids of the mature protein, and the design of the reverse primer was based on the region of *psLIF1.2.4* that encodes the last 5 amino acids and contains the stop codon (Section 3.2.9.2). The predicted RT-PCR product is 555 bp in length and includes the whole cDNA fragment (546 bp) and 9 bp of adaptor sequence containing *Bam*HI sites. Figure 4.29 shows the RT-PCR products obtained from the *S. crassicaudata* genomic DNA and cDNA templates.



(a) (b)

Fig. 4.29 PCR amplification of the region of *sLIF* expected to encode the mature protein. (a) PCR products obtained from genomic DNA; (b) PCR products from cDNA.

Tracks are:

(a) S - *SPP-1* DNA marker
1 - PCR products from *psLIF1.2*
2 - PCR products from genomic DNA

(b) λ – λDNA/*Hind*III marker
1 - PCR product from brain cDNA
2 - PCR products from ear cDNA
3 - PCR products from footpad cDNA
4 - PCR products from kidney cDNA
5 - PCR products from heart cDNA

A PCR fragment of the expected size (about 500 bp) was amplified from cDNA. The fragments amplified from genomic DNA and from the sLIF sub-clone *psLIF1.2* were about 2.8 kb, suggesting the presence of an intron of about 2.2 kb between exon 2 and 3. These results are consistent with the result from restriction mapping (Fig. 4.22).

The 555 bp cDNA fragment amplified by RT-PCR was extracted using phenol/chloroform, precipitated with ethanol, digested with *Bam*HI and electrophoresed on an agarose gel (Fig. 4.30A). This fragment was cut into two pieces (152 bp and 403 bp) by *Bam*HI. The location of this *Bam*HI site, near the beginning of exon 3, is indicated by underlining in Fig. 4.28.

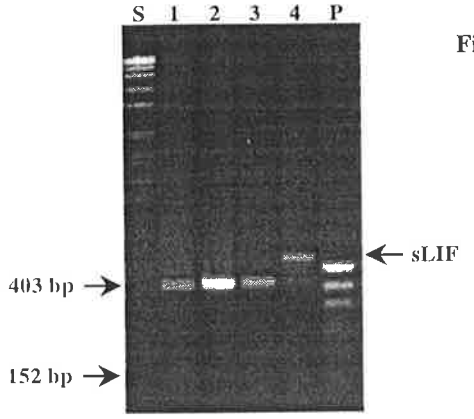


Fig. 4.30A Restriction analysis of sLIF RT-PCR products. The RT-PCR product was digested with *Bam*HI and electrophoresed on agarose gel.

Tracks are:
S - *SPP*-1 DNA marker
1 - Heart *sLIF* cDNA digested with *Bam*HI
2 - Intestine *sLIF* cDNA digested with *Bam*HI
3 - Intestine E3 fragment of *sLIF* cDNA
4 - Intestine *sLIF* cDNA undigested
P - pUC19/*Hap*II DNA marker

Oligonucleotide-directed PCR was used to remove this *Bam*HI site so that the entire fragment could be cloned into the *Bam*HI site of the plasmid vector for further analysis. Three separate PCR reactions were conducted to modify *sLIF* cDNA (see Fig. 4.30B). First, the 5' primer, *Exp1*, and the modifying primer, *3'E2*, were used in a PCR reaction to generate a fragment containing the mature protein coding sequence in exon2 with a protruding 5' end. Secondly, the 3'primer, *Exp2*, and the modifying primer, *5'E3*, were used in a PCR reaction to generate a cDNA fragment corresponding to the part of exon 3 that encodes the mature LIF protein, with a protruding 3' end. Gel slices containing the products of these two PCR reactions were purified using the Wizard PCR Preps System (Section 3.2.5.11), and the purified DNA was mixed in equal proportions. This mixed DNA (5 µl) was used in the third PCR reaction to generate the entire coding region for the mature sLIF protein. The resultant PCR product was purified, cloned in pBluescript, and sequenced to ensure that modification of the *Bam*HI restriction site had been successfully achieved.

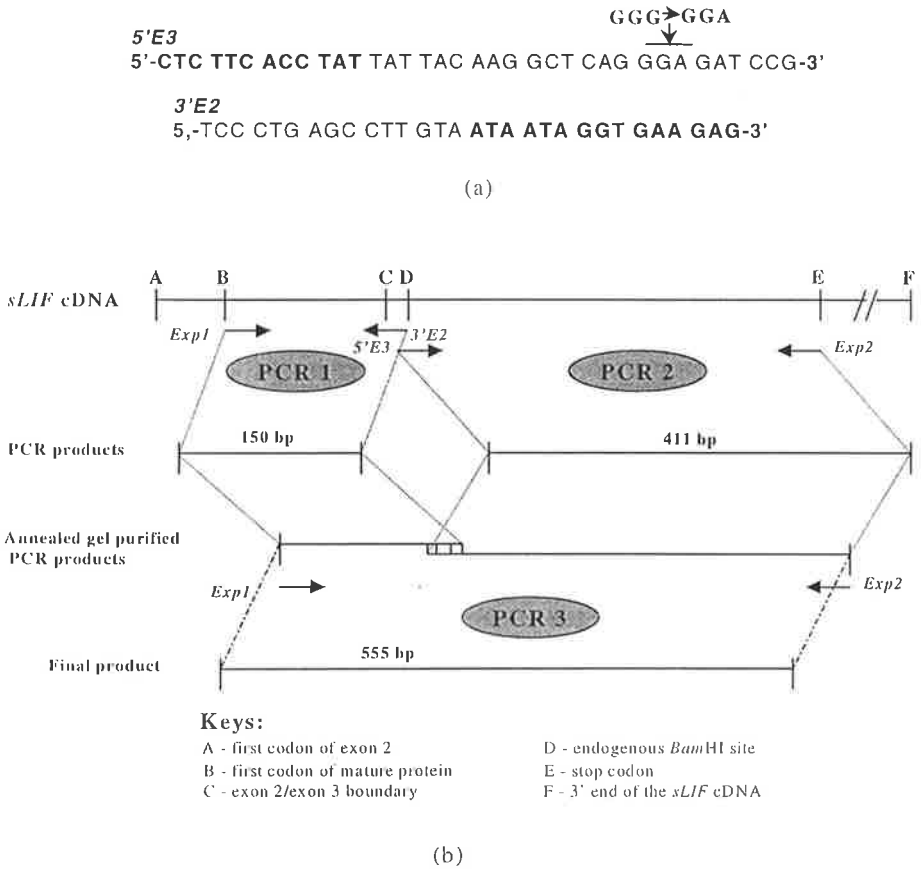


Fig. 4.30B Modification of *sLIF* cDNA.

(a) Primers designed from exon2/exon 3 boundary region. Overlapping sequences are shown in bold type.

(b) Strategy for the modification of *sLIF* cDNA fragment. The *Bam*HI restriction site within the cDNA was modified by substituting a single nucleotide to give a synonymous codon. A final product of 555 bp was archived with *Bam*HI sites at both ends.

The PCR product (555 bp) (Fig. 4.30A) was extracted by phenol/chloroform, precipitated with ethanol, resuspended in double distilled water and digested with *Bam*HI. After LMT agarose gel electrophoresis, a gel slice containing the cDNA fragment was excised and further purified through a *Wizard* column (*Promega*) from the gel slice and ligated into *Bam*HI linearized pBluescriptII KS^+ vector.

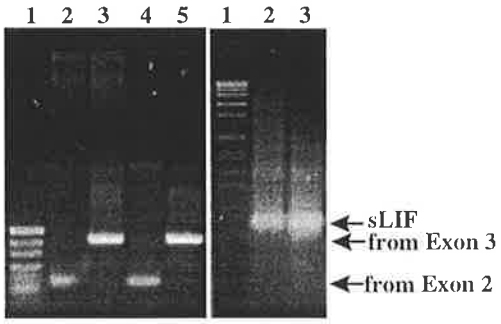


Fig. 4.31 Modification of *sLIF* cDNA (to "remove" a *Bam*HI site) by oligonucleotide-directed PCR. In the first step (a) fragments were amplified from exon 2 part and part of exon 3 and purified using a *Wizard* column to prevent contamination by carry-over primers. In the second step (b) the two fragments were mixed and amplified using sLIF gene-specific primers (Fig 4.30B). This procedure generated full-length cDNA encoding the mature sLIF protein but with a modified *Bam*HI site. Tracks are:

(a)
1 - pUC19/*Hap*II DNA marker
2 - Exon 2 cDNA amplified from brain
3 - Exon 3 cDNA amplified from brain
4 - Exon 2 cDNA amplified from heart
5 - Exon 3 cDNA amplified from heart

(b)
1 - *SPP*-I DNA marker
2 - cDNA amplified from brain
3 - cDNA amplified from heart

4.3.2 *sLIF* cDNA sequencing

pBluescriptII KS$^+$ containing *sLIF* cDNA (*psLIF*) in was transformed into XL-1 Blue bacteria strain and a single colony of the white transformants was picked and grown in LB medium in the presence of ampicillin. Plasmid DNA was isolated and purified according to the protocols described in Section 3.2.4.6. The nucleotide sequence of the insert in this clone was determined using the T7 and T3 promoters as sequencing primers. The sequence of *psLIF* cDNA is shown in Figure 4.32.

```
   1 GGGAGCCCCCTACCCATCACCCCCGATGATCCCAAATGCGAAATGCGCCACCAGTGCCCA 60
     G  S  P  L  P  I  T  P  D  D  P  K  C  E  M  R  H  Q  C  P

  61 GGCAACCTCACGTTCCAGATCCGGAACCAGCTGAATCAGCTCAATAGCAGCGCCCCGGAA 120
     G  N  L  T  F  Q  I  R  N  Q  L  N  Q  L  N  S  S  A  P  E
                                   A
 121 CTCTTCACCTATTATTACAAGGCTCAGGGGGATCCGTTCCCCAACAACCTGGACAAGCTG 180
     L  F  T  Y  Y  Y  K  A  Q  G  D  P  F  P  N  N  L  D  K  L

 181 TGCAACCCCAACGTGACGGACTTCCCGCCCTTCCACGCCAACGGGTCAAACAAGGAAAAG 240
     C  N  P  N  V  T  D  F  P  P  F  H  A  N  G  S  N  K  E  K

 241 CTGGTGGAGCTATACCGGATCATAGCCTACCTCAACGCCTCACTGGGCAACATCACGCGG 300
     L  V  E  L  Y  R  I  I  A  Y  L  N  A  S  L  G  N  I  T  R

 301 GACCAGAAGGCCCTCAACCCCAGTCGGCTCTACCTCCTCAGCCGGCATAACATGACCACG 360
     D  Q  K  A  L  N  P  S  R  L  Y  L  L  S  R  H  N  M  T  T

 361 GCCATAATGAGGGGCCTGCTCAACAACCTCACCTGTCGCCTGTGCAGGAAGTACCACGTG 420
     A  I  M  R  G  L  L  N  N  L  T  C  R  L  C  R  K  Y  H  V

 421 GCTCACGTCTCGGTCGCCTACGGCCCGGACACGGCCAACAAGGACACTTTCCAGAAGAAG 480
     A  H  V  S  V  A  Y  G  P  D  T  A  N  K  D  T  F  Q  K  K

481 AAGCTGGGCTGCCATTTGTTGGGGAAATACAAGCAGGTCATTTCTCTGGTGGCCCAGGCC 540
     K  L  G  C  H  L  L  G  K  Y  K  Q  V  I  S  L  V  A  Q  A

 541 TTCTAG 546
     F  *
```

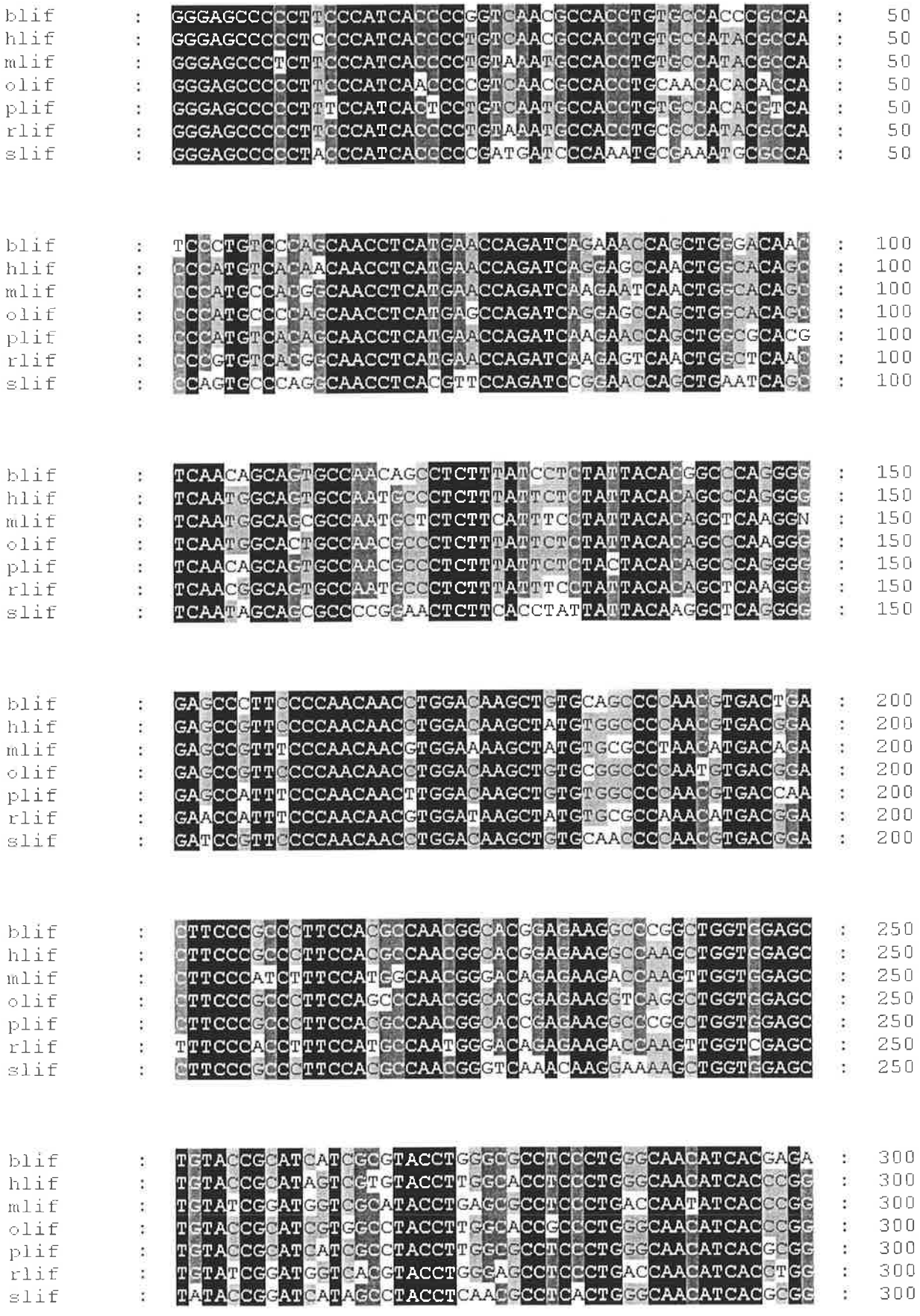
Fig. 4.32 The cDNA sequence encoding mature sLIF and the predicted amino acid sequence. The *Bam*HI restriction site that was removed by oligonucleotide-directed modification is underlined and the changed base (A) is indicated. "*" indicates the stop codon.

The amino acid sequence of the mature sLIF protein (Fig. 4.32) was deduced from the nucleotide sequence of *psLIF* cDNA. When compared to the LIF proteins from other species, sLIF contained no insertions and deletions, suggesting that the number of amino acid residues in the mature LIF protein of mammals is highly conserved.

4.4 Comparative Analysis of sLIF

4.4.1 Sequence alignments

The aligned *LIF* sequences (coding regions only) are shown in Figure 4.33 (overleaf).



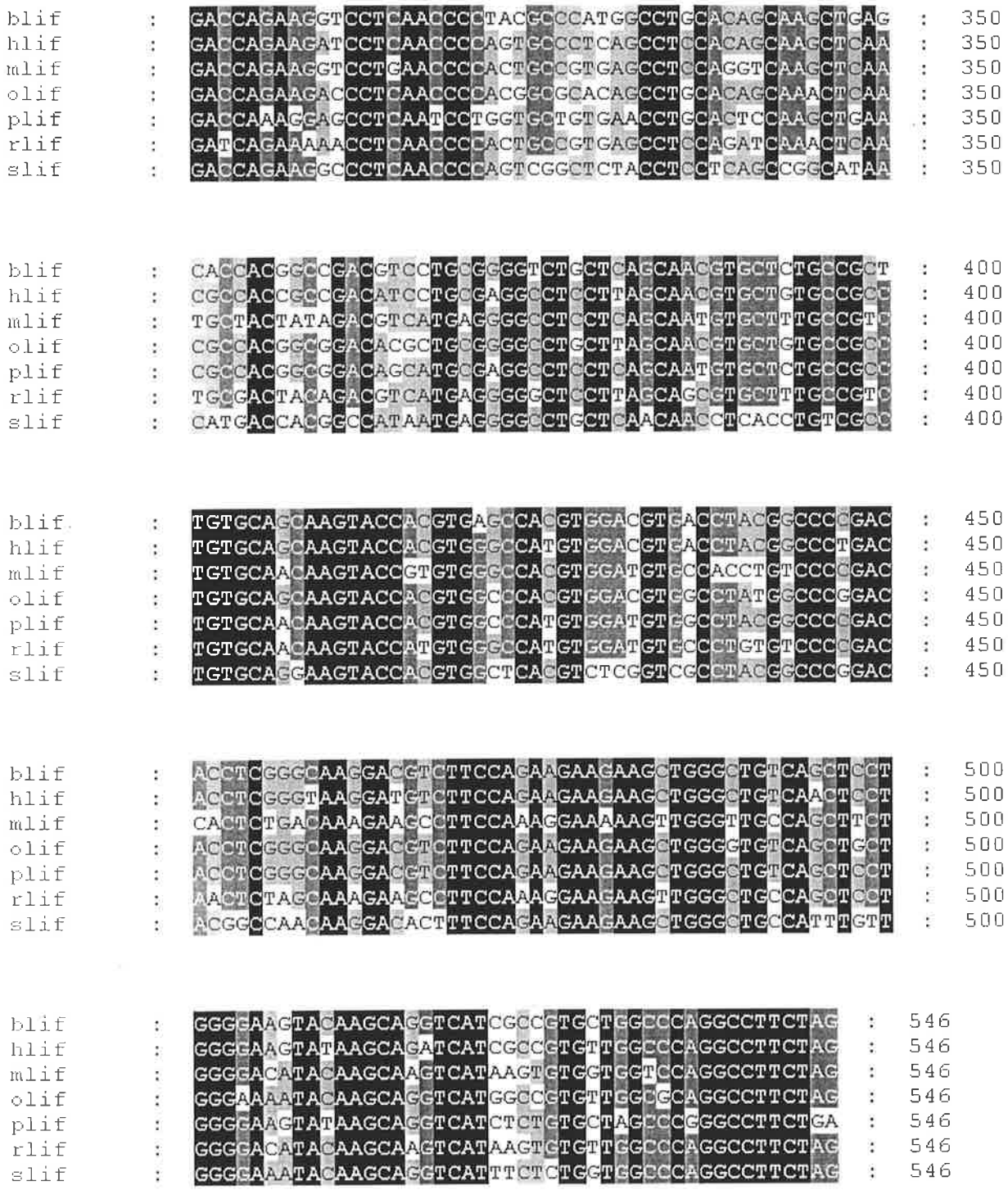


Fig. 4.33 Alignment of the mature protein coding region of *LIF* genes from seven mammalian species. b = bovine, h = human, m = mouse, o = ovine, p = porcine, r = rat and s = *S. crassicaudata*. The four levels of shading used are: Black – 100 % sequence identity; Grey - 80-99 % identity; Light grey – 60-79 % identity; no shading <60 % identity.

Comparison of the *sLIF* coding region for mature protein and its counterpart in bovine *LIF*, human *LIF*, murine *LIF*, ovine *LIF*, porcine *LIF* and rat *LIF* shows a high level of sequence identity across species (Fig. 4.33, Table 4.2B).

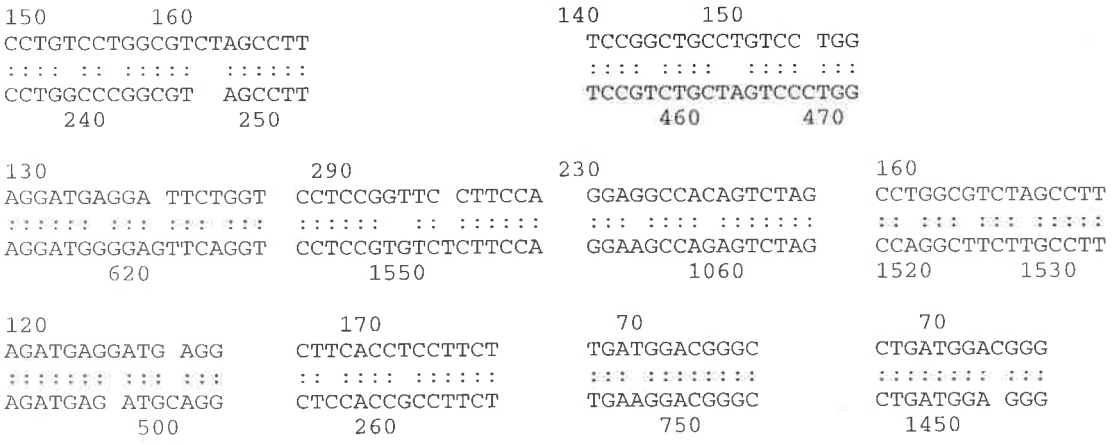
Table 4.2B *LIF* cDNA sequence identity between pairs of mammalian species. Identity levels are shown as percentages (upper matrix). The numbers of nucleotide residues compared was 546 in all cases. The number of nucleotides that matched exactly is shown in the lower matrix.

	blif	hlif	mlif	olif	plif	rlif	slif
	1	2	3	4	5	6	7
1	546	88%	76%	86%	86%	77%	75%
2	483	546	80%	89%	87%	82%	75%
3	416	439	546	77%	77%	90%	70%
4	474	489	421	546	84%	78%	76%
5	473	477	424	462	546	78%	73%
6	424	449	492	429	431	546	70%
7	413	411	386	416	401	384	546

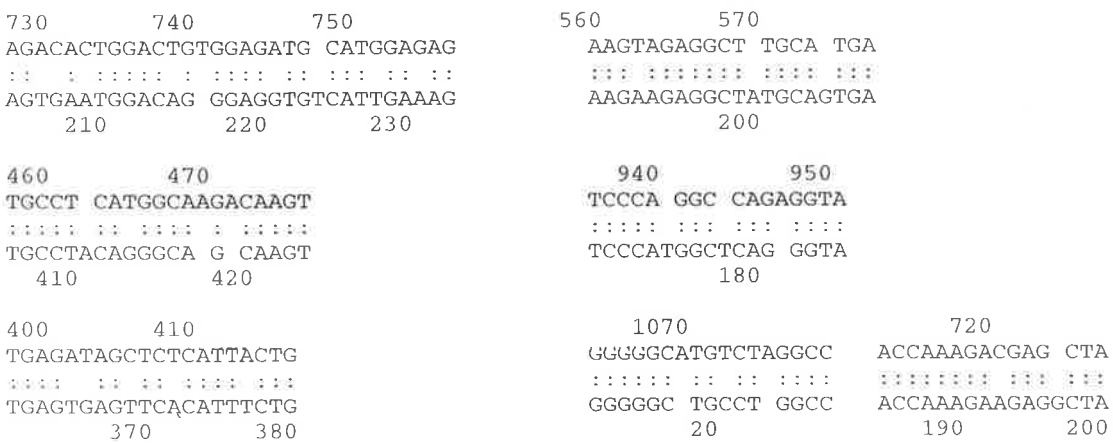
4.4.2 Intron sequence comparisons

Intron sequences were compared using the computer programs SEQH, CLUSTALW, COMPARE and DOTPLOT. Comparisons between Intron 1 of human (1730 bp) and mouse (1559 bp), and between Intron 2 of human (693 bp) and mouse (537 bp), revealed significant stretches of sequence identity (Fig. 4.35 (c) and (d)). The partial Intron 1 sequence available from *sLIF* (331 bp) came from the region immediately upstream of exon 2 (see Fig. 4.25 a & b). Comparisons between this sequence and the Intron 1 sequences from *hLIF* and *mLIF* showed only short stretches (about 20 bp) of significant sequence identity (Figure 4.34). These regions are scattered throughout the introns with no clear distributional pattern (Fig. 4.35a). Three non-overlapping sequence regions were available for intron 2 of *sLIF* (see Figs. 4.25 a & b). The first of these was from the 3' end of Region 1 and contained 627 bp immediately downstream of exon 2. The second was from Region 2 and contained 1214 bp located

in the central region of the intron. The third region was from Region 3 and contained 403 bp immediately upstream of exon 3. These sequences from *sLIF* intron 2 were compared individually and together with *mLIF* and *hLIF* intron 2 sequences (not all data shown). Again, only short stretches of sequence identity were revealed (Fig. 4.34 and 4.35). Taken overall, it can be concluded that the introns of *sLIF* show minimal levels of sequence identity with the corresponding introns of the orthologous genes from human and mouse. The possibility that the short regions of sequence identity that do exist, are due to chance rather than homology, can not be ruled out.

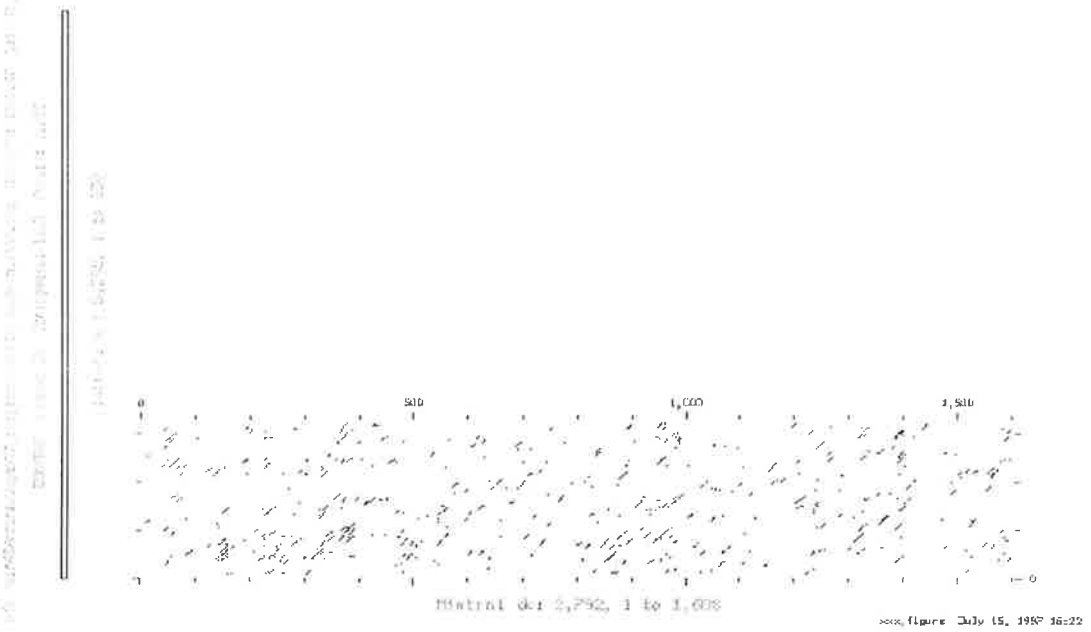


(a) *sLIF* (above) and *mLIF* (below) intron 1

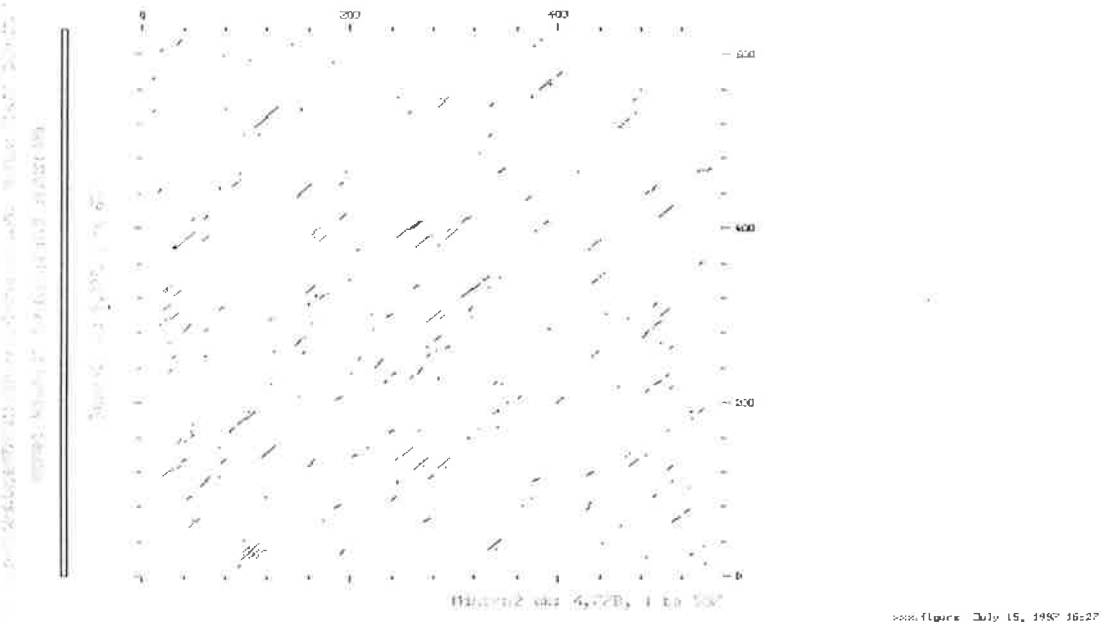


(b) *sLIF* (above) and *mLIF* (below) intron 2

Fig. 4.34 Examples of intron sequence alignments between *sLIF* and *mLIF*, obtained using the algorithm SEQH. (a) Intron 1, (b) Intron 2. In each case the *sLIF* sequences analysed were partial (see text). The *sLIF* intron 2 sequence is from Region 2 (see Fig. 4.25 (a)). For *mLIF* introns, numbering commences at the first nucleotide of the intron. For *sLIF* introns, numbering commences at the start of Region 1 (for intron 1) and Region 2 (for intron 2) see Fig. 4.24 (a). Note the presence of short regions of sequence identity scattered throughout the introns.

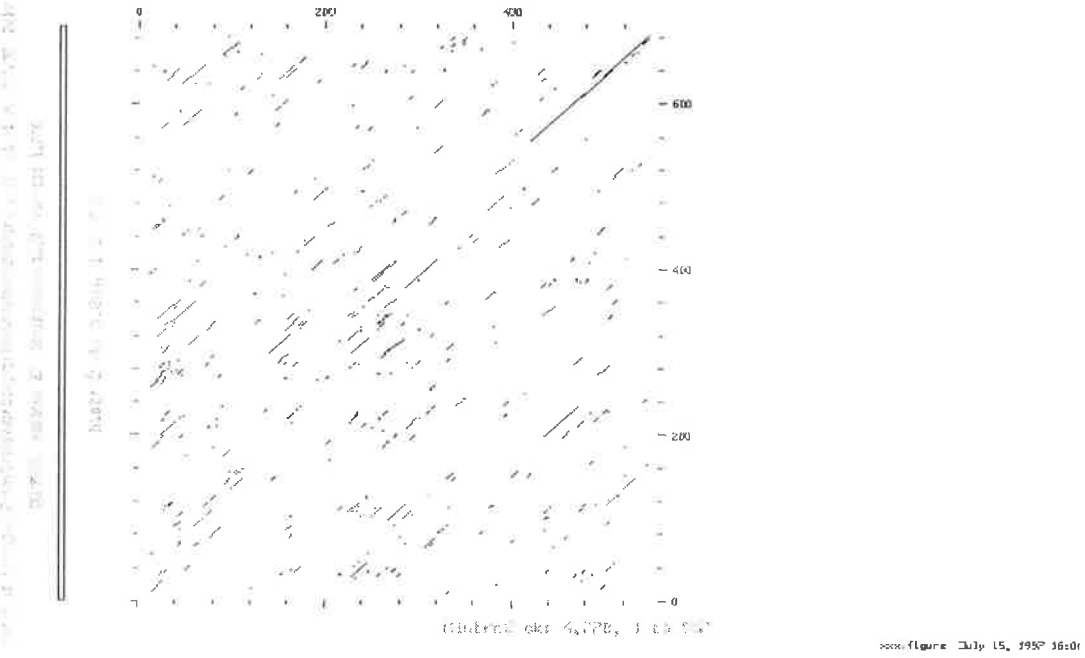


(a)



(b)

(c)



(d)

Fig. 4.35 Inter-species *LIF* intron sequence comparisons carried out using COMPARE and DOTPLOT. The comparison window used was 12. (a) *mLIF* and *sLIF* intron1, (b) *mLIF* and *sLIF* intron2, (c) *mLIF* and *hLIF* intron1 and (d) *mLIF* and *hLIF* intron 2.

4.4.3 Comparative study of predicted properties of sLIF

The inter-species comparisons of LIF protein sequences (Table 4.3A) demonstrate that the % sequence identity between *S. crassicaudata* and LIF protein from several eutherian species varies between 64 % and 71 %. It is doubtful if these differences are statistically significant. It can be seen that the eutherian LIF sequences are more similar to one another (average about 80% identity) than to the marsupial LIF sequence.

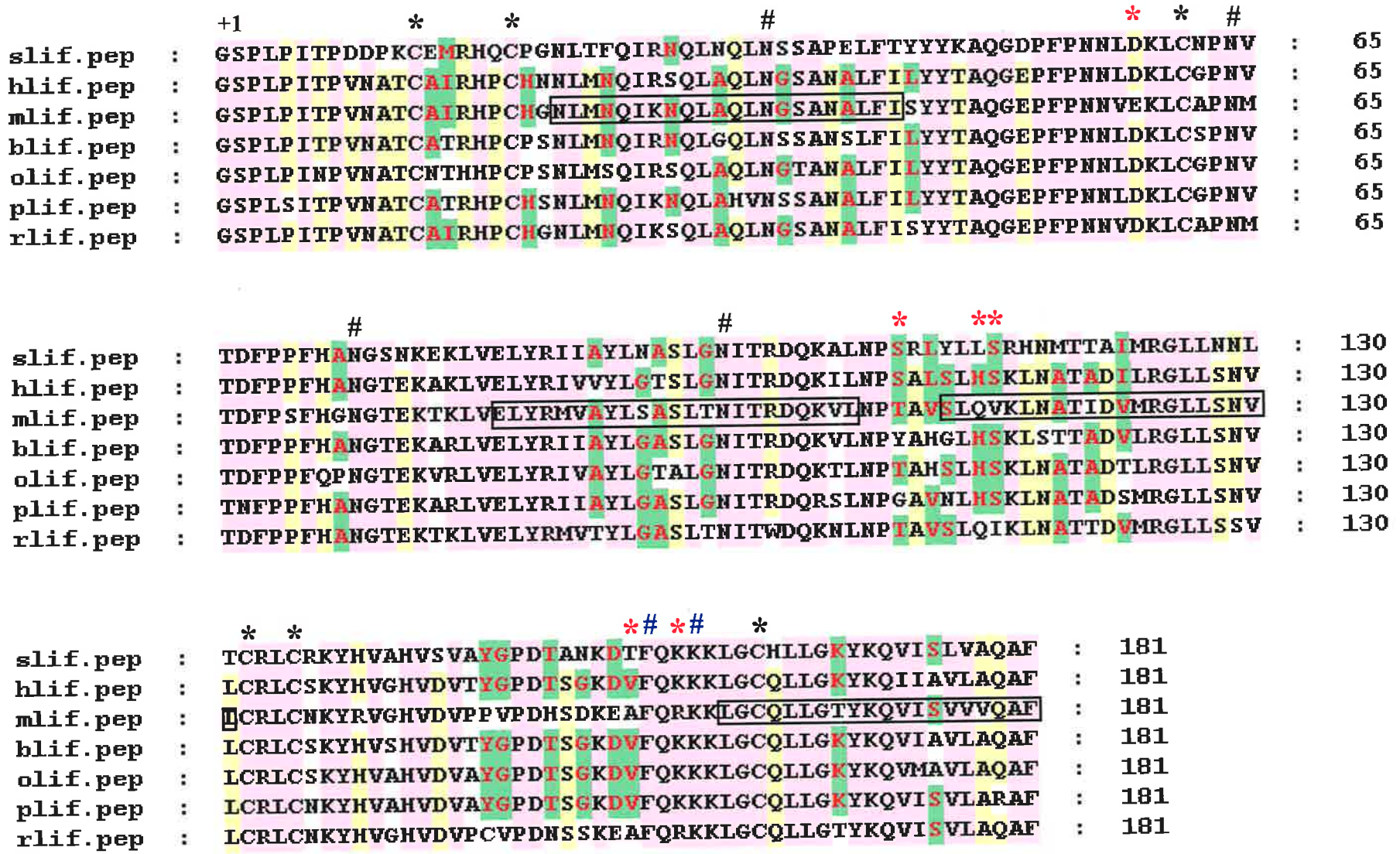
Table 4.3A Cross-species identity (%) of LIF proteins for pairs of species.

	bLIF	hLIF	mLIF	oLIF	pLIF	rLIF	sLIF
bLIF		88	75	86	87	76	71
hLIF			78	87	85	81	70
mLIF				73	76	90	64
oLIF					83	75	67
pLIF						78	70
rLIF							64

The alignment of LIF amino acid sequences from 7 mammalian species (Fig. 4.36) shows the presence of several blocks of residues (eg. 81- 86 inclusive) that are completely conserved. These conserved blocks are not clustered to any particular region of the protein. Overall, it can be seen that LIF is highly conserved.

All of the six cysteine residues which are conserved in location in eutherian species (Willson *et al.*, 1992) and have been shown to be involved in disulfide bond linkages (Robinson *et al.*, 1994), are found at the same positions in *S. crassicaudata* LIF (Fig. 4.36). Amongst the seven mammalian LIF sequences compared, only one cysteine residue occurs (rat, position 147) that is not conserved across all species. This suggests an important structural role for intracellular disulphide bridges in the integrity and activity of the LIF proteins.

Fig. 4.36 Sequence alignment of the mature LIF protein from 7 mammalian species. The four levels of shading used are: Pink background – 100 % sequence similarity; yellow - 80-99 % similarity; green – 60-79 % similarity; white - <60 % identity. Conservative substitution groups are taken to be: (D, N), (E, Q), (S, T), (K, R), (F, Y, W) and (L, I, V, M). Conserved cysteine residues are indicated by "*" (black). Conserved potential N-linked glycosylation sites are shown by "#" (black). Helices (A, B, C and D) identified by X-ray crystallography of mLIF are surrounded by boxes. +1 (serine) indicates the first amino acid of the mature native protein. Thrombin cleavage results in a additional glycine residue at position -1 in recombinant LIF. Residues in hLIF responsible for species-specific binding to the receptor (Owczarek *et al.*, 1993; Layton *et al.*, 1994; Hudson *et al.*, 1996) are indicated by "*" (red). Residues responsible for receptor binding (Hudson *et al.*, 1996) are indicated by "#" (blue). Unidentified residues in A and C helices contribute to binding the gp130 glycoprotein (Hudson *et al.*, 1996). The sequence from the mink was not available at the time this analysis was carried out.


+1
slif.pep : GSPLPITPDDPKCEMRHQCPGNLTFQIRNQLNQLNSSAPELFTYYYKAQGDPFPNNLDKLCNPNV : 65
hlif.pep : GSPLPITPVNATCAIRHPCHNNLMNQIRSQLAQLNGSANALFILYYTAQGEPFPNNLDKLCGPNV : 65
mlif.pep : GSPLPITPVNATCAIRHPCHGNLMNQIKNQLAQLNGSANALFISYYTAQGEPFPNNVEKLCAPNM : 65
blif.pep : GSPLPITPVNATCATRHPCPSNLMNQIRNQLGQLNSSANSLFILYYTAQGEPFPNNLDKLCSPNV : 65
olif.pep : GSPLPINPVNATCNTHHPCPSNLMSQIRSQLAQLNGTANALFILYYTAQGEPFPNNLDKLCGPNV : 65
plif.pep : GSPLSITPVNATCATRHPCHSNLMNQIKNQLAHVNSSANALFILYYTAQGEPFPNNLDKLCGPNV : 65
rlif.pep : GSPLPITPVNATCAIRHPCHGNLMNQIKSQLAQLNGSANALFISYYTAQGEPFPNNVDKLCAPNM : 65
slif.pep : TDFPPFHANGSNKEKLVELYRIIAYLNASLGNITRDQKALNPSRLYLLSRHNMTTAIMRGLLNNL : 130
hlif.pep : TDFPPFHANGTEKAKLVELYRIVVYLGTSLGNITRDQKILNPSALSLHSKLNATADILRGLLSNV : 130
mlif.pep : TDFPSFHGNGTEKTKLVELYRMVAYLSASLTNITRDQKVLNPTAVSLQVKLNATIDVMRGLLSNV : 130
blif.pep : TDFPPFHANGTEKARLVELYRIIAYLGASLGNITRDQKVLNPYAHGLHSKLSTTADVLRGLLSNV : 130
olif.pep : TDFPPFQPNGTEKVRLVELYRIVAYLGTALGNITRDQKTLNPTAHSLHSKLNATADTLRGLLSNV : 130
plif.pep : TNFPPFHANGTEKARLVELYRIIAYLGASLGNITRDQRSLNPGAVNLHSKLNATADSMRGLLSNV : 130
rlif.pep : TDFPPFHANGTEKTKLVELYRMVTYLGASLTNITWDQKNLNPTAVSLQIKLNATTDVMRGLLSSV : 130
slif.pep : TCRLCRKYHVAHVSVAYGPDTANKDTFQKKKLGCHLLGKYKQVISLVAQAF : 181
hlif.pep : LCRLCSKYHVGHVDVTYGPDTSGKDVFQKKKLGCQLLGKYKQIIAVLAQAF : 181
mlif.pep : LCRLCNKYRVGHVDVPPVPDHSDKEAFQRKKLGCQLLGTYKQVISVVVQAF : 181
blif.pep : LCRLCSKYHVSHVDVTYGPDTSGKDVFQKKKLGCQLLGKYKQVIAVLAQAF : 181
olif.pep : LCRLCSKYHVAHVDVAYGPDTSGKDVFQKKKLGCQLLGKYKQVMAVLAQAF : 181
plif.pep : LCRLCNKYHVAHVDVAYGPDTSGKDVFQKKKLGCQLLGKYKQVISVLARAF : 181
rlif.pep : LCRLCNKYHVGHVDVPCVPDNSSKEAFQRKKLGCQLLGTYKQVISVLAQAF : 181

Regions of hydrophobicity and potential N-linked glycosylation sites were predicted using the program PEPTIDESTRUCTURE (Wolf *et al. 1988).* Hydrophobicity values were calculated over a sliding window of seven residues, according to Kyte and Doolittle (1982). N-linked glycosylation was predicted for sites where the residues had the composition NXT or NXS. When X is D, W, or P, the site was taken to be a "weak" glycosylation site, otherwise it was taken to be a "strong" glycosylation site.

Analyses of the marsupial (sLIF) protein and LIF from six eutherian mammals (see Fig. 4.36) showed the presence of a number of potential N-linked glycosylation sites, some strong, others weak (Table 4.3B).

Table 4.3B Potential N-linked glycosylation sites in mammalian LIF. Ticks in small font size represent weak sites.

Site:	10	22	35	64	74	92	97	106	117	129	151
bLIF	√		√	√	√		√				
hLIF	√		√	√	√		√	√	√		
mLIF	√		√	√	√		√	√	√		
oLIF	√		√	√	√		√	√	√		
pLIF	√		√	√	√		√		√		
rLIF	√		√	√	√		√	√	√		√
sLIF		√	√	√	√	√	√	√	√	√	

It can be seen that residues 35, 64, 74 and 97 are strong potential glycosylation sites in all species including *S. crassicaudata*. (These sites are indicated in Fig. 4.36). It is also apparent that sLIF differs from all the eutherian LIF sequences in not having a strong site at 10, and in having extra sites at 22, 92 and 129.

The pI and molecular weight of LIF protein from seven species (Table 4.4) were estimated through SWISS-PROT, using Compute pI/Mw program. Protein pI was calculated using pK values of amino acids described in Bjellqvist *et al.* (1993, 1994),

and molecular weights were calculated by the addition of average isotopic masses of amino acids in the protein and the average isotopic mass of one water molecule.

The hydropathicity profile of the sLIF protein (Fig. 4.37) is similar to that of the LIF protein from mouse and human. Predictions of transmembrane regions and orientation of LIF proteins were conducted using the program TMpred (Hofmann and Stoffel, 1993). The four LIF proteins had similar transmembrane patterns (Fig.4.38). The 3D structure of sLIF was predicted using the computing program SWISS-MODEL (ProModII). This program predicts 3D structure based on comparisons with one or more template available in SWISS-PROT (a curated protein sequence database). The predicted 3D molecular structures of the sLIF and mLIF proteins were very similar (Fig. 4.39). It is concluded from above analyses that the *S. crassicaudata* LIF protein has similar structural elements and biochemical properties to eutherian LIF proteins.

Table 4.4 The theoretical pI and molecular weights of LIF proteins.

	pI	Molecular weight (kDa)
bLIF	9.37	21.43
hLIF	9.37	21.39
mLIF	9.30	21.67
oLIF	9.18	19.75
pLIF	9.51	19.75
rLIF	9.00	19.85
sLIF	9.49	22.12

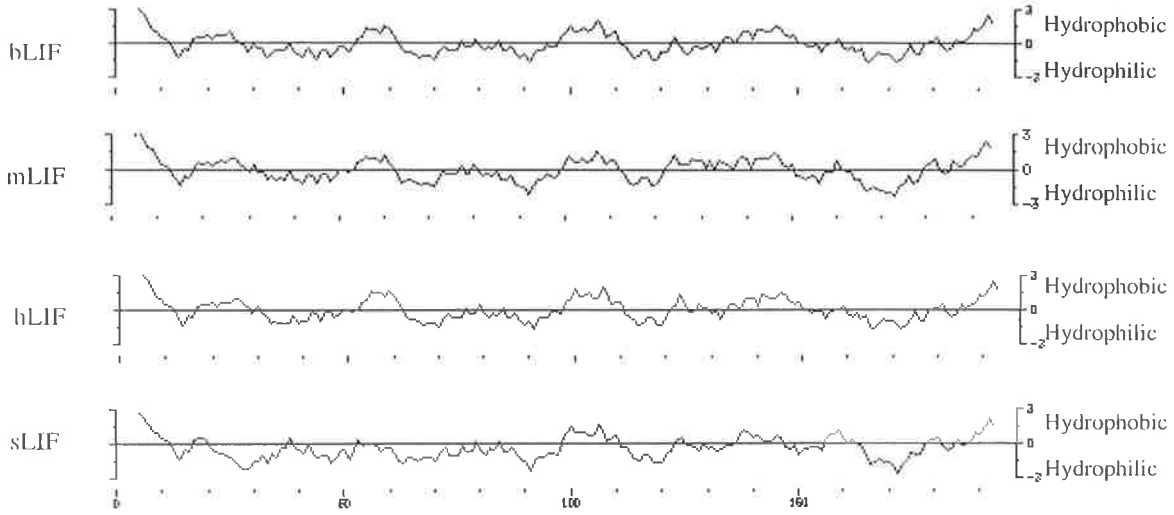
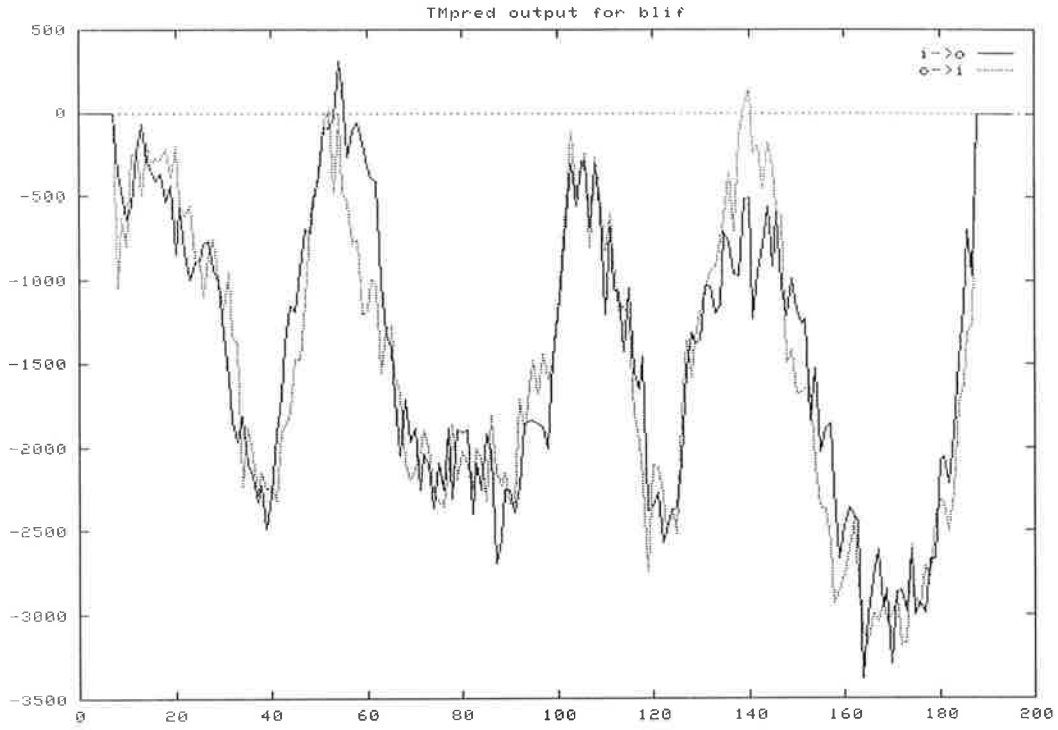
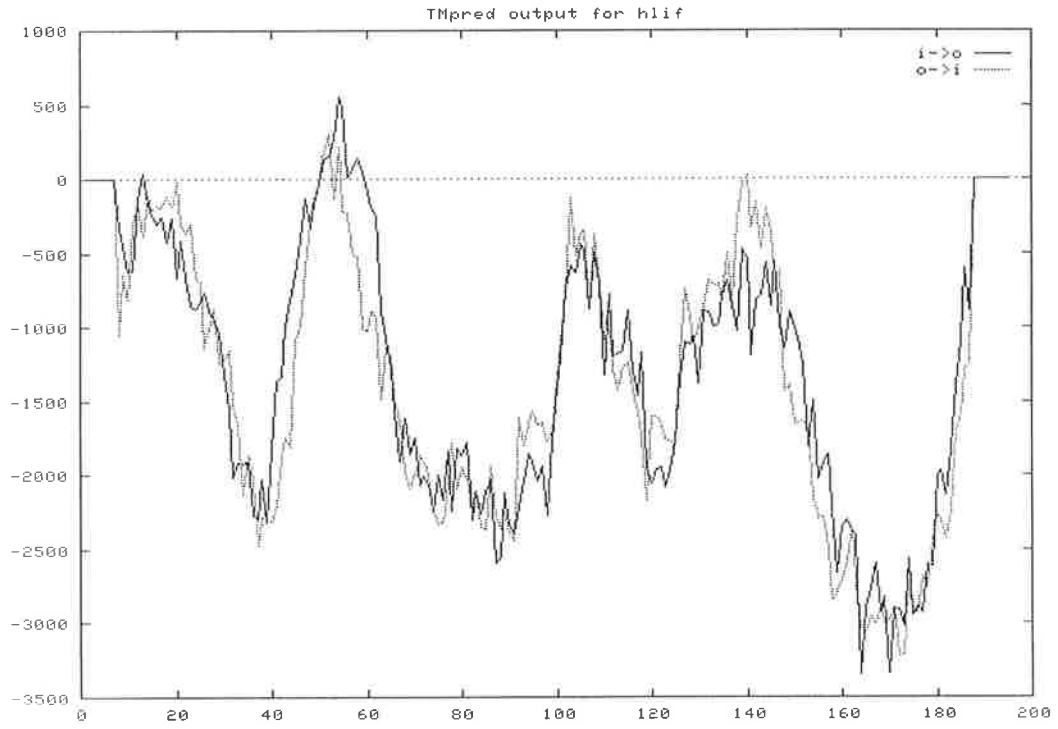


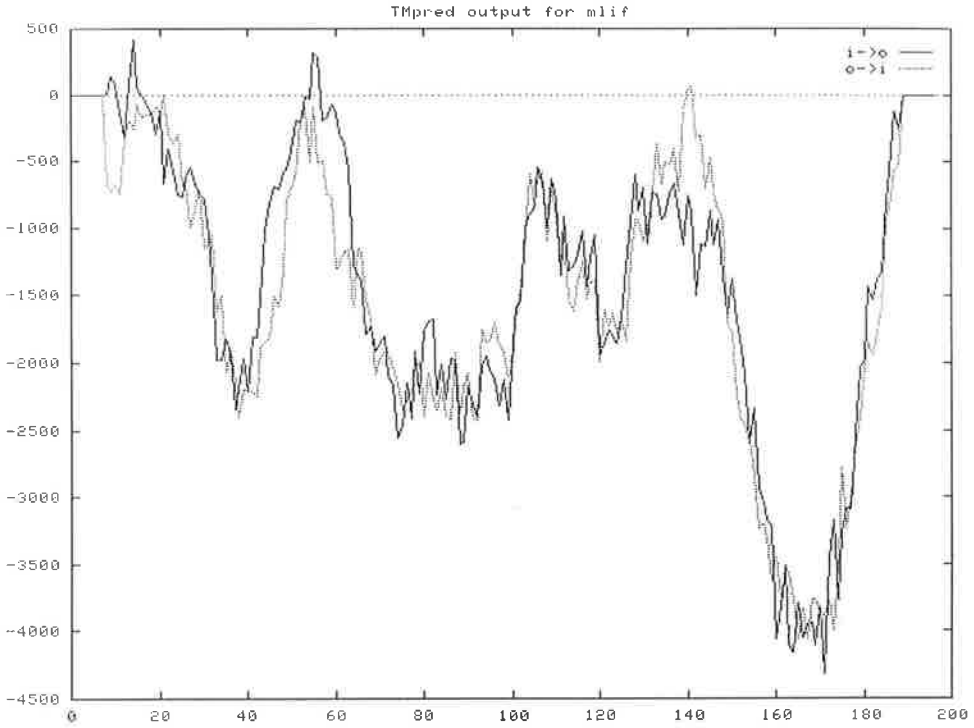
Fig. 4.37 Hydrophilicity calculated by the GCG software program PEPPLOT according to the method of Kyte and Doolittle (1982). Negative values indicate hydrophilic regions.


TMpred output for blif
i->o
o->i
500
0
-500
-1000
-1500
-2000
-2500
-3000
-3500
0
20
40
60
80
100
120
140
160
180
200

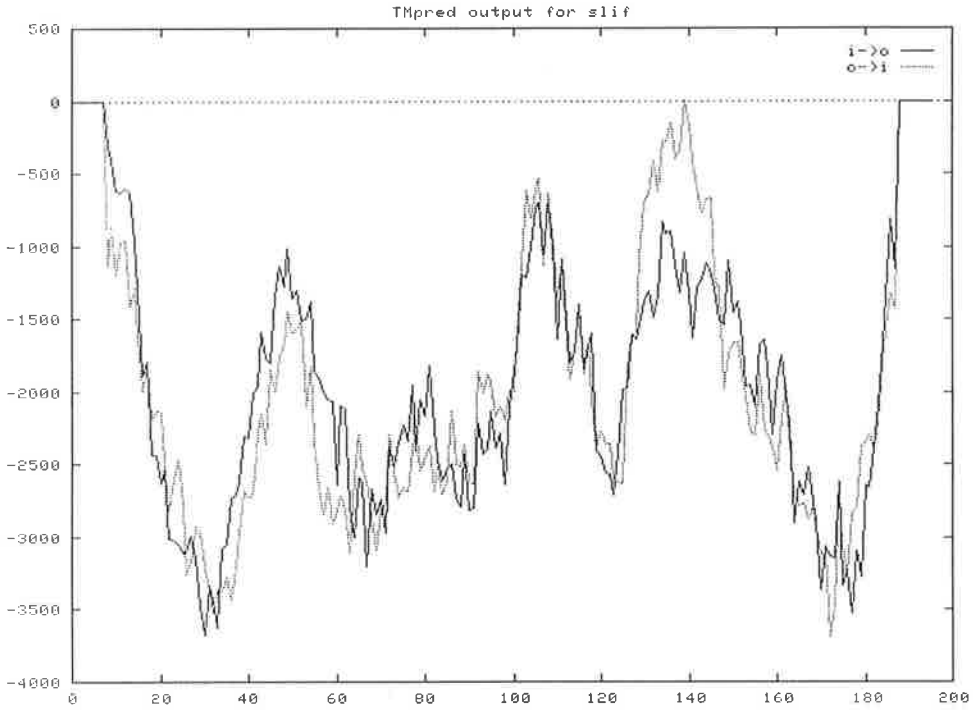
(a)


TMpred output for hlif
i->o
o->i
1000
500
0
-500
-1000
-1500
-2000
-2500
-3000
-3500
0
20
40
60
80
100
120
140
160
180
200

(b)



(c)



(d)

Fig. 4.38 Predicted transmembrane regions and orientation of LIF protein.
(a) bLIF, (b) hLIF, (c) mLIF and (d) sLIF.

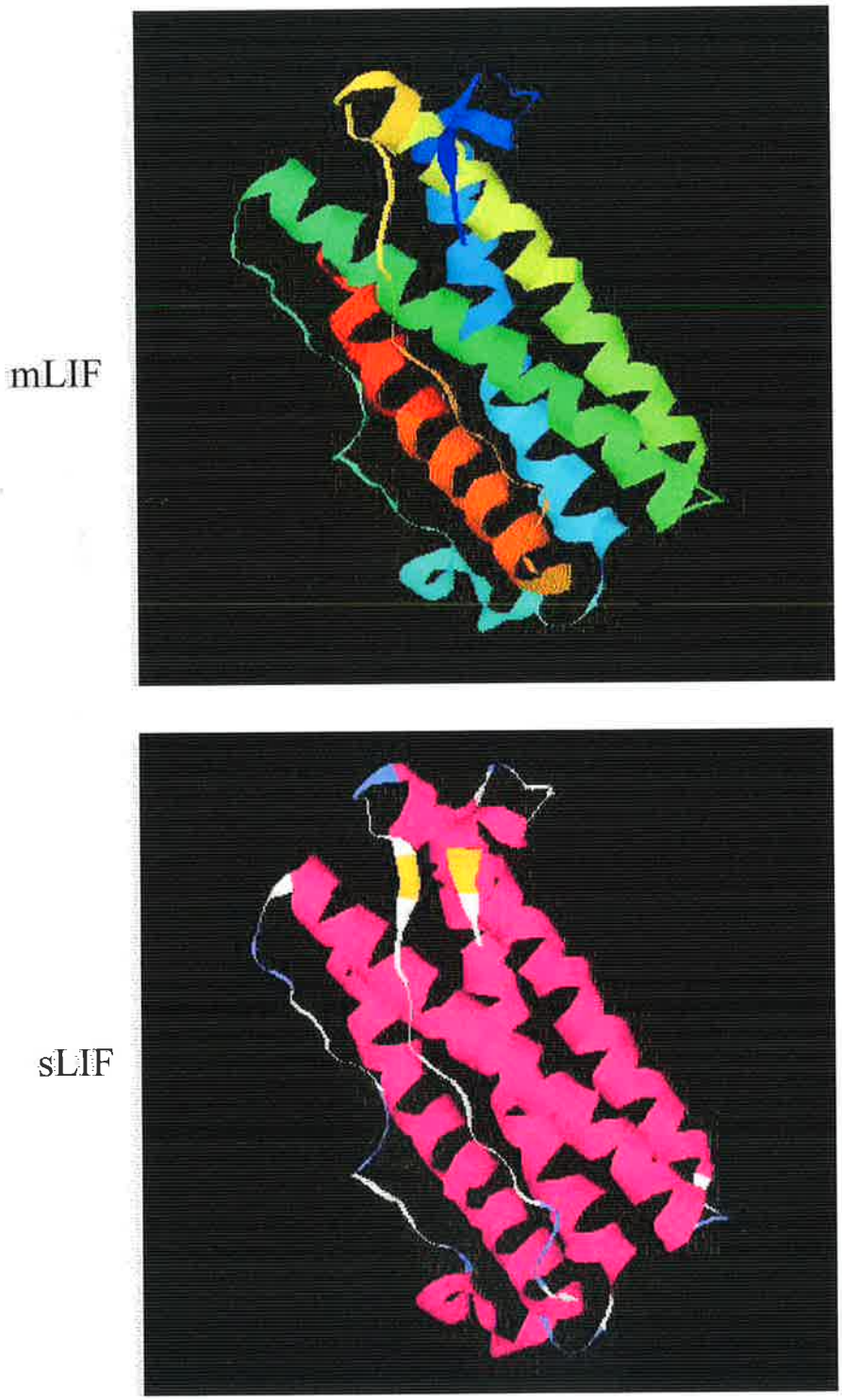


Fig. 4.39 3D structure of sLIF and mLIF viewed using RASMOL 3D viewer.

4.5 Evolutionary Analysis

4.5.1 Rates of nucleotide substitution.

The regions of DNA encoding the mature LIF protein in different species were compared, and the numbers of synonymous and non-synonymous nucleotide substitutions estimated (Table 4.5).

Table 4.5 Synonymous (above) and nonsynonymous (below) substitution values (%) for pairs of mammalian species.

	hLIF	*bLIF*	*mLIF*	*pLIF*	*oLIF*	*rLIF*	*sLIF*
hLIF		32.4	52.5	32.6	29.0	57.3	72.4
bLIF	6.8		77.2	32.3	39.8	81.2	79.7
mLIF	13.4	16.0		66.7	66.1	26.2	87.4
pLIF	7.9	8.8	15.3		42.6	72.8	82.6
oLIF	6.3	8.8	17.8	9.6		75.4	61.5
rLIF	10.5	14.4	5.4	13.2	14.3		96.9
sLIF	20.5	20.1	25.1	21.4	22.1	24.1	

4.5.2 Maximum parsimony analysis

Application of the algorithms DNAPARS, SEQBOOT and CONSENSE to the aligned coding regions of 7 mammalian *LIF* genes resulted in the tree shown in Fig. 4.40. The tree was rooted on the assumption that *LIF* from *S. crassicaudata* forms an appropriate outgroup, that is, the evolutionary branch point defining the separation of *S. crassicaudata* and the other mammalian *LIF* sequences is ancestral to all other branch points in the data set.

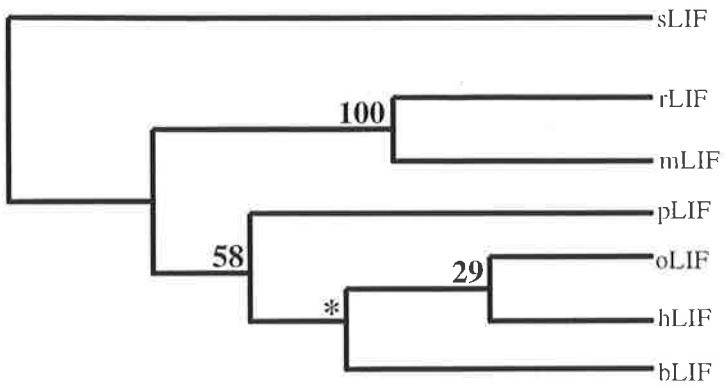


Fig. 4.40 Majority consensus bootstrap tree from a parsimony analyse of *LIF* coding regions. Numbers indicate percentage bootstrap values based on 1,000 pseudo-replications of the data. See Table 3.1 for species information. The asterisks (*) shows that this node was not resolved by bootstrap analysis. The tree is rooted with *sLIF*.

Aligned amino acid sequences of the mature LIF protein from 7 mammalian species were also analysed using PROTPARS; the results are shown in Figure 4.41.

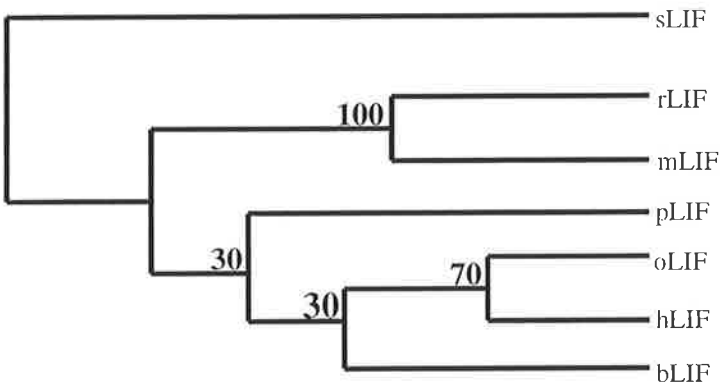
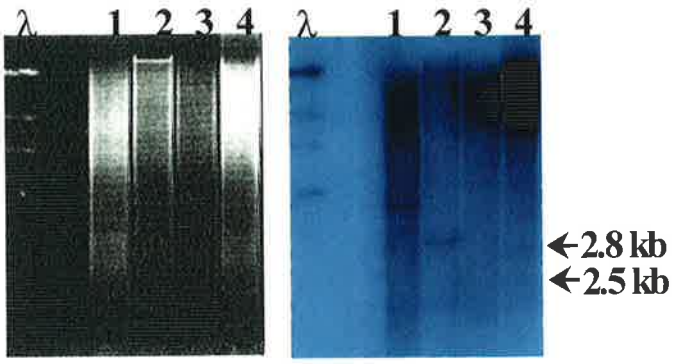


Fig. 4.41 Majority consensus bootstrap tree from a maximum parsimony analyse of mature LIF protein sequences. Numbers indicate percentage bootstrap values based on 1,000 pseudo replication of the data.

4.6 Southern Analysis

Southern analysis of genomic DNA from several eutherian species, using the mouse *LIF* cDNA clone *pLIF7.2b* as a probe, has shown that *LIF* is contained within a single

*Bam*HI fragment of size 3 kb in the mouse (Gearing *et al.*, 1987) and sheep (Willson *et al.*, 1992), and 2.6 kb in the pig (Willson *et al.*, 1992). Southern analysis of *Bam*HI digested *S. crassicaudata* genomic DNA was conducted, using the *Pst*I inserts of the human *LIF* clone HpGEM1 (*hLIF ORF*) and the *Eco*RI insert of the mouse *LIF* clone pDRI (*mLIF ORF*), as probes (Section 4.1.1). Both probes contain the entire open reading frame of the coding regions of *LIF*. High molecular weight genomic DNA (≈ 10 μg aliquots) from different mammalian species was completely digested with *Bam*HI, fractioned on 0.8% w/v agarose gel (Fig. 4.42a), transferred to nylon membrane Hybond-N^+, and hybridized with α-^{32}P-dATP labelled *hLIF ORF* probe (Section 3.2.5). The human *LIF ORF* probe detected a single *Bam*HI fragment in each species, with the exception of *S. crassicaudata* where two bands were observed (Fig. 4.42b). An identical result was obtained when murine *LIF ORF* probe was employed (data not shown).



(a) (b)

Fig. 4.42 Southern analysis of genomic DNA from different mammalian species. (a) Genomic DNA (≈ 10 μg) was digested with *Bam*HI and the fragments were separated by electrophoresis on a 0.8 % w/v agarose gel, viewed under UV light and transferred to Hybond-N+ membrane. (b) Autoradiograph of the membrane probed with human LIF cDNA containing the entire ORF of the coding regions for the mature LIF protein. *S. crassicaudata* DNA shows two bands as indicated by arrows. Tracks are: λ - λDNA/*Hind*III marker; 1 - Fox genomic DNA digested with *Bam*HI; 2 - Mouse genomic DNA digested with *Bam*HI; 3 - Rabbit genomic DNA digested with *Bam*HI; 4 - *S. crassicaudata* genomic DNA digested with *Bam*HI.

The hybridizing bands were very weak even though the filter was exposed to X-ray film for more than one month at -70°C. Hybridization was observed in a 4.0 kb *Bam*HI fragment in fox, a 3.0 kb fragment in mouse and a 1.6 kb fragment in rabbit.

In *S. crassicaudata*, two *Bam*HI fragments, 2.8kb and 2.5kb hybridized to both *hLIF ORF* and *mLIF ORF* probes, suggesting the presence of a *Bam*HI restriction site between exon 2 and exon 3. (This *Bam*HI site is presumed to be the site that was later shown to reside near the 5' end of exon 3 (see Fig. 4.28)).

Southern analysis of restricted DNA from different marsupial species failed to show hybridization to the human and mouse probes, even under conditions of low stringency (data not shown). Southern analysis using *sLIF* cDNA (*psLIF*) as probe was carried out under identical conditions to those described above. The results of probing genomic DNA from various marsupial species, and a species of monotreme, are shown in Figure 4.43.

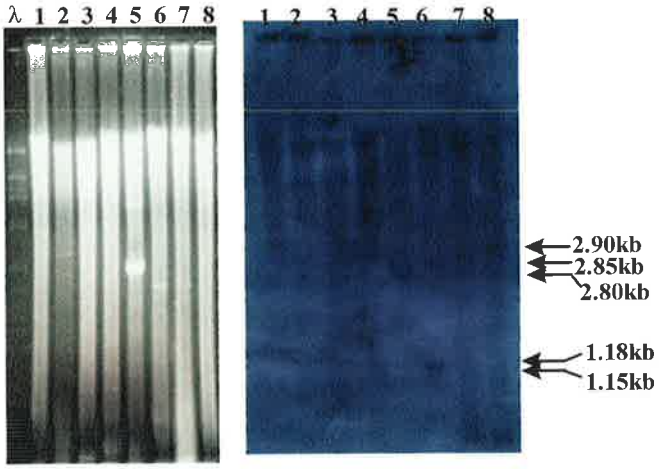


Fig. 4.43 Southern analysis of genomic DNA from different marsupial and monotreme species. (a) Genomic DNA (≈10 μg) was digested with the restriction enzyme *Bam*HI. Fragments were separated by electrophoresis on a 0.8 % w/v agarose gel and transferred to Hybond-N+ membrane. (b) Autoradiograph of the membrane probed with *S. crassicaudata LIF* cDNA (*psLIF*).

Track are: λ - λDNA/*Hind*III marker

1 - *Trichosurus vulpecula* (brush tailed possum)

2 -*Tachyglossus aculeatus* (echidna, a monotreme)

3 - *Macropus eugenii* (tammar wallaby)

4 – *Mus musculus* (mouse)

5 – *Macropus rufogriseus* (red necked wallaby)

6 - *S. crassicaudata* (fat-tailed dunnart)

7 - *Sminthopsis macroura* (stripe-faced dunnart)

8 – *Dasyurus viverrinus* (Tasmanian native cat)

Weak hybridization of *sLIF* to a 3.0 kb fragment was observed in track 4 (mouse DNA). Hybridizing DNA fragments were also detected in the DNA from marsupial species: 3.3 kb (brush-tailed possum), 4.0 kb (Kangaroo Island wallaby), 2.5 kb (red neck wallaby), and 2.9 kb (Tasmanian native cat). Of particular interest was the presence of a 2.7 kb fragment in the echidna. This important observation suggests the presence of a *LIF* gene in monotremes. (Before the work described in this thesis was undertaken, LIF had not been described in any non-eutherian mammal). Two bands were revealed in each of the *Sminthopsis* species, 2.8 kb, 1.18 kb in *S. crassicaudata* and 2.85 kb, 1.15 kb in *S. macroura*. The reason for the discrepancy between the *Bam*H1 fragment sizes (from S. *crassicaudata*) reported here, and those referred to earlier (see Fig. 4.42) is not known. It is possible that the *Bam*H1 restriction site is polymorphic in this species. These Southern results suggest that the *sLIF* cDNA probe could be used to further study *LIF* genes in other marsupial and monotreme species.

4.7 Investigation of *sLIF* Gene Expression *In Vivo*

4.7.1 Isolation of RNA from animal tissues

Total RNA was prepared as described in Section 3.2.7.2, using the TRIZOL™ RNA isolation reagent. The quantity and quality of RNA samples was determined by spectrophotometry and formaldehyde agarose gel electrophoresis. About 50 µg total RNA was recovered per 100 mg adult tissues.

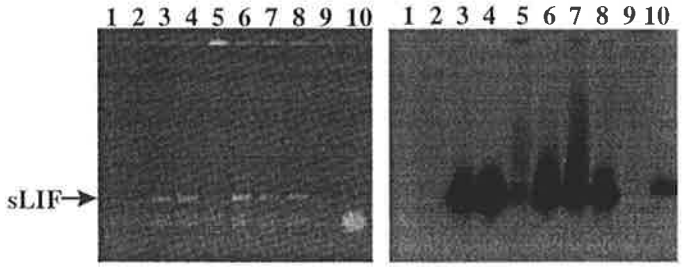
4.7.2 Northern hybridization of *S. crassicaudata* RNA with different *LIF* probes

Total RNA (15 µg) from brain, intestine, kidney, liver and uterus of the same animal was electrophoresed on a 1.4 % w/v formaldehyde agarose gel and Northern transferred to Hybond-N^+ membrane (Section 3.2.7.4). The membrane was probed with *Eco*RI insert of pDRI (*mLIF ORF*, Fig. 4.2a) or *Pst*I insert of HpGEM1 (*hLIF ORF*, Fig. 4.2b). Standard hybridization conditions were used (Section 3.2.2.3). Post-hybridization washes were performed initially at low stringency (twice in 2 X SSPE/0.1 % w/v SDS for 10 minutes at room temperature) and then at high stringency

(in 0.1 X SSPE/0.1 % w/v SDS for 10 minutes). No significant hybridization was detected using either probe. Similar experiments were repeated several times and in each case no evidence of specific hybridization was obtained. These filters (or new filters prepared as above) were stripped and re-probed with *psLIF* cDNA, and again they failed to display specific hybridization.

4.7.3 RT-PCR amplification of *sLIF* from total RNA of adult tissues

Total RNA samples were isolated from different adult tissues and the first-strand cDNA was synthesized using M-MLV reverse transcriptase and oligo (dT) $_{12\text{-}18}$ or random hexamer as described in Section 4.2.4.1. The first-strand cDNA was used as a template for PCR amplification. Two pairs of *sLIF* gene-specific primers were designed for amplifying the 180 bp cDNA fragment of exon 2 and the 411 bp fragment of exon 3 of the mature sLIF protein. The forward primer designed from 5' end of exon 2 (5'- TAGGATCCCCCCTACCCATCAC-3') and the reverse primer designed from 3' end of exon 3 (5'-TGGATCCTAGAAGGCCTGGGCCACCA-3') were expected to amplify the whole coding region for the mature LIF protein. PCR reactions were performed either as described in Section 3.2.5.11, or as recommended by the commercial manufacturer (GIBCO BRL). RT-PCR products were analyzed by agarose gel electrophoresis, transferred to a nylon membrane (*Amersham*) and hybridized with *psLIF* cDNA (Fig. 4.44).



(a) (b)

Fig. 4.44 *S. crassicaudata LIF* mRNA detected by RT-PCR using *sLIF* specific primers.

(a) A 555bp cDNA fragment amplified from different adult tissues electrophoresed on agarose gel.

(b) Autoradiograph of RT-PCR bands hybridized with α-^{32}P-dATP labelled *sLIF* probe.

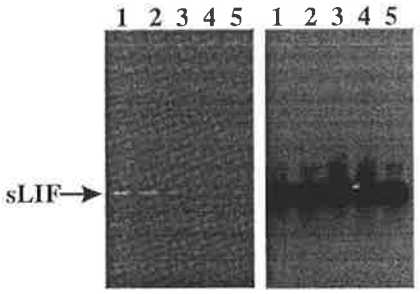
Tracks are: 1- diH_2O negative control
2 - Spleen RNA
3 - Spleen cDNA
4 - Lung cDNA
5 - Liver cDNA
6 - Intestine cDNA
7 - Heart cDNA
8 - Footpad cDNA
9 - Kidney cDNA
10 - Uterus cDNA

A DNA fragment of the predicted size (555 bp) was amplified by RT-PCR from each of the following adult tissues: spleen, lung, liver, intestine, heart, footpad (Fig. 4.44a). This result suggests that LIF is expressed in these tissues at least at a low level. These same adult tissues have been reported to express *LIF* mRNA in other species (Bhatt *et al.*, 1991; Robertson *et al.*, 1993). *S. crassicaudata* brain and ear tip tissues also express *LIF* mRNA (Fig. 4.29b). When the RT-PCR products were Southern transferred to nylon membrane and probed with *psLIF* cDNA labelled with α-^{32}P-dATP, hybridization signals were revealed (Fig. 4.44b). The cDNA fragment amplified from liver gave a weak signal on the autoradiograph, suggesting *LIF* expression in liver tissue may be at low level. Even the relatively weak cDNA band from uterus tissue detected by staining with EtBr, hybridized strongly to the probe, indicating the expression of *LIF* in adult uteri.

4.7.4 RT-PCR amplification of *sLIF* transcripts from uterus/embryo tissue derived during embryogenesis

Jiang *et al.* (1995) and Dakhama *et al.* (1996) have studied *LIF* expression during embryogenesis in mouse and human using RT-PCR on material recovered from tissue sections of the uterus/embryo complex. In the present study, tissue sections made from *S. crassicaudata* were similarly used to investigate *LIF* expression. The tissue sections, which had been prepared for use in a study of embryological development, were kindly provided by Dr. Claire Roberts, Department of Obstetrics and Gynaecology, University of Adelaide.

The intact RNA from paraffin-embedded tissue sections was isolated (see Section 3.2.9.1) and the RNA samples were used as templates for RT-PCR amplification as described in Section 3.2.9.3. Figure 4.45a shows the RT-PCR products amplified using the same primers as those used for the adult tissues.



(a) (b)

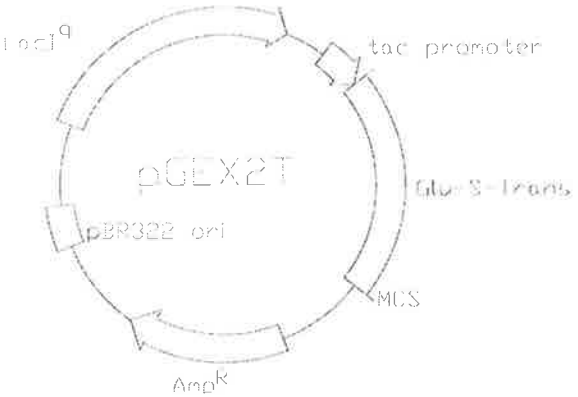
Fig. 4.45 *S. crassicaudata LIF* mRNA detected by RT-PCR and electrophoresed on agarose gel. (a) A 555bp cDNA fragment. (b) Southern blot of RT-PCR bands hybridized with α-^{32}P-dATP labelled *sLIF* probe.

Track are: 1 - *sLIF* mRNA RT-PCR products from unilaminar blastocysts
2 - *sLIF* mRNA RT-PCR products from pre-somite embryo time
3 - *sLIF* mRNA RT-PCR products from late pregnancy
4 - *sLIF* mRNA RT-PCR products from later pregnancy

A unique band of the size expected for *sLIF* cDNA was obtained from each of the tissue section (Fig. 4.45a), and in each case the band strongly hybridized to *psLIF* cDNA probe (Fig.4.45b). The presence of *LIF* transcripts is not confined specifically to the time of implantation of the embryo, but occurs throughout the early stages of embryogenesis, suggesting a possible role for LIF in early embryogenesis in marsupials.

4.8 *In Vitro* Expression of *sLIF*

To investigate possible conservation of LIF function between marsupial and eutherian mammals, *sLIF* cDNA was cloned in an expression vector and the mature LIF protein was produced and purified and its properties examined *in vitro*. The expression vector used was pGEX2T (Fig. 4.46). The C-terminus of *sLIF* is in-frame if sLIF is inserted into the *Bam*HI site of the vector (Fig. 4.46).



Site of thrombin digestion

Leu Val Pro Arg Gly Ser Pro Gly Ile His Arg

5'-CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG ACG-3'

*Bam*HI *Sma*I *Eco*RI Stop codons

Fig. 4.46 The multiple cloning site (MCS) and other major features of the glutathione *S*-transferase fusion vector pGEX2T.

4.8.1 Construction of a GST-sLIF expression plasmid

Before insertion into the *Bam*HI site of the cloning vector, the 555 bp cDNA fragment amplified by RT-PCR from *S. crassicaudata*, had to be modified to remove the *Bam*HI restriction site it contained. This was achieved by nucleotide substitution without reading frame alteration. The strategy used is illustrated in Figure 4.30B.

pGEX2T vector DNA was linearized by digestion with *Bam*HI, and the dephosphorylated vector DNA fragments were ligated to *Bam*HI digested *sLIF* cDNA, forming a plasmid construct *pGEX2T-sLIF* (546 bp) (Fig. 4.47). The ligated plasmid DNA was transformed into JM109 competent cells. Transformants were screened on ampicillin selective plates and single colonies isolated from the culture plate were grown in ampicillin complemented LB medium.

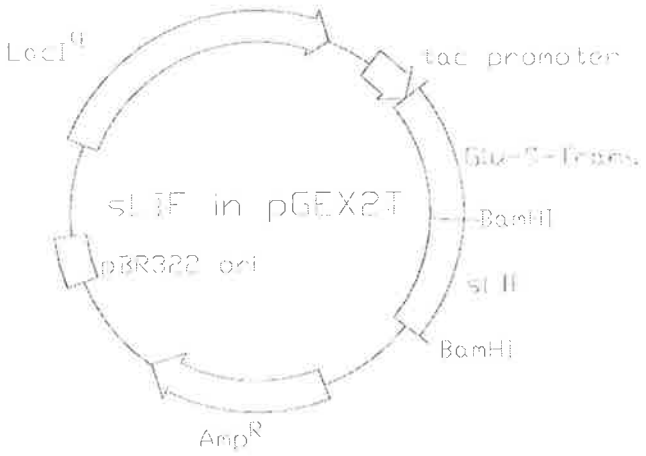


Fig. 4.47 The recombinant plasmid *pGEX2T-sLIF* used for bacterial expression of *sLIF*. A 546bp *sLIF* fragment was cloned into the *Bam*HI site of pGEX2T. *S. crassicaudata* LIF, expressed as a fusion protein with GST, was induced by IPTG through the *tac* promoter.

Plasmid DNA was prepared from overnight cultures of these single colonies, digested with *Bam*HI and analysed by agarose gel electrophoresis. No *sLIF* inserts of the expected size were observed. The bacterial colonies on LB-agar selective plates was

blotted to nylon membrane, Hybond-N^+ and probed with the *Hind*III insert of *psLIF1.2.4* (which contains *sLIF* exon 3 coding region). Positive colonies were displayed on an X-ray film (Fig. 4.48).

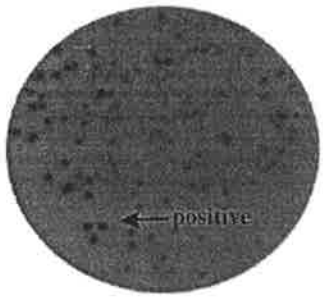


Fig. 4.48 Hybridization analysis of colony-lifts of the *pGEX-sLIF* transformants. Colonies were blotted onto nylon membrane and probed with α-^{32}P-dATP labelled *Hind*III insert of *psLIF1.2.4*.

Several positive colonies were picked and grown in ampicillin-containing liquid LB medium, from which plasmid DNA samples were prepared, digested with *Bam*HI and electrophoresed on agarose. Thirteen samples were shown to have inserts of about 0.5 kb and they were analysed by sequence determination. Three sub-clones were found to contain *sLIF* in the correct reading frame and one of these sub-clones, named *pGEX2T-sLIF* (Fig. 4.47) was chosen for further analysis.

Using a 5' pGEX2T sequencing primer (5'-TGGACCCAATGTGC-3') at position 765 of the vector (ie. about 150 bp upstream of the *Bam*HI cloning site), the cloning junctions between the GST and *sLIF* cDNA of *pGEX2T-sLIF* were sequenced to verify that the *sLIF* cDNA fragment had been inserted in the correct reading frame. Nucleotide sequence determination revealed that *sLIF* cDNA had been inserted in-frame with the GST coding region (Fig. 4. 49).

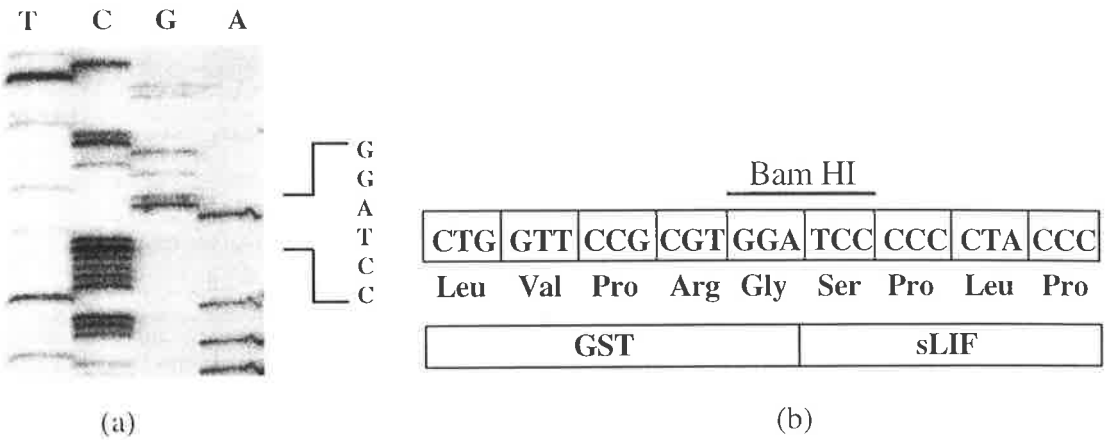


Fig. 4.49 Nucleotide sequence analysis of GST-*sLIF* cloning junction in the *pGEX2T-sLIF* clone. (a) Sequence analysis of the *pGEX2T-sLIF* cloning junction. The DNA sequence of the *Bam*HI site is displayed, indicating that the sLIF insert is in-frame with the GST open reading frame. (b) Schematic representation of the GST-*sLIF* cloning junction.

4.8.2 Expression of the GST-sLIF fusion protein

pGEX2T-sLIF was transformed into competent *E.coli* bacterial cells (BL21) (this strain was recommended for the expression of GST fusion proteins since it is protease deficient (Studier *et al.*, 1990)) and the expression of GST-fusion protein was induced by IPTG.

A single colony from ampicillin selective plate was used to inoculate 25 ml of LB medium and, after incubation overnight at 37°C, the culture was diluted in ampicillin and IPTG-enriched (0.4 mM) LB medium to a final concentration of 1.0 % v/v. The cells grown at 37°C for a further 3.5 hours. Proteins were prepared from the culture as described in Section 3.2.10.6 and separated on 12 % w/v SDS-PAGE gel (Fig. 4.50).

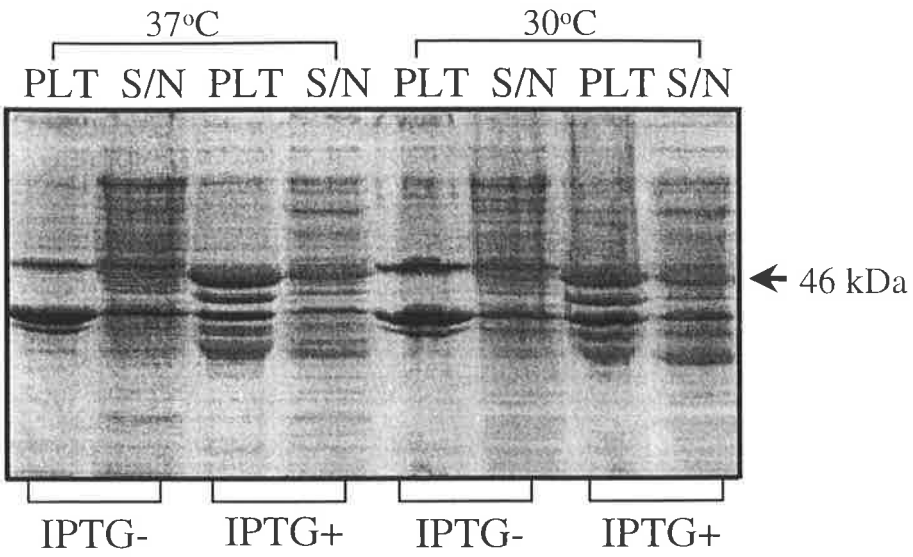


Fig. 4.50 Induction of GST-sLIF fusion protein. 8µl of the IPTG induced and non-induced total protein samples from crude cell lysates were electrophoresed on 12% w/v discontinuous SDS-PAGE. The gel was stained with Coomasie brilliant blue. S/N = supernatant, PLT= pellet.

Comparison of the non-induced samples with induced samples indicates that induction resulted in over-expression of a protein of Mr 46,000. The quantity of the induced protein increased as the incubation time and concentration of the inducer (IPTG) was increased. A comparison of supernatant and pellet of the lysates revealed that most of the induced proteins were present in the post-sonication pellet, indicating a low solubility of the fusion protein.

4.8.3 Solubility of GST-sLIF fusion protein

Low concentration of soluble protein described above could have been caused by insufficient sonication. However, various intensities and times of sonication and the addition of lysozyme to cell lysate, had no effect on solubility (data not shown).

The original description of the pGEX system suggested that most GST fusion proteins would be soluble (Smith and Johnson, 1988). In practice, over-expression of a

number of proteins in bacterial cells results in the formation of large, insoluble protein aggregates known as inclusion bodies (Schein, 1989) and many GST fusion proteins, even those of relatively low molecular weights (40-50 kDa), are partially or completely insoluble after lysis in nonionic detergents (Frangioni and Neel, 1993). Proteins from inclusion bodies can be solubilized using a variety of treatments (Schein, 1990; Marston, 1986; Gentry and Burgess, 1990; Frangioni and Neel, 1993). The protein samples prepared in these ways must be properly re-folded to regain biological function. Fusion protein solubility can also be archived by altering the bacterial growth conditions (Schein, 1989; Schein and Noteborn, 1988). Solubility may be dramatically increased by lowing the culture temperature, decreasing inducer IPTG concentration to <0.1 mM, changing the time of induction and increasing aeration. A combination of the above approaches is sometimes needed to obtain high percentage yields of soluble fusion protein.

Various methods were used to improve the solubility of the LIF fusion protein. Therefore, the BL21 bacterial cells transformed with *pGEX2T-sLIF* were grown overnight at 37°C in LB medium containing ampicillin (100 µg/ml) with agitation. The overnight culture was then diluted (1:5) in pre-warmed (30°C) fresh LB medium containing 100 µg/ml ampicillin and 0.1 mM IPTG. The diluted culture was incubated at 30°C for 3 hours with agitation and the GST fusion proteins were prepared (Section 3.2.10.6) and examined using 12 % (w/v) SDS-PAGE (Fig. 4.51). The fusion proteins induced with 0.4 mM IPTG at 30°C showed no significant difference in solubility from those prepared at 37°C (Fig. 4.50), most of the fusion proteins were retained in the post-sonicate pellet, indicating that the solubility of GST-sLIF proteins can not be improved by lowing incubating temperature only. When the fusion protein was induced with 0.1 mM IPTG, the 37°C-incubated culture did not release the fusion protein from the inclusion bodies into the supernatant. However, lowering the incubation temperature of the culture to 30°C did affect the solubility of the fusion protein. Comparison of pellet and supernatant lanes of the 30°C culture (Figure 4.51) shows that about 70 % of the fusion protein was released from the pellet into the supernatant. Therefore, induction of bacterial cells at 30°C with 0.1 mM of IPTG resulted in the release of the fusion protein from inclusion bodies.

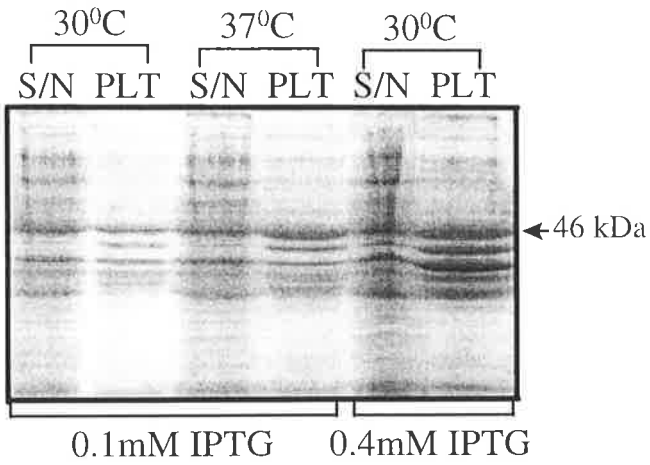


Fig. 4.51 Optimization of the conditions for fusion protein production. 12% w/v SDS-PAGE gel stained with Coomasie brilliant blue. GST fusion protein samples induced with different concentration of IPTG at 37°C and 30°C. S/N = supernatant, PLT= pellet.

4.8.4 *In vitro* solubilization of fusion protein using non-ionic detergent

In an attempt to improve the yield of soluble fusion protein, a procedure described by Frangioni and Neel (1993) was trialed. Bacterial strain BL21 cells transformed with *pGEX2T-sLIF*, were grown as described previously and fusion protein expression was induced with 0.1 mM IPTG for 4 hours at 37°C. The cells were pelleted and washed once with ice-cold STE (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) and resuspended in STE solution with 100 µg/ml lysozyme followed by incubation on ice. Bacteria were lysed by the addition of N-laurylsarcosine to the final concentration of 1.5 % w/v and sonicated for 1 minute. The lysate was clarified by centrifugation and the supernatant was transferred to a centrifugation tube followed by the addition of Triton X-100 to a final concentration of 2% v/v. Comparison of pellet and supernatant lanes of the fusion protein samples on the gel (Figure 4.52) showed that more than 95% of the fusion protein was released from the pellet into the supernatant.

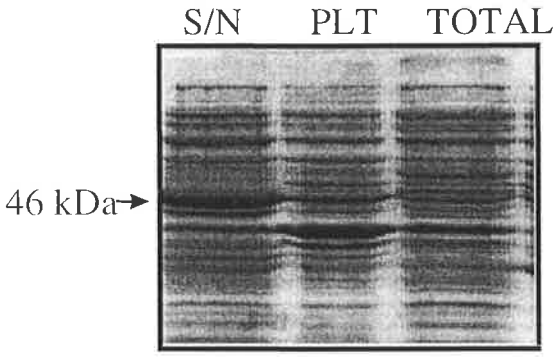


Fig. 4.52 12% w/v SDS-PAGE gel stained with Coomasie brilliant blue. GST fusion protein samples were induced with 0.1mM IPTG at 37^{0}C and prepared using sarkosyl. S/N = supernatant, PLT = pellet, TOTAL = uninduced cell lysate denatured by boiling.

4.8.5 Affinity chromatographic purification of recombinant GST-sLIF

Large scale preparations of the soluble GST-sLIF protein were carried out using Glutathione Sepharose® 4B (*Pharmacia Biotech*), a reagent designed specially for rapid single step purification of recombinant derivatives of glutathione S-transferase (Section 4.8.4). Fusion proteins were eluted from glutathione Sepharose 4B by competition with the reduce form of glutathione. Mild, non-denaturing conditions were used to preserve protein antigenicity and functionality. Rapid purification of pGEX-derived sLIF from GST-sLIF fusion proteins was conducted using the method described in Section 3.2.11. sLIF protein samples were analysed by SDS-PAGE (Fig. 4.53).

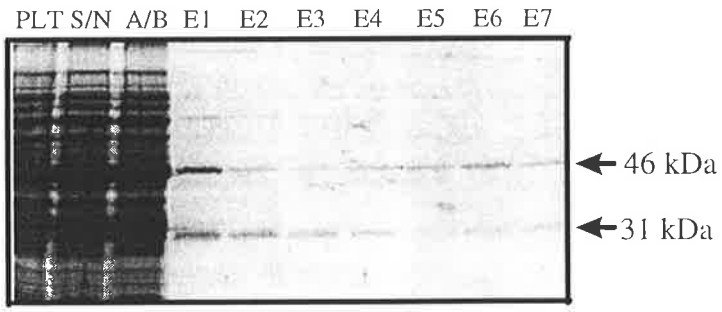


Fig. 4.53 Purification of GST-sLIF fusion proteins. 10µl of each sample was analysed on 12% w/v SDS-PAGE. PLT = pellet, S/N = supernatant, A/B = sample of S/N after binding, E1-E7 = elute 1 to elute 7.

Comparisons of the first three tracks in Figure 4.53 (pellet, supernatant and total protein after binding to the glutathione Sepharose 4B) show that a considerable amount of the fusion protein was retained in the solution after mixing with glutathione Sepharose 4B beads, even though incubation at room temperature was for a longer time than that recommended by the manufacturers. This result suggests the binding of GST-sLIF fusion protein to glutathione Sepharose 4B had reached saturation.

Two proteins (46 kDa and 31 kDa) from each of the GST fusion protein samples, were displayed on SDS-PAGE (Fig. 4.58, E1-E7). The 31 kDa protein is assumed to be a truncated form of the fusion protein with deleted residues at its C-terminus. The higher molecular weight protein was assumed to present full length purified GST-sLIF protein. The truncation could result from the isolation and purification procedures. For example, one or more of the ingredient used in these steps could possibly disrupt covalent bonds.

Purified fusion protein samples were digested with thrombin and the resulting material was analysed on 12 % w/v SDS-PAGE (Fig.4.54). Purified fusion proteins consisted of both full-length and truncated forms (sizes 46 Kda and 31 Kda respectively). The truncated form of the protein was in excess. On treatment with thrombin, these two forms of the fusion protein generated GST (26 Kda) peptides, releasing the 6 Kda C-terminal part (Fig. 4.54) which was too small to be seen on the gel. It was concluded that truncation occurred at the C-terminus of the fusion protein.

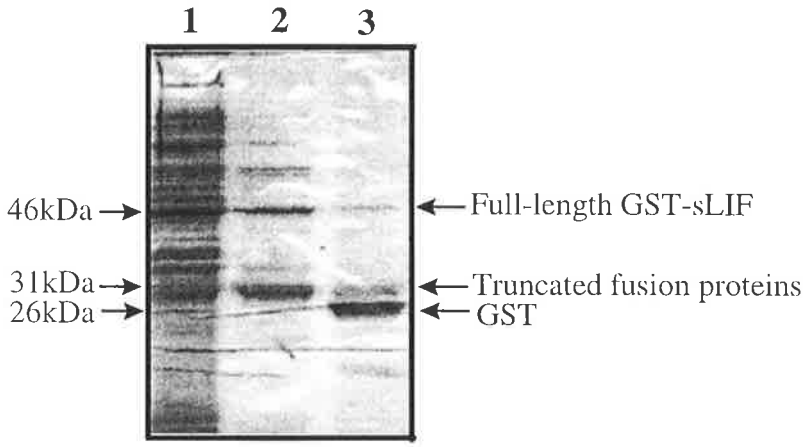


Fig. 4.54 Protease digestion analysis of purified GST-sLIF fusion proteins. Samples were electrophoresed on 12 % w/v PAGE gel and stained with Coomasie brilliant blue.

Lane 1 - Supernatant of the cell lysates

Lane 2 - Purified GST-sLIF fusion protein without digestion

Lane 3 - Purified GST-sLIF fusion protein digested with thrombin

To reduce the truncation of fusion protein, lysozyme was added to the cell suspension in order to reduce the amount of sonication required, and protease inhibitor (PMSF) was added to suppress any possible endogenous protease degradation due to proteases. Non-ionic detergent (Triton X-100) and DTT were used to solubilize the protein. It was found that higher concentration of Triton X-100 significantly reduced truncation (Fig. 4. 55).

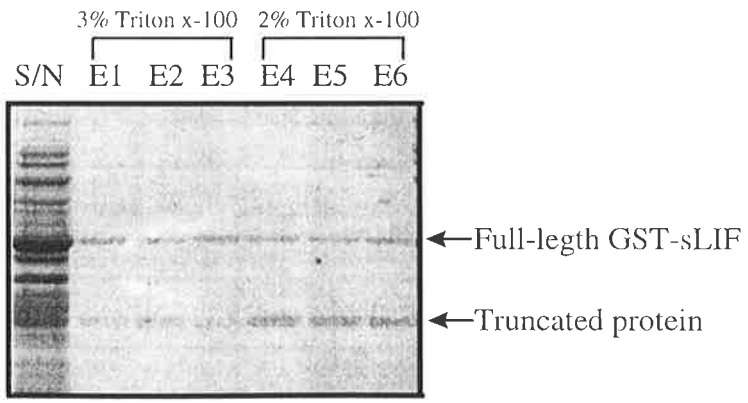


Fig. 4.55 Elution of the GST-sLIF fusion protein by modified conditions. Cell lysate containing 3 % v/v Triton X-100 in the cleavage/storage solution results in less truncated fusion proteins than 2 % Triton X-100 solution. S/N = supernatant, E1-E6 = elute 1 to elute 6

4.8.6 Purification of recombinant sLIF polypeptides

The pGEX2T expression vector is designed to express GST-insert fusion proteins that can be separated by thrombin cleavage (Fig. 4.46). Thrombin digestion was carried out at room temperature for >3 hours (normally overnight) on a rotating shaker. Thrombin was added to the column eluate to a concentration of 0.4 cleavage units per millilitre eluate. The thrombin digested crude bacterial cell lysates were shown to contained the expected 26kDa and a 20kDa portions on the PAGE (Fig. 4.56).

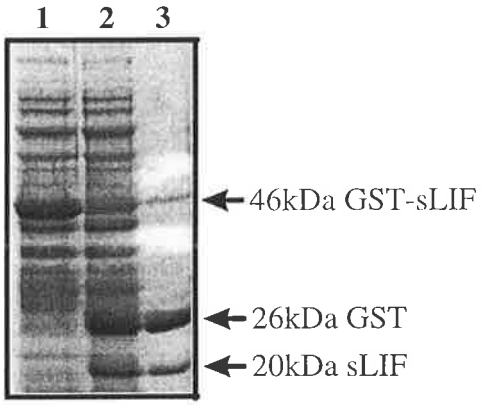


Fig. 4.56 Results of proteolytic digestion of over-expressed GST-sLIF fusion protein. The crude bacterial cell lysates and glutathione Sepharose 4B purified GST-sLIF protein were digested with thrombin and electrophoresed on 12 % w/v SDS-PAGE gel.

Lane 1 - Total protein of crude lysates

Lane 2 - Total protein of crude lysates digested with thrombin

Lane 3 - Purified GST-sLIF fusion protein digested with thrombin

Comparison of tracks 1 and 2 (Fig. 4.56) show that an over-expressed 46 kDa protein (lane 1) was removed by thrombin digestion and generated two polypeptides, 26 kDa (Glutathione S-transferase) and 20 kDa (lane 2, *S. crassicaudata* LIF). The purified fusion proteins digested with thrombin (lane 3) contained two fragments identical to those in lane 2.

The initial procedure for the pGEX expression and purification of expressed protein involved several steps, including the preparation of crude bacterial lysates, purification on a glutathione-agarose or glutathione Sepharose 4B affinity matrix,

elution of the bound protein from the affinity matrix, protease digestion and separation of the fusion protein and recovery of the interest C-terminal portion. The eluted fusion proteins were cleaved with thrombin and the C-terminal moiety was recovered by removing the glutathione competitor by dialysis and re-absorbing the N-terminal GST portion (26 KDa) to an affinity matrix, leaving the C-terminal protein in the solution (Smith and Johnson, 1988). This procedure was later modified and simplified by carrying out thrombin digestion directly on the initial affinity matrix, thus releasing the C-terminal portion into the supernatant (Gearing *et al.*, 1989). Cleavage using thrombin proved to be efficient, and sLIF was released by application of this simplified procedure. The result is shown in Figure 4.57.

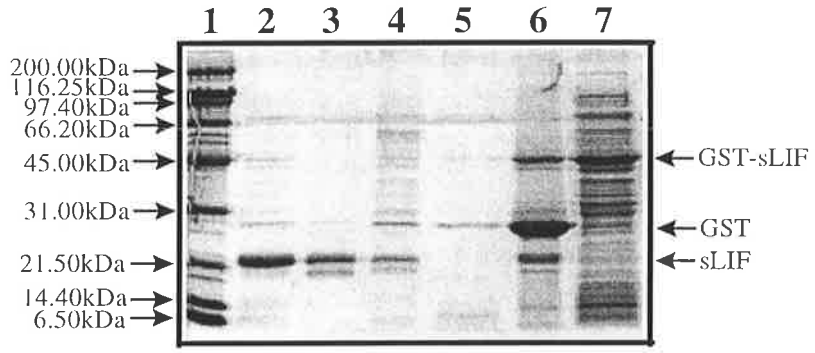


Fig. 4.57 Direct release of recombinant sLIF protein from fusion protein bound to glutathione Sepharose 4B beads by thrombin digestion. Coomasie brilliant blue stained 15 % w/v SDS-PAGE gel of 10µl samples taken from the different stages of the purification as indicated.

1 = Molecular weight markers (sizes indicated next to arrows)
2 = First 2ml mLIF-released supernatant sample
3 = First 2ml sLIF-released supernatant sample
4 = Second 2ml sLIF-released supernatant sample
5 = Third 2ml sLIF-released supernatant sample
6 = Eluted GST sample
7 = Post-sonication supernatant sample

A bacterial cell lysate was prepared as described in Section 3.2.11.7 using a incubation volume (1 litre) of LB medium. Cell lysate (50 ml) was incubated with 2

ml of glutathione Sepharose 4B beads at room temperature for at least 30 minutes to allow the fusion protein to bind to the beads. This was followed by series washes using 10 X bed volumes of TTBS and TBS buffers. After the washes, the glutathione Sepharose 4B beads with the fusion protein bound were diluted in 2 ml of 1 X TBS buffer, and thrombin solution was added to a final concentration of 3 cleavage units per millilitre volume. The mixture was incubated overnight at room temperature with low speed shaking, followed either by centrifugation at low speed (500 X g) or by column chromatography to collect the sLIF protein. Most of the released sLIF protein came through in the first three washes and, as observed on the gel, more and more undigested fusion protein come off, although there was still a small amount of sLIF protein trapped in the matrix (lane 6, Fig. 4.57). The protein samples were concentrated using a *Centricon* centrifugal concentrator (Centricon-3, *Amicon, Inc.*). These LIF protein preparations of sLIF were available for use in a variety of biological assays, since they were prepared under non-denaturing conditions.

4.9 Bioassay of *S. crassicaudata* Recombinant LIF Using Murine ES Cell Cultures

(This part of the work was carried out in collaboration with Professor Peter Rathjen and Dr. Joy Rathjen, Department of Biochemistry, University of Adelaide)

LIF proteins expressed and purified from mouse, human and sheep have been demonstrated to prevent the differentiation of murine ES cells *in vitro* (Gearing *et al.*, 1987; Smith *et al.*, 1988;Williams *et al.*, 1988; Gearing *et al.*, 1989; Piquet-Pellorce *et al.*, 1994). High affinity LIF receptors have been found on ES cells (Gough and Williams, 1989) and the addition of recombinant LIF proteins to the cell culture medium can substitute for feeder cells and allow the long-term maintenance of undifferentiated ES cells.

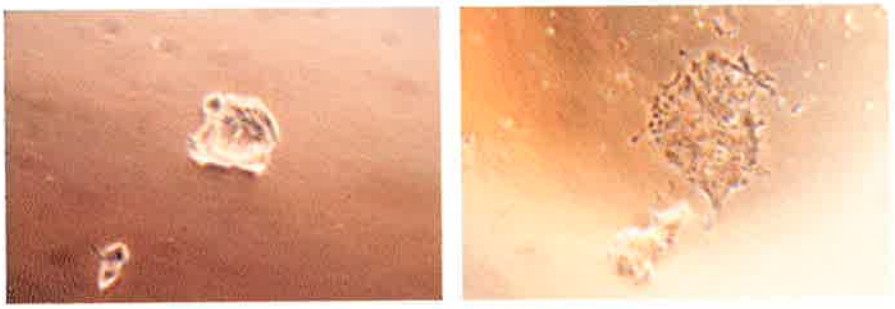
E14 murine embryonic stem cells were routinely maintained in culture in the presence of 1,000 u/ml mLIF (Willaims *et al.*, 1988). These cells were washed and plated at low density (2.8 x 10^2 cells/cm^2) in the presence of recombinant sLIF and mLIF (Section 3.2.11.13). After fixing and staining with alkaline dye mixture (dilute 2 ml of

diazonium salt solution mix of 1 ml sodium nitrite, *Sigma No. 91-4*, and 1 ml FRV-alkaline, *Sigma No. 82-1*, with 1ml Naphthol AS-Bl solution, *Sigma No.86-1*), undifferentiated cells, which express alkaline phosphatase, stain red, and can be distinguished from the unstained differentiated cells. Wells containing dilutions of recombinant mLIF and sLIF were compared to ES cells seeded into medium containing either no LIF or 1,000 u/ml commercially supplied mLIF (ESGRO: AMRAD) (Fig. 4.58).

Initial trials indicated that concentrations of sLIF in the range 100 ng/ml to 0.1 ng/ml showed complete inhibition of ES cell differentiation. Therefore, further trails were carried out using concentrations of LIF in the range of 1 ng/ml to 0.0008 ng/ml. The results (Table 4.6 and Fig. 4.59) show that the effects of mLIF and sLIF are very similar. Cytokines from both species inhibited the differentiation of about 50% of ES cells at a concentration of 0.02 ng/ml. Thus sLIF and mLIF are comparable in activity in terms of their effect on ES cell differentiation, implying a high degree cross-species identities of LIF receptors and/or receptor components.

Table 4.6 The effect of varying concentrations of recombinant mLIF and sLIF on the differentiation of murine ES cells. The commencing concentration was 1 ng / ml. The degree of inhibition of differentiation is indicated by: + = complete inhibition, +/- = partial inhibition, - = lack of inhibition.

Number of doubling dilutions of LIF	0	1	2	3	4	5	6	7	8	9	10	11
Approx. LIF concentration (ng/ml)	1	.5	.25	.125	.063	.031	.016	.008	.004	.002	.001	.0005
mLIF	+	+	+	+	+	+/-	+/-	+/-	-	-	-	-
sLIF	+	+	+	+	+	+/-	+/-	+/-	-	-	-	-



(a) (b)

Fig. 4.58 Morphology of ES cells and their differentiated progeny. (a) undifferentiated murine embryonic stem cell colony cultured for 5 days in the presence of 1000 u/ml mLIF. (b) differentiated colony after 5 days culture in the absence of LIF (diffuse).

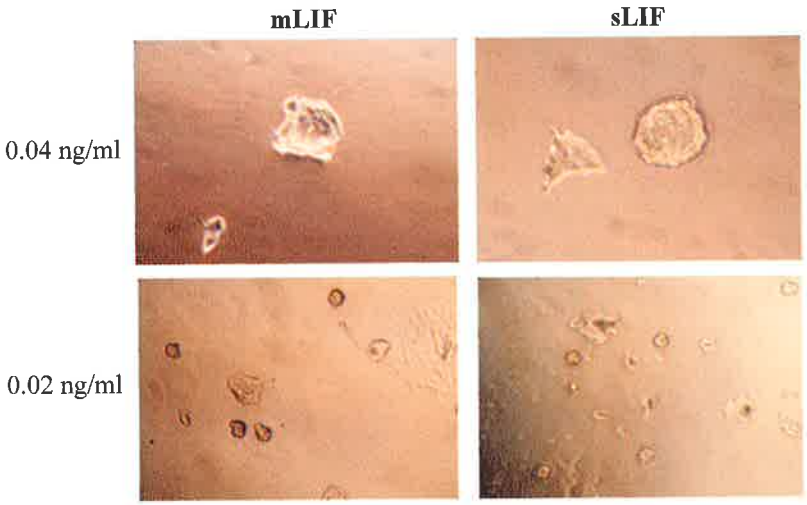


Fig. 4.59 The effect of recombinant LIF effect on the differentiation of murine ES cells. ES cells cultured for 5 days were stained for alkaline phosphatase. The concentration of the LIF proteins in each well was given aside the picture. Consistent results were obtained in three repeated experiments.

Chapter 5 Discussion

5.1 Southern Analysis, Identification and Isolation of *sLIF* Genomic and cDNA Clones

Considering the fundamental function of LIF in mouse embryo implantation, and the high degree of sequence conservation of LIF protein and cDNA in eutherian mammals, it was expected that *LIF* genes would also occur in marsupial mammals. However, at the commencement of the research described in this thesis, no direct evidence for the presence of *LIF* in a non-eutherian mammal had been published; although, as previously mentioned, Willson *et al.* (1992) had cited unpublished observation by Spencer *et al.*, that low hybridisation signals, with high backgrounds, had been observed on Southern blots of marsupial DNA probed with mouse *LIF* cDNA. Hybridisation with murine and human *LIF* cDNA probes was used in this project to identify and clone *LIF* in *S. crassicaudata.* The assumption was that marsupials and eutherians possessed homologous *LIF* genes that traced their evolutionary origins back to a single *LIF* gene in a common ancestral species, and that sufficient sequence identity remained to use the eutherian *LIF* probes to detect their marsupial orthologues.

It was found that a murine *LIF* cDNA strongly hybridised, on Southern blots, to human and mouse control DNA. Initial analysis, however, showed an absence of hybridisation to marsupial genomic DNA. Weak (but nevertheless specific) hybridisation to *S. crassicaudata* DNA was eventually detected using abundant (> 15 μg) DNA, low stringency washes and long exposure times (Fig. 4.42). Even under these conditions, DNA from other species of marsupial failed to show hybridisation to the eutherian LIF probes.

These results provided preliminary evidence for the existence of a *LIF* gene, or at least a *LIF*-like DNA sequence, in *S. crassicaudata.* The results also suggested that *sLIF* could be identified by hybridisation to *mLIF* cDNA, in a genomic library of *S.*

crassicaudata. A region of the dunnart *LIF* gene was subsequently cloned and sequenced, and a cDNA encoding the mature LIF protein was also obtained.

As an aside, it is worth noting that specific hybridisation of *mLIF* and *hLIF* cDNAs also occurred with the DNA from several other eutherian mammals including the fox and rabbit. This is the first report of putative *LIF* sequences in these two species.

It was surprising that Southern analysis of DNA from a variety of marsupial species, with the exception of *S. crassicaudata,* failed to show detectable hybridisation to mouse or to human *LIF* cDNA probes, even under conditions of low stringency. It was concluded, initially, that the degree of sequence conservation between eutherian and marsupial *LIF* was low, and that, fortuitously, the *sLIF* gene was more similar in sequence to the eutherian probes than the *LIF* gene from other marsupial species. Subsequent isolation and sequencing showed that *sLIF* cDNA has 75% sequence identity with *hLIF* cDNA and 70% sequence identity with mLIF cDNA (Table 4.2B). At these levels of sequence identity, the detection of DNA/DNA hybridisation by Southern analysis is problematical, and this is likely to account for the weak hybridisation signals observed, and also for the differences between different marsupial species.

Searches of the data bases, and alignment of the *sLIF* DNA and cDNA with human and murine *LIF* sequences, left little doubt that the *S. crassicaudata* clones were correctly identified as *LIF*. By sequence comparisons, it was concluded that regions of DNA encoding the mature *S. crassicaudata* LIF protein were present as part of two exons separated by an intron of about 2.6 kb (Fig. 4.26). The presence of open reading frames in the two *sLIF* exons, makes it unlikely that the *LIF*-like sequence represented a pseudogene.

Although the 5'-region of *sLIF* remains to be characterised, it seemed that *sLIF* has a similar overall genomic organisation to the *LIF* genes that have been characterised in eutherian mammals. A notable feature, however, was the presence in *sLIF* of a second intron that was about 4-times longer than its counterpart in human and mouse. The significance of this observation is completely unknown. *sLIF* cDNA and deduced

amino acid sequences, when compared with corresponding sequences from other eutherian species, showed a high degree of conservation across all seven species examined (Fig. 4.33 and Fig. 4.36).

To strengthen the identification of the isolated gene as *LIF*, its cDNA was expressed *in vitro* and the biological properties of the protein product examined. The demonstration that recombinant sLIF inhibits the differentiation of cultured murine ES cells, provides strong evidence that *sLIF* is indeed a marsupial homologue to *mLIF* and *hLIF*.

The use of the *sLIF* cDNA clone as a probe in Southern analysis against DNA from a variety of other marsupial and monotreme species gave strong hybridisation signals to DNA from these species but weak hybridisation to human and mouse control DNA samples. These results are partly expected and partly surprising. Given the long evolutionary time span since marsupials and eutherian mammals last shared a common ancestor, it would be expected that marsupial and eutherian *LIF* sequences may have diverged to such an extent that only weak hybridisation would occur between them. What is unexpected, however, is that the eutherian probes showed no detectable hybridisation to monotreme DNA, yet the marsupial probe, *sLIF*, did show detectable hybridisation to monotreme DNA (Fig 4.43).

The generally accepted model of mammalian evolution places eutherians and marsupials in a monophyletic group to the exclusion of monotremes. It is thought that the monotremes separated from the stem therians about 200 MYA (Clemens, 1989) whereas the marsupial/eutherian lineages diverged some 100 -150 MYA (Air *et al.*, 1971; Beard and Thompson, 1971; Cifilli and Eaton, 1987; Kirsch *et al.*, 1997). If the rates of molecular substitution have been approximately constant over time for *LIF* sequences (and the data suggests that they have - Table 4.5), in other words, if the molecular clock was assumed to be operating as it does for many if not most DNA sequences (Easteal *et al.*, 1995), then it would be expected that marsupial and eutherian *LIF* sequences would share more identity than either would with monotreme *LIF* sequences. The Southern results reported here suggest that this may not be the case. Indeed, these results are consistent with (although do not provide strong support

for) the recently proposed (but controversial) evolutionary model that marsupials and monotremes last shared a common ancestor more recently than either did to eutherian mammals (Janke *et al.*, 1996; 1997).

The results of this research provide evidence for the presence of a single *LIF* gene in *S. crassicaudata,* a finding that is consistent with the results obtained from a number of eutherian species. It is possible, of course, that additional, paralogous, *LIF*-like genes are present in eutherian and marsupials mammals and that these genes have diverged in sequence to such an extent that they can not be detected using available probes.

Further studies are needed to fully characterise the *LIF* gene in *S. crassicaudata*, including:

- analysis of the 5' end of the *LIF* gene which is expected to contain the up-stream regulatory elements of the promoter, the 5' untranslated region of the transcript, and the leader sequence of the protein;
- analysis of the 3' end of the *LIF* gene, which is expected to contain the 3' untranslated region of the transcript;
- chromosomal mapping *sLIF*.

Human *LIF* has been localised to chromosome 22q 12.1-12.2 (Budarf *et al.*, 1989; Sutherland *et al.*, 1989), and mouse *LIF* to chromosome 11 (11A1) (Kola *et al.*, 1990). This mouse chromosomal region is known to contain genes homologous to those on human 22q (Kola *et al.*, 1990). LIF is therefore part of a conserved cluster of syntenic genes in at least these two mammalian species. It would be interesting to determine if *sLIF* is part of the same syntenic group that has been conserved in the human and mouse genomes (Budarf *et al.*, 1989; Kola *et al.*, 1990).

The *S. crassicaudata LIF* probes and sequences obtained in this study, open the way to the efficient isolation of *LIF* genes from other marsupial and monotreme species. An important consequence of this study is the availability, now, of a set of LIF sequences from mammals that separated as long ago as 100 million years. Regions of

DNA sequence conserved over such a long time are likely to provide excellent sites for the design of PCR primers. An initial strategy for isolating *LIF* from other marsupial species would be to use direct PCR on genomic DNA, or RT-PCR on RNA from appropriate tissues.

5.2 *sLIF* Expression *In Vivo*

In this project, Northern blot analysis, using *LIF* cDNA probes from eutherian species, failed to detect *LIF* transcripts in adult tissues of *S. crassicaudata.* A similar result was obtained when the *sLIF* cDNA clone was used as a probe, even if large quantities of target RNA were used. These results, which suggest that the adult level of *LIF* expression in this marsupial is low (or possibly absent) are consistent with results obtained previously from eutherians, using Northern analysis (Hilton *et al.*, 1988b; Bhatt *et al.*, 1991).

The RT-PCR technique was used to search for expression of *sLIF* transcripts in adult tissues. For each tissue, a pair of gene specific primers, derived from the *sLIF* sequence, was used to amplify a cDNA fragment spanning the sequence encoding the mature protein, and the PCR products were analysed by Southern hybridisation using *sLIF* as a probe. Using this technique, *LIF* was found to be expressed in variety of tissues, including spleen, lung, intestine, heart, footpad, and uterus (Fig. 4.44) and brain and ear tip (Fig. 4.29). The finding that *LIF* is expressed in most adult tissues as detected by RT-PCR, is consistent with previous studies on eutherian mammals (Bhatt *et al.*, 1991; Patterson *et al.*, 1992; Robertson *et al.*, 1993; Charnoch-Jones *et al.*, 1994; Estrov *et al.*, 1995; Patterson and Fann, 1992). The evidence for relatively strong *LIF* expression in *S. crassicaudata* uterus tissue, is consistent with previous studies in the mouse (Bhatt *et al.*, 1991), and implicates *LIF* as having an important function in this tissue. The finding that *LIF* is expressed in a wide range of tissues and organs is presumed to reflect the poly-functionality of this cytokine.

LIF expression is absent from adult kidney and liver in range of eutherian mammals (Patterson and Fann, 1992; Robertson *et al.*, 1993). In *S. crassicaudata*, low levels of

LIF expression were detected in the liver but no expression was detected in the kidney. These results suggest that the adult kidney and liver are unlikely to be normal sites for LIF action.

In comparison to the relatively low levels of basal expression found in a broad range of adult tissues in eutherian mammals, high levels of LIF are found in murine and human uterine tissues at the time implantation, (Bhatt *et al.*, 1991; Robertson *et al.*, 1993; Yang *et al.*, 1995a; Nachtigall *et al.*, 1996). Uterine *LIF* expression has been localised to the endometrial glands of mouse (Conquet and Brulet, 1990; Bhatt *et al.*, 1991; Nichols *et al.*, 1996), and human (Kojima *et al.*, 1994; Cullinan *et al.*, 1996), and shown to be essential for embryo implantation in the mouse (Stewart *et al.*, 1992).

RT-PCR products from formalin-fixed and paraffin-embedded uterus/embryo tissue sections of *S. crassicaudata* (Fig. 4.45a) were identified as *LIF* transcripts by Southern analysis using the *sLIF* cDNA probe (Fig. 4.45b). Since the tissue sections were prepared from pregnant females at the time that their blastocysts would be expected to implant, the strong hybridisation signals observed indicate that *LIF* expression in the marsupial uterus/embryo tissue complex parallels that found in eutherians, and is compatible with the hypothesis that maternal expression of *LIF* in marsupials is essential for blastocyst implantation.

It would be desirable to clarify the identity of the tissue and cell types where the *sLIF* transcripts are located. To do this, RNA samples from isolated tissues should be examined by RT-PCR or RNase protection analysis. Spatial and temporal expression patterns could be detected using *in situ* hybridisation or the combination of PCR technique and *in situ* hybridisation (*in situ* PCR amplification). Such studies could be focused, initially, on *LIF* expression in the embryonic and maternal tissues at the time of implantation. The *sLIF* cDNA clone (*psLIF*) is ideally suited for use as an *in situ* probe because sense and anti-sense RNA transcripts can be produced using the T7 and T3 promoters flanking the insert in this clone.

Sense and antisense cRNA probes, generated from *sLIF,* were labelled with digoxigenin and hybridised to formalin-fixed, paraffin-embedded uterus/embryo tissue

sections. No hybridisation signals were observed (data not shown), probably because the tissue sections had been used previously for histochemical research, and unknown factors applied to the slides had interfered with the procedure. Furthermore, the tissue sections had been stored at room temperature for several years so it is possible that RNA degradation had occurred to such an extent that the RT-PCR technique yielded results, but that the *in situ* hybridisation technique did not. It proved to be impossible, in the time period available, to obtain fresh tissue sections from *S. crassicaudata* uteri that could be used for further *in situ* hybridisation studies.

In *S. macroura* and *S. crassicaudata*, implantation of the trilaminar blastocyst occurs at day eight and the young is born at day eleven, about two-thirds the way through the relatively short gestation period (Selwood and Woolley, 1991; Roberts and Breed, 1994a). In *S. crassicaudata*, about one quarter of captive paired animals copulate, and about one quarter of mated animals become pregnant; however, there is no efficient method for monitoring progress of the pregnancy (Claire Roberts, personal communication) and this makes it extremely difficult to collect suitable tissue from this species. Close collaboration with marsupial embryologists, in the preparation of fresh tissue sections, would be beneficial to the investigation of *sLIF* gene expression during the stage of blastocyst implantation.

5.3 *sLIF* Expression *In Vitro*

The *sLIF* cDNA clone, *pGEX2T-sLIF*, was used for the production of sLIF protein *in vitro*. Initially BL-21 bacterial cells transformed with *pGEX2T-sLIF* were grown at 37°C and induced with 0.4 mM IPTG. A protein of the expected size, about 46 kDa, was induced. Most of this protein was present in the post-sonication pellet (Fig. 4.50), indicating that the fusion LIF protein had a low solubility under these conditions. This result was anticipated, since it was consistent with other attempts to express LIF fusion proteins *in vitro* (Tricia A. Pelton, Department of Biochemistry, University of Adelaide. Personal communication).

Various procedures (eg. the use of non-ionic detergents, low culture temperatures and low concentrations of inducer) were used, successfully, to solubilise the fusion protein and to improve its yield (Fig. 4.52). Preparations were subjected to Glutathione Sepharose 4B affinity chromatography to isolate GST-sLIF complex and the eluates were analysed by PAGE. Two fusion polypeptides were present (Fig. 4.53). The 46 kDa polypeptide was presumed to be the full-length GST-sLIF fusion protein, but the second isolated polypeptide was about 31 kDa in size, which is larger than GST (26 kDa), proved to be a C-terminal truncated form of the GST-sLIF fusion protein (Fig. 4.54). No such truncations have been reported in previous attempts at GST-LIF fusion protein purification. These truncations might be caused by a unique fragmentation of the molecule by chemical ingredients during the process of preparation, or by endogenous site-specific proteolysis. The truncation could also have been caused by premature termination of synthesis of the GST-sLIF fusion protein. Some codons, commonly used in mammalian species, are known to be infrequently used in *E. coli* (Spanjaard *et al.*, 1990). Such codons, if present in the *sLIF* transcript, may have blocked translation due to specific tRNA shortage. However, because truncated protein was not observed from the cell lysates prepared with the initial protocol, it seemed likely that truncation resulted from the process of purification rather than from premature termination during synthesis. A higher than normal (Frangioni and Neel, 1993) concentration of non-ionic detergent Triton X-100 was found to protect the fusion protein from truncation (Fig. 4.55).

The protocol for the GST Gene Fusion System (*Pharmacia Biotech*) provides a GST detection method for fusion proteins using colorimetric assays or immunoassays based on the biological activity of the N-terminal glutathione S-transferase. This technique has been successfully used to isolate enzymatically active chicken muscle pyruvate kinase fused with GST (Frangioni and Neel, 1993). However, it has been suggested that LIF in the fusion product is conformationally restrained with little or no biological activity and that a proteolytic cleavage to separate LIF from fusion protein is essential for full biological function, despite the fact that biological activity of LIF can be restored by endogenous cleavage (Gearing *et al.*, 1989; Peter Rathjen, personal communication). GST-sLIF fusion protein remains to be examined for its LIF activity, *in vitro*.

In general then, GST-sLIF fusion protein was over-expressed using the pGEX2T expression vector and the polypeptide displayed the expected size (46 kDa) on PAGE. The conditions for a high yield of soluble fusion protein were optimised to avoid purification in a denaturing environment. The C-terminal truncation of GST-sLIF, presumably caused by chemical ingredients in the preparation buffer, was overcome by using a high concentration of the non-ionic detergent Triton X-100 in solution. The sLIF polypeptide was released from the fusion protein by protease digestion with thrombin, and a high quality sLIF protein was obtained use in further biological assays. Either the sarkosyl-solubilizing procedure (Section 4.8.4) or the initial protocol with modifications (Section 3.2.10.9) could be used for large scale sLIF preparation, but when using the latter, a large volume of bacterial culture would be needed, since it produces a low yield of soluble fusion protein.

5.4 Biological Properties of Recombinant sLIF

The results obtained in the present study show that recombinant sLIF is biologically active *in vitro*, in the sense that it is able to suppress the differentiation of cultured murine ES cells. The fact that sLIF can substitute for the species-compatible mLIF suggests that LIF receptors on the mouse ES cells can "recognise" and interact with sLIF to produce the effect of differentiation suppression. This, in turn, suggests that sLIF and mLIF are not sufficiently different in structure and conformation to nullify the specificity of the receptor/ligand interaction. It should be pointed out that the suppression of differentiation of murine ES cells is only one of the many properties that have been assigned to LIF in eutherian mammals. The extent to which some or all of these additional properties apply to sLIF remains to be determined.

In the mouse, and probably in all eutherian mammals, maternal *LIF* expression is necessary for implantation of the developing blastocyst. An important question, therefore, is whether maternal expression of *LIF* is necessary for implantation of the marsupial blastocyst, and possibly for other processes in embryogenesis. The findings in this project that:

- sLIF is strongly expressed in tissues from the embryo/uterus complex,
- the *LIF* genes of *S. crassicaudata* and mouse (and other eutherians) are highly conserved in their overall organisation and coding sequence,
- the LIF proteins are highly conserved in their sequence and probably 3D conformation, and
- sLIF can suppress the differentiation of cultured ES cells,

suggest that LIF has a similar function in marsupial and eutherian mammals.

5.5 Additional Evolutionary Considerations

Previous studies in species of eutherian mammal, which separated from one another no more than about 50 MYA, have shown that the coding region *LIF* and the LIF protein are conserved in their sequence. The availability of LIF sequence data from a marsupial permitted sequence comparisons to be made between species of mammal that separated at least 100 MYA. Such comparisons showed that the coding region of *LIF* and the LIF protein have been highly conserved, even over this extremely long period of time (Fig. 4.33). The finding that *sLIF* cDNA hybridises, on Southern blots, to DNA from marsupial species belonging to the families Dasyuridae, Phalangeridae, and Macropodidae (Fig 4.43) provides a strong indicator that a *LIF* gene is universally found in marsupials.

A significant outcome of this project was the observation that a *LIF*-like DNA sequence exists in the monotreme *Tachyglossus aculeatus* (Australian echidna), as judged by Southern hybridisation using *sLIF* cDNA as a probe. This observation is significant for two main reasons. First, monotremes are thought to have separated from the stem therian (marsupial + eutherian) lineage about 200 MYA (Clemens, 1989) and the confirmed presence *LIF* in a monotreme would extend the time during which a *LIF* gene has been known to exist. Secondly, the monotreme blastocyst does not implant in the uterus (monotremes lay eggs) so the absolute requirement of maternal *LIF* expression for embryo implantation, that has been demonstrated in the mouse, can not apply in monotremes. This raises the intriguing question as to the function of LIF in monotremes. It is likely that key information on the roles of LIF in

eutherian mammals, including humans, may come from studies on the molecular biology, expression and function of LIF in a monotreme.

The overall similarity in amino acid sequence (Table 3.3A & Fig. 4.36), pI and molecular weight (Table 4.4), between sLIF and LIF from eutherian mammals suggests that the three dimensional conformation and properties of this cytokine have been conserved in these species. Certain features of human and murine LIF are known to be important in the structure and function of this protein and it is potentially informative to examine the extent to which these features are conserved in the LIF protein in *S. crassicaudata.*

The four alpha helices (A, B, C and D) identified by Robinson *et al.* (1994) in mLIF are indicated on the aligned sequences in Fig. 4.36, and the predicted 3-D structure of sLIF is compared to mLIF in Fig. 4.39. There is no evidence from these comparisons to indicate that mLIF and sLIF have significantly different molecular topologies.

Robinson *et al.* (1994) has shown that all six cysteine residues in mLIF participate in disulphide links and are important in the formation of the structural core mLIF. The presence of these cysteine residues at identical positions in all other eutherian LIFs and in sLIF (Fig. 3.36) suggests that these molecules have similar molecular structures.

Hydrophilicity plots obtained from the program PEPPLOT and outputs from a program (TMpred) that predicts membrane-spanning regions and their orientation show that, overall, bLIF, mLIF, hLIF and sLIF have similar profiles (Figs. 4.37 and 4.38), particularly in the carboxyl half of the molecule. However, while the amino terminal segments of LIF in the three eutherian species exhibit similar profiles, sLIF has a different profile in this region, being more strongly hydrophilic. While the effect of this difference on function is unknown, it is suggested that it is unlikely to affect the interaction between sLIF and its receptor.

sLIF contains 9 potential N-linked glycosylation sites (Table 4.3B and Fig. 4.36), one of them being a "weak" site. Other eutherians LIFs range from 5 sites (bLIF) to 8 sites

(rLIF). This provides some evidence that sLIF may be more heavily glycosylated than LIF in eutherian mammals. It is also apparent that sLIF differs from all the eutherian LIF sequences in not having a strong site at 10, and in having extra sites at 22, 92 and 129. The effect of these differences on the properties of the molecules *in vivo* is impossible to predict at present. However, recombinant mouse and human LIF, which lack the normal degree of glycosylation, have been shown to function in *in vitro* assays at the same level as native LIF (Gearing *et al.*, 1989). Aikawa *et al.* (1998) analysed the contribution of glycosyl moieties at each of the N-linked glycosylation sites of rat LIF, to LIF function as defined by bioassays *in vitro* using mouse leukemic cell lines. They found that although glycosylation at individual sites reduced somewhat the capacity of LIF to promote proliferation of DA-1a cells, glycosylation was non-essential for this function.

mLIF is unable to bind to the human LIF receptor, but hLIF is able to bind to both high- and low-affinity mLIF receptors, and having done so is fully biologically active as judged by *in vitro* assays on cultured mouse cells (Layton *et al.*, 1992). Therefore, two important properties of LIF are i) its capacity in binding specifically to the LIF receptor and receptor component molecule gp130, and ii) its species specificity in receptor binding. Regions of LIF responsible for these properties have been identified using human-mouse chimeric molecules (Owczarek *et al.*, 1993; Layton *et al.*, 1994; Hudson *et al.*, 1996) and conservation of these regions in sLIF was therefore of interest.

Residues that were identified as being, at least in part, responsible for general receptor binding (F156 and K159) were found to be conserved across all seven species including sLIF (Fig. 4.36).

Functional similarity between marsupial and eutherian LIF was demonstrated by cross-species interaction between sLIF and the mLIF receptor, and the capacity of sLIF to act on murine ES cells to prevent their differentiation. This result indirectly demonstrates a similarity in molecular conformation and function between the sLIF and mLIF receptors. It is tempting to speculate that cytokine receptors, because of their capacity to bind more than one specific cytokine molecule, and their capacity to

interact with common components, such as gp130, in forming an active receptor complex, have a molecular structure that combines specificity and flexibility. The molecular evolution of such receptors would be restrained by a complex set of interacting selective factors. It would be interesting to examine further the co-evolution of LIF and its receptor complex. In the dunnart, it should be possible to alter the sLIF protein by targeted mutagenesis of *sLIF* cDNA, and examine the effects of such alterations on LIF function *in vitro*.

Residues shown to be responsible for species-specific binding of hLIF to the hLIF receptor (D57, S107, H112, S113, V155, K158), together with residues at the corresponding sites in mLIF and sLIF, are shown in the following table and indicated on the sequence alignment in Fig. 4.36.

Residue:	**57**	**107**	**112**	**113**	**155**	**158**
hLIF	D	S	H	S	V	K
sLIF	D	S	L	S	T	K
mLIF	E	T	Q	V	A	R

Of the six sites, four have identical residues in hLIF and sLIF, whereas all the sites have different residues when comparing sLIF and mLIF. That is to say, at these key sites involved in species-specific receptor binding, sLIF resembles hLIF more closely than in resembles mLIF. This observation parallels the finding that both sLIF and hLIF bind to the mLIF receptor. It is also noted that *sLIF* cDNA has a higher level of sequence identity with hLIF cDNA than with mLIF cDNA (Table 4.2B).

Given that the marsupial *sLIF* cDNA sequence forms a outgroup to the 6 eutherian *LIF* cDNA sequences, and assuming that nucleotide substitutions in *LIF* occur at an approximately constant rate during evolution, then the number of nucleotides separating sLIF from the various eutherian LIF sequences should be approximately the same. For non-synonymous substitutions, it can be seen (Table 4.5) that the values occur in the range of 20.1 (±2.5) to 25.1 (±2.9). Assuming confidence limits attached to each value extend and diminish that value by approximately twice the standard

5.5 (b) Possible Ancestral Role(s) of LIF

Southern hybridisation revealed the presence in the monotreme, *Tachyglossus aculeatus* (Australian echidna), of DNA sequences that hybridise to the probe *sLIF* (Fig 4.43). While this result does not prove the existence of a functional *LIF* gene in *T. aculeatus*, it does at least suggest the presence of regions of DNA in this monotreme that are orthologous to the *LIF* genes of eutherian and marsupial mammals. When we look outside Mammalia, the existence of LIF is problematical. Murine ES cells can be maintained in a medium conditioned with chicken liver cell line (LMH-CM) or when cultured over a feeder layer of primary chicken embryonic fibroblast (CEF) (Yang and Petitte, 1994). It has been proposed, therefore, that avian cells can produce an LIF-like cytokine. Nevertheless, such a cytokine and the gene that encodes it are yet to be isolated and characterised from the chicken. Furthermore, exhaustive searches of DNA and protein databases failed to obtain any evidence for the presence of LIF genes or proteins in non-mammalian species. It seems probable that LIF is present in all three major mammalian groups (eutherians, marsupials and monotremes) but is absent from non-mammals. If confirmed, this would suggest that LIF evolved in a mammalian progenitor, after separation from Sauropsids some 300 MYA.

We know that LIF has a number of functions in eutherian mammals including a critical role in normal implantation of the blastocyst (Stewart *et al.*, 1992). There is no definitive evidence that LIF is necessary for implantation in marsupials. However, it has been shown, in this study, that LIF is expressed in a variety of adult tissues, including uteri during early embryonic development in *S. crassicaudata*, so one may speculate that LIF is also involved in early stages of marsupial development. Monotremes lay eggs and no implantation occurs. Therefore, the function of LIF in monotremes cannot be to control implantation. It follows that a study of temporal and spatial LIF expression patterns in monotremes may shed light on important functions of LIF, apart from its role in implantation. Because LIF is involved in maintenance of a pluripotent state, it may also be involved in maintenance of diapuse in marsupial. LIF plays a role in many regulatory cellular processes and perhaps its role in mouse blastocyst implantation is through coordinating the production of other hormonal factors that prepare the maternal environment for implantation. Cloning and characterization of a LIF gene from egg-laying mammals would give more insights into the ancestral role of LIF and a better understanding of mammalian development.

error, it can be concluded that none of the comparisons differ significantly and that non-synonymous sites in *LIF* evolve at an approximately constant rate over time. As expected from the fact that they are less likely to come under the constraints of natural selection, substitution rates at synonymous sites are considerably higher than those at non-synonymous sites (Table 4.5) and range from 61.5% (±10.9) to 96.9% (±15.5). Again, it appears that the different vales do not differ significantly. It can be concluded that synonymous and non-synonymous sites in the coding region of mammalian *LIF* evolve at an approximately constant rate over time.

Because genes orthologous to mammalian *LIF* have not been isolated or characterised from any non-mammalian species, it is necessary to use *sLIF* as an outgroup in the phylogenetic analyses if the aim is to produce a rooted phylogenetic tree. Therefore, the maximum parsimony tree (Fig. 4.40) provides no direct evidence for the phylogenetic affinities of *sLIF*. The cloning and sequencing of an *LIF* ortholog from a non-mammalian species would be useful in that it would enable such affinities to be determined. The tree does, however, enable the relationships of the eutherian sequences to be examined. An important feature of the Tree (Fig. 4.40) is that the bootstrap values associated with several of the nodes have a low value, suggesting that the accuracy of the monophyletic groups formed by these nodes is problematical. However, several of the monophyletic groups (eg *LIF* from Mouse and Rat) have high bootstrap values, indicating their robust nature.

5.6 Concluding remarks and future prospects

The key findings in this research project have been:

- *S. crassicaudata,* and probably all marsupials, possesses a functional *LIF* gene that encodes a protein that is able to suppress the differentiation of mouse ES cells in culture;
- A monotreme (the Australian echidna) contains a *LIF*-like DNA sequence in its genome suggesting that LIF was present in a common mammalian ancestor that probably existed over 150 MYA;

- LIF cDNA and protein sequences have been highly conserved over a very long period of time;
- *sLIF* can be expressed *in vitro* to obtain purified recombinant LIF, and this material will be useful for further studies on marsupial reproductive biology and development.

To fully characterise *sLIF* and gain further insights into its evolution, regulation and function, more work needs to be undertaken, possibly along the following lines:

Firstly, the determination of the full nucleotide sequence of *psLIF-1* (Fig. 4.22) should be pursued, so that comparisons between the full sequence of the gene and its cDNA can be made to define the organisation of this gene. The cloning and characterization of its 5' non-coding region will facilitate the study of expression and regulation.

Secondly, further investigation of the temporal and spatial distribution of *sLIF* transcripts and sLIF protein, could be carried out using RNase protection assays, and *in situ* hybridisation (with DNA and antibody probes) to tissue sections. These experiments would enable the gene expression pattern to be determined, particularly at the time of blastocyst implantation. This would assist the further elucidation of the role of sLIF during embryogenesis in marsupials.

Thirdly, in light of the intriguing result that sLIF has the ability to maintain pluripotency of the murine ES cells, it would be very interesting to attempt to establish an ES (or ES-like) cell line from *S. crassicaudata*. This would not only facilitate the functional study of sLIF but also be of great scientific significance for the mammalian developmental biology. Recombinant sLIF protein could be used in the attempts to establish and maintain marsupial ES cell lines so that techniques such as gene targeting, could be used to gain further insights into *LIF* gene expression and function.

Sufficient data has been obtained in this project to indicate that LIF in marsupials is likely to have similar properties and biological functions to LIF in the mouse. One of these functions is the requirement of LIF for blastocyst implantation. Procedures that

interfere with normal maternal *LIF* expression in marsupials, are therefore likely to interfere with marsupial fertility, and this possibility could be exploited in the development of immuno-contraceptive strategies for artificial population control of marsupials, in situations where these animals form ecological and economical "pests".

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Appendix

A1. *mLIF* ORF (probe) cDNA sequence

FT CDS join(962--980,2541--2722,3280--3690)

MMALIFA Length: 612 Fri Apr 24 13:20:45 1998

ATGAAGGTCT TGGCCGCAGG GATTGTGCCC TTACTGCTGC TGGTTCTGCA
CTGGAAACAC GGGGCAGGGA GCCCTCTTCC CATCACCCCT GTAAATGCCA
CCTGTGCCAT ACGCCACCCA TGCCACGGCA ACCTCATGAA CCAGATCAAG
AATCAACTGG CACAGCTCAA TGGCAGCGCC AATGCTCTCT TCATTTCCTA
TTACACAGCT CAAGGNGAGC CGTTTCCCAA CAACGTGGAA AAGCTATGTG
CGCCTAACAT GACAGACTTC CCATCTTTCC ATGGCAACGG GACAGAGAAG
ACCAAGTTGG TGGAGCTGTA TCGGATGGTC GCATACCTGA GCGCCTCCCT
GACCAATATC ACCCGGGACC AGAAGGTCCT GAACCCCACT GCCGTGAGCC
TCCAGGTCAA GCTCAATGCT ACTATAGACG TCATGAGGGG CCTCCTCAGC
AATGTGCTTT GCCGTCTGTG CAACAAGTAC CGTGTGGGCC ACGTGGATGT
GCCACCTGTC CCCGACCACT CTGACAAAGA AGCCTTCCAA AGGAAAAAGT
TGGGTTGCCA GCTTCTGGGG ACATACAAGC AAGTCATAAG TGTGGTGGTC
CAGGCCTTCT AG

A2. *hLIF ORF* (probe) cDNA sequence

FT CDS join(721--739,2471--2649,3343--3753)

HSALIFA Length: 609 Fri Apr 24 13:33:29 1998

ATGAAGGTCT TGGCGGCAGG AGTTGTGCCC CTGCTGTTGG TTCTGCACTG
GAAACATGGG GCGGGGAGCC CCCTCCCCAT CACCCCTGTC AACGCCACCT
GTGCCATACG CCACCCATGT CACAACAACC TCATGAACCA GATCAGGAGC
CAACTGGCAC AGCTCAATGG CAGTGCCAAT GCCCTCTTTA TTCTCTATTA
CACAGCCCAG GGGGAGCCGT TCCCCAACAA CCTGGACAAG CTATGTGGCC
CCAACGTGAC GGACTTCCCG CCCTTCCACG CCAACGGCAC GGAGAAGGCC
AAGCTGGTGG AGCTGTACCG CATAGTCGTG TACCTTGGCA CCTCCCTGGG
CAACATCACC CGGGACCAGA AGATCCTCAA CCCCAGTGCC CTCAGCCTCC
ACAGCAAGCT CAACGCCACC GCCGACATCC TGCGAGGCCT CCTTAGCAAC
GTGCTGTGCC GCCTGTGCAG CAAGTACCAC GTGGGCCATG TGGACGTGAC
CTACGGCCCT GACACCTCGG GTAAGGATGT CTTCCAGAAG AAGAAGCTGG
GCTGTCAACT CCTGGGGAAG TATAAGCAGA TCATCGCCGT GTTGGCCCAG
GCCTTCTAG

A3. pGEX2T expression vector sequence

```
ID   PGEX2T     preliminary; circular DNA; SYN; 4948 BP.
DE   E. coli plasmid vector pGEX-2T - complete.
OC   Artificial sequences; Cloning vehicles.
RA   Smith D.B., Johnson K.S.;
RT   "Single-step purification of polypeptides expressed in Escherichia
RT   coli as fusions with glutathione S-transferase";
RL   Gene 67:31-40(1988).
CC   thrombin or factor Xa protease sites to cleave protein from fusion.
CC   NA (ds-DNA)
CC   TP (circular)
CC   TY (plasmid)
CC   SP (Pharmacia)(ATCC)
CC   FN (expression)(cloning directional)
FT                   /note="GEN Schistosoma japonicum
FT                   glutathione S-transferase (GST)"
FT                   /note="MCS unique BamHI-SmaI-EcoRI"
FT   misc_feature    0..0
FT                   /note="stop codons"
FT                   /note="ANT E. coli beta-lactamase gene (bla)
FT                   ampicillin resistance gene (apr/amp)"

SQ   Sequence 4948 BP; 1223 A; 1193 C; 1284 G; 1248 T; 0 other;
     ACGTTATCGA CTGCACGGTG CACCAATGCT TCTGGCGTCA GGCAGCCATC GGAAGCTGTG
     GTATGGCTGT GCAGGTCGTA AATCACTGCA TAATTCGTGT CGCTCAAGGC GCACTCCCGT
     TCTGGATAAT GTTTTTTGCG CCGACATCAT AACGGTTCTG GCAAATATTC TGAAATGAGC
     TGTTGACAAT TAATCATCGG CTCGTATAAT GTGTGGAATT GTGAGCGGAT AACAATTTCA
     CACAGGAAAC AGTATTCATG TCCCCTATAC TAGGTTATTG GAAAATTAAG GGCCTTGTGC
     AACCCACTCG ACTTCTTTTG GAATATCTTG AAGAAAAATA TGAAGAGCAT TTGTATGAGC
     GCGATGAAGG TGATAAATGG CGAAACAAAA AGTTTGAATT GGGTTTGGAG TTTCCCAATC
     TTCCTTATTA TATTGATGGT GATGTTAAAT TAACACAGTC TATGGCCATC ATACGTTATA
     TAGCTGACAA GCACAACATG TTGGGTGGTT GTCCAAAAGA GCGTGCAGAG ATTTCAATGC
     TTGAAGGAGC GGTTTTGGAT ATTAGATACG GTGTTTCGAG AATTGCATAT AGTAAAGACT
     TTGAAACTCT CAAAGTTGAT TTTCTTAGCA AGCTACCTGA AATGCTGAAA ATGTTCGAAG
     ATCGTTTATG TCATAAAACA TATTTAAATG GTGATCATGT AACCCATCCT GACTTCATGT
     TGTATGACGC TCTTGATGTT GTTTTATACA TGGACCCAAT GTGCCTGGAT GCGTTCCCAA
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     ATCCTCCAAA ATCGGATCTG GTTCCGCGTG GATCCCCGGG AATTCATCGT GACTGACTGA
     CGATCTGCCT CGCGCGTTTC GGTGATGACG GTGAAAACCT CTGACACATG CAGCTCCCGG
     AGACGGTCAC AGCTTGTCTG TAAGCGGATG CCGGGAGCAG ACAAGCCCGT CAGGGCGCGT
     CAGCGGGTGT TGGCGGGTGT CGGGGCGCAG CCATGACCCA GTCACGTAGC GATAGCGGAG
     TGTATAATTC TTGAAGACGA AAGGGCCTCG TGATACGCCT ATTTTTATAG GTTAATGTCA
     TGATAATAAT GGTTTCTTAG ACGTCAGGTG GCACTTTTCG GGGAAATGTG CGCGGAACCC
     CTATTTGTTT ATTTTTCTAA ATACATTCAA ATATGTATCC GCTCATGAGA CAATAACCCT
     GATAAATGCT TCAATAATAT TGAAAAAGGA AGAGTATGAG TATTCAACAT TTCCGTGTCG
     CCCTTATTCC CTTTTTTGCG GCATTTTGCC TTCCTGTTTT TGCTCACCCA GAAACGCTGG
     TGAAAGTAAA AGATGCTGAA GATCAGTTGG GTGCACGAGT GGGTTACATC GAACTGGATC
     TCAACAGCGG TAAGATCCTT GAGAGTTTTC GCCCCGAAGA ACGTTTTCCA ATGATGAGCA
     CTTTTAAAGT TCTGCTATGT GGCGCGGTAT TATCCCGTGT TGACGCCGGG CAAGAGCAAC
     TCGGTCGCCG CATACACTAT TCTCAGAATG ACTTGGTTGA GTACTCACCA GTCACAGAAA
     AGCATCTTAC GGATGGCATG ACAGTAAGAG AATTATGCAG TGCTGCCATA ACCATGAGTG
     ATAACACTGC GGCCAACTTA CTTCTGACAA CGATCGGAGG ACCGAAGGAG CTAACCGCTT
     TTTTGCACAA CATGGGGGAT CATGTAACTC GCCTTGATCG TTGGGAACCG GAGCTGAATG
     AAGCCATACC AAACGACGAG CGTGACACCA CGATGCCTGC AGCAATGGCA ACAACGTTGC
     GCAAACTATT AACTGGCGAA CTACTTACTC TAGCTTCCCG GCAACAATTA ATAGACTGGA
     TGGAGGCGGA TAAAGTTGCA GGACCACTTC TGCGCTCGGC CCTTCCGGCT GGCTGGTTTA
     TTGCTGATAA ATCTGGAGCC GGTGAGCGTG GGTCTCGCGG TATCATTGCA GCACTGGGGC
     CAGATGGTAA GCCCTCCCGT ATCGTAGTTA TCTACACGAC GGGGAGTCAG GCAACTATGG
     ATGAACGAAA TAGACAGATC GCTGAGATAG GTGCCTCACT GATTAAGCAT TGGTAACTGT
     CAGACCAAGT TTACTCATAT ATACTTTAGA TTGATTTAAA ACTTCATTTT TAATTTAAAA
     GGATCTAGGT GAAGATCCTT TTTGATAATC TCATGACCAA AATCCCTTAA CGTGAGTTTT
     CGTTCCACTG AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTTT
     TTCTGCGCGT AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG GTGGTTTGTT
     TGCCGGATCA AGAGCTACCA ACTCTTTTTC CGAAGGTAAC TGGCTTCAGC AGAGCGCAGA
     TACCAAATAC TGTCCTTCTA GTGTAGCCGT AGTTAGGCCA CCACTTCAAG AACTCTGTAG
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CACCGCCTAC ATACCTCGCT CTGCTAATCC TGTTACCAGT GGCTGCTGCC AGTGGCGATA
AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC GGATAAGGCG CAGCGGTCGG
GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG AACGACCTAC ACCGAACTGA
GATACCTACA GCGTGAGCTA TGAGAAAGCG CCACGCTTCC CGAAGGGAGA AAGGCGGACA
GGTATCCGGT AAGCGGCAGG GTCGGAACAG GAGAGCGCAC GAGGGAGCTT CCAGGGGGAA
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TGTGATGCTC GTCAGGGGGG CGGAGCCTAT GGAAAAACGC CAGCAACGCG GCCTTTTTAC
GGTTCCTGGC CTTTTGCTGG CCTTTTGCTC ACATGTTCTT TCCTGCGTTA TCCCCTGATT
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CCGAGCGCAG CGAGTCAGTG AGCGAGGAAG CGGAAGAGCG CCTGATGCGG TATTTTCTCC
TTACGCATCT GTGCGGTATT TCACACCGCA TAAATTCCGA CACCATCGAA TGGTGCAAAA
CCTTTCGCGG TATGGCATGA TAGCGCCCGG AAGAGAGTCA ATTCAGGGTG GTGAATGTGA
AACCAGTAAC GTTATACGAT GTCGCAGAGT ATGCCGGTGT CTCTTATCAG ACCGTTTCCC
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TGGCGGAGCT GAATTACATT CCCAACCGCG TGGCACAACA ACTGGCGGGC AAACAGTCGT
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CGATTAAATC TCGCGCCGAT CAACTGGGTG CCAGCGTGGT GGTGTCGATG GTAGAACGAA
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CCATGTCCGG TTTTCAACAA ACCATGCAAA TGCTGAATGA GGGCATCGTT CCCACTGCGA
TGCTGGTTGC CAACGATCAG ATGGCGCTGG GCGCAATGCG CGCCATTACC GAGTCCGGGC
TGCGCGTTGG TGCGGATATC TCGGTAGTGG GATACGACGA TACCGAAGAC AGCTCATGTT
ATATCCCGCC GTTAACCACC ATCAAACAGG ATTTTCGCCT GCTGGGGCAA ACCAGCGTGG
ACCGCTTGCT GCAACTCTCT CAGGGCCAGG CGGTGAAGGG CAATCAGCTG TTGCCCGTCT
CACTGGTGAA AAGAAAAACC ACCCTGGCGC CCAATACGCA AACCGCCTCT CCCCGCGCGT
TGGCCGATTC ATTAATGCAG CTGGCACGAC AGGTTTCCCG ACTGGAAAGC GGGCAGTGAG
CGCAACGCAA TTAATGTGAG TTAGCTCACT CATTAGGCAC CCCAGGCTTT ACACTTTATG
CTTCCGGCTC GTATGTTGTG TGGAATTGTG AGCGGATAAC AATTTCACAC AGGAAACAGC
TATGACCATG ATTACGGATT CACTGGCCGT CGTTTTACAA CGTCGTGACT GGGAAAACCC
TGGCGTTACC CAACTTAATC GCCTTGCAGC ACATCCCCCT TTCGCCAGCT GGCGTAATAG
CGAAGAGGCC CGCACCGATC GCCCTTCCCA ACAGTTGCGC AGCCTGAATG GCGAATGGCG
CTTTGCCTGG TTTCCGGCAC CAGAAGCGGT GCCGGAAAGC TGGCTGGAGT GCGATCTTCC
TGAGGCCGAT ACTGTCGTCG TCCCCTCAAA CTGGCAGATG CACGGTTACG ATGCGCCCAT
CTACACCAAC GTAACCTATC CCATTACGGT CAATCCGCCG TTTGTTCCCA CGGAGAATCC
GACGGGTTGT TACTCGCTCA CATTTAATGT TGATGAAAGC TGGCTACAGG AAGGCCAGAC
GCGAATTATT TTTGATGGCG TTGGAATT
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A4. pBluescriptII KS(+) cloning vector sequence

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ID   PBLUEKSP   preliminary; circular DNA; SYN; 2958 BP.
DE   E. coli phagemid vector pBluescript KS(+) - complete.
KW   cloning vector.
OC   Artificial sequences; Cloning vehicles.
RL   Thomas E.A., Stratagene Cloning Systems,
RL   11099 North Torrey Pines Rd., La Jolla, CA 92037, USA.
CC   The KS designation indicates the polylinker is oriented such
CC   that beta-galactosidase (lacZ) transcription proceeds through the KpnI
CC   site first and the SacI site last.
CC   pBluescript KS(+) carries an F1 origin of replication,
CC   oriented such that transcription proceeds in
CC   the same direction as beta-galactosidase (lacZ) transcription.
CC   NA (ds-DNA)
CC   TP (circular)

SQ   Sequence 2958 BP; 749 A; 734 C; 751 G; 724 T; 0 other;
     CACCTAAATT GTAAGCGTTA ATATTTTGTT AAAATTCGCG TTAAATTTTT GTTAAATCAG
     CTCATTTTTT AACCAATAGG CCGAAATCGG CAAAATCCCT TATAAATCAA AAGAATAGAC
     CGAGATAGGG TTGAGTGTTG TTCCAGTTTG GAACAAGAGT CCACTATTAA AGAACGTGGA
     CTCCAACGTC AAAGGGCGAA AAACCGTCTA TCAGGGCGAT GGCCCACTAC GTGAACCATC
     ACCCTAATCA AGTTTTTTGG GGTCGAGGTG CCGTAAAGCA CTAAATCGGA ACCCTAAAGG
     GAGCCCCCGA TTTAGAGCTT GACGGGGAAA GCCGGCGAAC GTGGCGAGAA AGGAAGGGAA
     GAAAGCGAAA GGAGCGGGCG CTAGGGCGCT GGCAAGTGTA GCGGTCACGC TGCGCGTAAC
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CACCACACCC GCCGCGCTTA ATGCGCCGCT ACAGGGCGCG TCCCATTCGC CATTCAGGCT
GCGCAACTGT TGGGAAGGGC GATCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA
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TTGTAAAACG ACGGCCAGTG AATTGTAATA CGACTCACTA TAGGGCGAAT TGGAGCTCCA
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CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
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AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA
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GAAAACGTTC TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA
TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG
GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT
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TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA
CATTTCCCCG AAAAGTGC