Mechanisms of Intracellular and Extracellular Cytokine Production from the Human Leukaemia Inhibitory Factor Gene

A thesis submitted to the University of Adelaide for the degree of Doctor of Philosophy

by

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Statement

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Roger B. Voyle
December 1999

Publications

Work associated with that presented in this thesis is also reported in the following scientific papers:

Haines, B.P.*, **Voyle, R.B.*,** Pelton T.A., Forest, R. and Rathjen P.D. (1999). Complex conserved organisation of the mammalian LIF gene: A novel mechanism for regulated expression of intracellular and extracellular cytokines. *J. Immunol.* 162, 4637-4646

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and was presented orally [**Voyle, R.B.**, Haines, B.P., Pera, M.F., Forrest, R.F., Pelton, T.A. and Rathjen, P.D. (1998) Novel leukaemia inhibitory factor mRNAs in human embryonal carcinoma cell lines.] at the 37th National Scientific Conference of the Australian Society for Medical Research, Hobart, Australia where it received an ASMR-AMRAD Young Investigator Award

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Summary

The polyfunctional cytokine leukaemia inhibitory factor (LIF) has been implicated in the maintenance of many stem and progenitor cell populations, including primordial germ cells and embryonic stem cells. There is also evidence that LIF and other members of the IL-6 family of cytokines can play a role in tumourigenesis. Embryonal carcinomas (EC) are among the best characterised subsets of the human germ cell tumours and a common form of early-onset cancer in males. Malignant EC are sustained by stem cells resembling the pluripotential cell populations of the early embryo. Cell lines derived from EC provide an *in vitro* system for the study of germ cell tumourigenesis and stem cell differentiation / self-renewal. Since it had been determined that two independently isolated human EC cell lines expressed novel LIF transcripts, it seemed possible that LIF expression could be important in the embryonal carcinoma stem cells.

An initial aim of these studies was to characterise the LIF transcripts and proteins expressed by these two EC cell lines. Cloning of LIF cDNAs from the GCT 27C4 EC cell line yielded three distinct human LIF cDNAs representing the previously characterised transcript, hLIF-D, and two novel transcripts, termed hLIF-M and hLIF-T. The three cloned cDNAs diverged in sequence upstream of the exon1-2 boundary, indicating that they represented alternate transcripts containing different first exons spliced to common second and third exons. Comparison of these cDNA sequences to the hLIF gene sequence positioned the LIF-M and LIF-T first exons between the previously reported first and second exons. Interspecies comparison of the LIF gene sequences of five eutherian mammals determined that this represented an arrangement of the LIF gene in which the positioning and sequence of these exons was well conserved in evolution. There were also conserved potential transcriptional regulatory elements upstream of the alternate first exons. The conservation of these putative regulatory sequences, together with the demonstration by others of transcriptional origins in these regions, suggested that the three alternate LIF transcripts arose from independently regulated promoters lacking TATA boxes.

Diverse human cell lines were surveyed for expression of the alternate hLIF transcripts. It was found that the five EC cell lines surveyed had a consistent hLIF transcription profile which was distinct from that of other germ cell tumour lines. Furthermore, hLIF-M and hLIF-T

were the predominant LIF transcripts in the EC cell lines. In contrast, independently regulated expression of each alternate hLIF transcript was seen in other cell lines of haematopoietic and tumour origin, suggesting that each transcript served a distinct biological function.

Immunoprecipitation of LIF proteins from Cos cells separately overexpressing the alternate hLIF transcripts revealed that they encoded distinct but overlapping sets of proteins. Secreted glycoproteins based on a 20 kD mature polypeptide were encoded by the hLIF-D and hLIF-M transcripts whereas proteins based on a 17 kD, N-terminally truncated polypeptide were encoded by the hLIF-M and hLIF-T transcripts. The N-terminally truncated proteins lacked a signal sequence and were retained within the cell. Since the hLIF-M and hLIF-T transcripts lacked translation initiation codons in their first exons, translation of the truncated proteins originated at the first in-frame AUG in the common exon 2 located downstream of sequences encoding the secretion signal peptide. Intriguingly, translation of the secreted hLIF-M encoded proteins appeared to initiate at a non-AUG codon(s) and was dependent upon sequences in the hLIF-M first exon. Levels of LIF biological activity released into the media of Cos cells separately overexpressing alternate hLIF transcripts were consistent with the levels of protein immunoprecipitated, indicating that all of the alternate hLIF proteins retained extracellular biological activity. Since transcripts encoding intracellular LIF proteins consistently predominated in EC cell lines, it is possible that cell-autonomous LIF signalling is involved in the maintenance of the EC stem cell pool.

Immunolocalisation of hLIF proteins expressed by transfected Cos cells suggested that secreted proteins were present in the Golgi apparatus and endoplasmic reticulum of hLIF-D and hLIF-M transfected cells and demonstrated that the truncated proteins encoded by hLIF-M and hLIF-T were present in both the cytoplasm and nucleus. Furthermore, Cos cells overexpressing the intracellular, truncated LIF proteins underwent apoptosis while cells expressing secreted LIF proteins did not. Apoptosis of these cells could be antagonised by the caspase inhibitor CrmA but not by Bcl-2, indicating induction of a specific apoptotic pathway by the intracellular hLIF proteins. This represented a distinct and novel biological activity of the intracellular hLIF proteins. Since this activity was mediated by a distinct region of the protein, LIF provides the first example of a an intracellular cytokine that is distinct from its extracellular counterpart in terms of its means of production, activity and mechanism of action

Oncostatin M (OSM) is a cytokine closely related to LIF and the genes encoding both cytokines appear to have arisen through the recent duplication of an ancestral gene. The possibility that the two genes underwent similar alternate transcription processes was therefore investigated. Expression studies using the ribonuclease protection assay indicated production of multiple alternate transcripts from the murine OSM gene. A novel mOSM transcript was cloned and found to be expressed at relatively high levels in the bone marrow and spleen. This transcript lacked the second exon present in the previously characterised mOSM transcript and potentially encoded an intracellularly-retained protein able to antagonise OSM signalling.

These findings establish LIF, and possibly OSM, as new members of a small but growing class of cytokines produced in an intracellularly active form and also suggest that the production of alternate transcripts and intracellularly-retained proteins may be a common and important feature of cytokines of the IL-6 and other families.

Chapter 1 :

General Introduction

1.1 Complex Organisms Require Sophisticated Mechanisms of Intercellular Communication

1.1.1 Complex Organisms are Cellular Communities Which Make Regulated and Collective Decisions

Complex organisms are communities of interdependent cells existing in a regulated state of flux. A single fertilised human egg will give rise to a complex adult organism that can be grouped by simple histological classification into over 200 cell types (Alberts et al., 1994). The developing human organism is thus a diversifying yet mutually-interdependent cellular community. As a whole, this community must provide for the changing specific and general requirements of all its constituent cell types, and maintain their numbers at appropriately balanced levels. The development of the human organism into a healthy adult and its maintenance thereafter is consequently an extraordinarily complex and remarkable occurrence.

The processes that give rise to and maintain all complex organisms involve the concerted regulation of cellular "choices" and can be thought of as a "decision making" process. Decisions may be made cell-autonomously (involving just one cell), collectively (involving populations of cells within a tissue), or systemically (involving cell populations in multiple tissues of an organism). These various levels of decision making can all be examined at their most fundamental level: the choice(s) made by individual cells involved in the process. Some examples of the more important choices facing cells living in community include whether to divide or differentiate, whether to persist or die, whether to adhere at one location or migrate to another, and whether or not to instruct other cells to do any of the above.

Any decision making process occurring in the context of a healthy organism, regardless of whether it is a cell-autonomous, collective, or systemic decision, must be engineered to favour outcomes which promote the long-term viability of the organism (or, strictly speaking, its germ line). Thus, in a dynamic and sometimes deleterious environment, homeostasis must be promoted and disease suppressed.

1.1.2 The Molecular Basis of Cellular Decision Making

Implicit in all controlled decision making processes are sophisticated mechanisms of intercellular communication and these have been a major focus of research in the area of molecular and cellular biology. Although evidence is accumulating for an important role for direct cell to cell contacts such as adherens junctions and gap junctions in intercellular communication (Goodenough et al., 1996; Lo & Chen, 1994) as well as cell to extracellular matrix contacts (Juliano & Haskill, 1993; Adams & Watt, 1993), investigative efforts have historically focussed on diffusible extracellular signalling molecules that are exchanged between cells *in vivo*. These are extremely diverse and include eicosinoid fatty acids, nitric oxide, steroid hormones, thyroid hormones, retinoids, neurotransmitters, amino acids and various classes of polypeptides (Alberts et al., 1994). All of these factors mediate a signal by interaction with proteins on the surface of, or inside, cells. This interaction then sets in motion chains of molecular changes and catalysis that propagate the signal that, ultimately, can influence cell behaviour and / or gene expression. Collectively, signalling by diffusible molecules and through direct cell to cell and cell to extracellular matrix contacts creates the complexity and plasticity in cellular decision making necessary for the development and maintenance of complex organisms.

In the interests of brevity, only the role of the class of extracellular signalling polypeptides known as cytokines will be further elaborated.

1.2 Cytokines Can Mediate Decision Making in the Cellular Community

1.2.1 Cytokines Defined

Prominent among the diffusible molecules identified as participants in intercellular signalling have been polypeptides (Jessell & Melton 1992). Historically, these have been classified in relation to their sites of production and action and were usually named with reference to their first reported biological action. In recent years, a greater understanding of the nature of these polypeptides and their modes of action has been attained. For instance, it is now known that similarities in protein structure between these polypeptides often transcend systems of classification (Callard and Gearing, 1993). For these reasons, such categorisations and types of

nomenclature as hormone, growth factor, neurotrophin and interleukin are no longer as meaningful.

Cytokines can be defined as soluble polypeptide factors produced by leucocytes that act as chemical mediators of intercellular communication but not as effector molecules in their own right (Callard & Gearing, 1993). In practice, this classification is often extended to polypeptides that are structurally-related to those produced by leucocytes. The term cytokine is, however, often used more narrowly by some authors. Cytokines are distinct from hormones in that: they are always proteinaceous, their production is not confined to glandular cell populations, and they tend to act locally rather than systemically. It is noteworthy, however, that some polypeptide hormones such as growth hormone and prolactin show structural relatedness to cytokines (Boulay & Paul, 1993). It should be evident from this discussion that there is a degree of arbitrariness in the classification of some polypeptides as cytokines.

1.2.2 Cytokine Sub-Classification

Different approaches have been used to arrive at sub-classifications of cytokines, however, sub-classifications based on protein tertiary structures perhaps have the greatest general utility (Sprang & Bazan, 1993; Boulay & Paul, 1993). Table 1.1 provides a classification of cytokines into seven families on this basis (adapted from Callard & Gearing, 1993) and this classification will be used throughout the present discussion.

1.2.3 Overview of Cytokine Action

In vertebrate organisms at least, cytokines are known to be involved in all types of cellular decisions and their action seems to underpin a great many biologically important processes (Callard & Gearing, 1993).

1.2.3.1 Modes of Cytokine Action

Distinct modes of intercellular cytokine action have been defined that relate the site of production to the site of action. Telocrine action refers to cytokine signalling in cell populations that are remote from the cells producing the cytokine: IL-4 and many other cytokines are transported in mammalian serum and presumed to be capable of telocrine action (Fernandez-Botran & Vitetta, 1991; Heaney & Golde, 1996). Paracrine cytokine action describes situations

List of Abreviations Used in Table 1.1

BDNF	Brain-derived neurotrophic Factor
BMP	Bone Morphogenetic Protein
CNTF	Ciliary Neurotrophic Factor
CSF	Colony Stimulating Factor
CT	Cardiotrophin
EGF	Epidermal Growth Factor
EPO	Erythropoietin
FGF	Fibroblast Growth Factor
G-CSF	Granulocyte CSF
GH	Growth Hormone
GM-CSF	Granulocyte / Macrophage CSF
gp	Glycoprotein
GRO	Growth Related Gene
IFN	Interferon
IGF	Insulin-like Growth Factor
IL	Interleukin
IP	Inflammatory Protein
LIF	Leukaemia Inhibitory Facctor
M-CSF	Macrophage CSF
MCP	Monocyte Chemotactic Protein
MIP	Macrophage Inflammatory Protein
NGF	Nerve Growth Factor
NT	Neurotrophin
OSM	Oncostatin M
PDGF	Platelet-Derived Growth Factor
PRL	Prolactin
R	Receptor
RANTES	Regulated Activation, Normal T Cell Expresed, Secreted
SCF	Stem Cell Factor
SDGF	Schwanoma-Derived Growth Factor
Ser./Thr. Kin.	Serine / Threonine Kinase
TGF	Transforming Growth Factor
Tm.	Transmembrane
TNF	Tumour Necrosis Factor
TPO	Thrombopoietin
Tyr. Kin.	Tyrosine Kinase
VEGF	Vascular Endothelial Growth Factor

Table 1.1 Cytokine Classification on the Basis of Protein Structure (See over for a list of abbreviations used herein) (Adapted from Callard & Gearing, 1993)

Cytokine Family (Structural Classification)	Examples of Member Cytokines	Cytokine Receptor Family (Type)	Examples of Member Cytokine Receptor Subunits
Haematopoietins (4 α-Helical Bundle)	GH, PRL IL-3, IL-5, GM-CSF IL-2, IL-4, IL- 7, IL-9, IL-13, IL-6, IL-11, LIF, OSM, CNTF, G-CSF Epo, Tpo	Haematopoietin Receptor (HR I)	GHR, PRLR, IL-3Rα, AI2CA, AI2CB, IL-2Rα, IL-2Rβ, IL-2Rγ, IL-6Rα, CNTFRα, gp190, gp130, EpoR, TpoR / c-*mpl*
	IL-10, IFNα, IFNβ, IFNγ	Haematopoietin Receptor (HR II)	IL-10R, IFNα/βR, IFNγR,
	M-CSF, SCF	MCSFR (Split Tyr. K. III)	M-CSFR / c-*fms*, SCFR / c-*kit*
Epidermal Growth Factor (β-Sheet)	EGF, TGFα, Amphiregulin, SDGF	EGFR (Tyr. K. I)	EGFR / TGFR
Insulin (α-Helical / β-Sheet)	Insulin, IGF-1, IGF-2, Relaxin	IGFR (Tyr. K. II)	Insulin R, IGFR I, IGFR II
Fibroblast Growth factor (β-Trefoil)	FGFs 1 to 9 IL-1α, IL-1β, IL-1Ra	FGFR (Split Tyr. K. IV); IL-1R (Single-Tm. G-Protein)	FGFRs 1 to 4 IL-1R I, IL-R II
Tumour Necrosis Factor (Jelly Roll Motif)	TNFα, TNFβ, FasL	NGFR (Tyr. K. V)	TNFR I, TNFR II, Fas
Nerve Growth Factor (Cysteine Knot)	NGF, NT-3, BDNF, TGFβ1, BMP-2, Activin, NODAL PDGF-A, PDGF-B, VEGF	NGFR (Tyr. K. V); TGFβR (Ser./Thr. K.); MCSFR (Split Tyr. K. III)	NGFR, *trk* A, TGFβR I, TGFβR II, TGFβR III, PDGFRα, PDGFRβ, flt-1, flk-1
Chemokines (Greek Key Motif)	GRO-α, γIP-10, IL-8, MIP-1α, MIP-2, RANTES, MCP-1, I-309	Rhodopsin-like (Seven-Tm G-Protein)	IL-8RA, IL-8RB, MCP-1R, MIP1αR, Duffy Antigen

where the cell populations producing the cytokine and the cell populations receiving the cytokine signal are distinct but in proximity to one another: TGF α is produced by macrophages at sites of wounding and is thought to stimulate keratinocyte and fibroblast proliferation (Rappolee et al., 1988). Juxtacrine cytokine action describes paracrine signalling occurring between cell populations that are in direct contact with each other: FGF 7 produced in the dermis stimulates epidermal keratinocyte proliferation during wound healing (Werner et al., 1993). Autocrine cytokine action occurs when cytokine production and signalling take place within a homogeneous cell population: some cultured keratinocyte cell lines produce TGF β as an autocrine growth inhibitor in a cell density-dependent manner (Kato et al., 1995). Cell-autonomous cytokine action occurs when the cytokine is produced by and acts on the same cell: IL-2 appears to stimulate the proliferation of LSA T-cell lymphoma cells by acting within the cell in which it is produced (Hassuneh et al., 1997).

1.2.3.2 Cellular and Molecular Effects of Cytokine Action

The cellular effects of cytokine signalling are many and varied. In many cases cytokines seem to be required simply to preserve cell populations. Signalling by particular cytokines, often in combination, can be required to prevent apoptosis and this may be important in enforcing the compartmentalisation of cells (Collins, 1993). There is also evidence that differentiation is, in some instances, a default process which is able to occur because cytokine signalling has enabled continued cell survival (Fairbairn et al., 1993; Williams & Smith, 1993). While ephemeral cellular effects such as the cytoskeletal remodelling associated with FGF 2-mediated chemotaxis (Mignatti et al., 1992) may sometimes be important, the best studied cellular effects of cytokine action have been longer-term ones that are at least partly manifest as changes in gene expression.

Cytokine action can, in principle, influence gene expression at any level from the initiation of transcription to the turnover of an encoded protein. While cytokine signalling is known to affect some post-transcriptional events such as mRNA degradation (Dani et al., 1985), most of the cytokine-mediated effects on gene expression documented to date have involved the modulation of transcription factor activities that govern the transcriptional initiation rates of specific genes. Cytokine signalling is known to affect the cellular levels of transcription factor activities as well as their activation by modification, complexation and localisation (Hill & Treisman, 1995). Additionally, cytokine

gene expression is itself affected by cytokine action (Callard & Gearing, 1993). In its entirety, cytokine action is therefore extremely complex.

1.2.4 Receptors for Cytokines

All cytokines can bind to cell surface receptors and generally multiple receptor subunits must oligomerise upon ligand binding before signalling can occur (Heldin, 1995). Some cytokines form homo-oligomeric signalling complexes with a single receptor subunit type, while others utilise hetero-oligomeric receptor complexes consisting of multiple receptor subunit types. Modular conserved structural and functional domains have been identified in homo- and hetero-oligomerising cytokine receptor subunits and used to group them into classes which show some correspondence to the cytokine protein structural classification system (see Table 1.1 and Callard & Gearing, 1993). The fibroblast growth factor and fibroblast growth factor receptor families are one of the more striking examples of this phenomenon (Mason, 1994). This correspondence has been taken to imply that cytokines and their receptors have co-evolved through processes involving the duplication of whole ancestral genes as well as regions within them (Shields et al., 1995; Boulay & Paul, 1993). However, the individual receptor subunits that form cytokine receptor signalling complexes are frequently unrelated structurally. The three structurally distinct receptor subunits comprising the IL-2 receptor complex are a case in point (Minami et al., 1993).

1.2.4.1 Cytokine Receptor Assembly

Mechanisms of receptor complex assembly appear to vary with the type of cytokine ligand and the type of receptor complex. The ligand may be either monovalent or multivalent and the receptor complex may be hetero- or homo-oligomerising. Ligation-induced conformational changes, such as those occuring when EGF binds its receptor (Ullrich & Schlessinger, 1990), may result in the recruitment of other receptor subunits when a monovalent cytokines binds their first receptor subunit. Alternatively , additional receptor subunits may be recruited to complexes formed by monovalent cytokines by ligation of the first receptor subunit with a multimeric form of the cytokine, as occurs with PDGF (Ullrich & Schlessinger, 1990). With multivalent ligands, oligomerisation results from the binding of multiple receptor subunits to single ligands and,

sometimes, from ligation induced conformational changes in the bound receptor subunit(s), as appears to occur in the case of growth hormone (Wells & de Vos, 1996).

Among hetero-oligomeric receptor complexes, not all of the constituent receptor subunits are specific to, or even capable of binding separately to, the cytokine ligand. Such receptor subunits are often found in receptor complexes for multiple cytokine ligands and are referred to as shared or "public" receptor subunits to distinguish them from the "private" ligand-specific receptor subunits. Receptor subunit sharing is widespread among the haematopoietin receptor complexes: gp130 is shared by LIF, OSM, CNTF, IL-6, IL-11, and CT-1; gp190 by LIF, OSM, CNTF and CT-1; IL-2Rγ by IL-4, IL-7, IL-9, and IL-15; IL-2Rβc by IL-2 and IL-15; and AI2CB / KH97 by GM-CSF, IL-3, and IL-5 (Table 1.1) (Hibi et al., 1996, Kishimoto, 1994). Cytokine signalling complexes seem to form in an ordered and step-wise fashion from pools of private and public receptor subunits. Public receptor subunits are sometimes the only signal-transducing elements in cytokine receptor complexes and the non-signalling private receptor subunits simply facilitate assembly of the complex by the ligand (Nicola & Metcalf, 1991). Indeed, studies of conservation among haematopoietin cytokine receptor subunits have indicated that signalling and non-signalling receptor subunits diverged very early in the evolution of cytokine receptor complexes (Shields et al., 1995).

These generalisations about cytokine receptor complexes are by no means universal. Some receptor subunits originally classified as public also have the ability to bind some ligands specifically: Oncostatin M first binds to gp130 and then recruits either OSMR or gp190 to form a signalling complex (Mosley et al., 1997). Also, some receptor subunits originally classified as ligand-binding, presumably private, subunits were subsequently found in the receptor complexes of other cytokines: gp190 subunit, originally shown to specifically bind LIF (Gearing et al., 1991), subsequently appeared in signalling complexes for oncostatin M (Gearing et. al., 1992), ciliary neurotrophic factor (Davis et al., 1993), and cardiotrophin-1 (Pennica et al., 1995b). Only in the LIF receptor complex is gp190 known to act as a ligand-specific subunit or to initiate assembly of the complex.

1.2.4.2 Non-Signalling and Souluble Cytokine Receptor Subunits

As discussed above, many of the private receptor subunits in hetero-oligomerising cytokine receptor complexes appear to be incapable of transducing a signal. Most non-signalling receptor subunits contain transmembrane regions but either lack or possess an extremely short cytoplasmic region. Such receptors include: IL-6Rα, GM-CSFRα, and IL-2Rα (Kishimoto, 1994). The CNTFRα subunit, however, appears to be unique in that it is bound to the cell membrane via a lipid-soluble anchor moiety which is added post-translationally (Davis et al., 1991).

In addition to non-signalling cell surface receptor subunits, soluble forms of a great many cytokine receptor subunits containing only their extracellular region have been identified (Heaney & Golde, 1996). These may result from: protease action on cell-surface receptors, as occurs with growth hormone receptor (Sadeghi et al., 1990); phospholipase action on cell-surface receptors, as occurs with CNTFRα (Davis et al., 1993b); or alternate splicing of the transcript encoding the receptor subunit, as occurs with GM-CSFRα (Heaney et al., 1995). These soluble cytokine receptors are generally diffusible and can be found in the serum of healthy mammals as seen with CNTFRα (Davis et al., 1993b). In none of these cases is the function of these soluble receptor subunits entirely clear. Roles that have been suggested include: down-regulation of cell surface receptor signalling by proteolytic "shedding" of the ligand binding domain (Downing et al., 1989); action as antagonists through competition for binding with cell surface receptors (Fanslow et al., 1991); allowing cytokines to act on cell populations that express all the receptor complex-forming subunits except the ligand-binding one (Nicholls et al., 1990; Davis et al. 1993b); and acting to protect cytokines from protease action while they are in the extracellular space (Fernandez-Botran & Vitetta, 1991). The p35/p40 IL-12 complex (Gearing & Cosman, 1991; Chua et al., 1994) is exceptional in that it seems to represent a cytokine (p35) whose action has evolved to become intrinsically associated with its soluble receptor (p40) such that they have become cross-linked to it by disulphide bonds.

1.2.5 Signal Transduction by Cytokine Receptors

The conformational changes in the extracellular regions of cytokine receptor subunits associated with complex formation have been suggested to induce conformational changes in the cytoplasmic domains of the receptor subunits that promote signalling (Heldin, 1995; Wells & de

Vos, 1996). The signalling pathways activated by these allosteric changes are thought to involve the induction of cascades of transient catalytic activity that, usually in combination, exert particular effects on cell behaviour and / or gene expression.

Many types of catalytic activity have been shown to play a role in cytokine signal transduction including: nucleotide cyclases, lipid kinases, protein kinases, and protein phosphatases. Often these catalytic activities cross-regulate, and many are influenced by the intracellular levels of "second messengers" such as metal ions, cyclic adenosine mono-phosphate and other nucleotides, and fatty acid metabolites (Clapham, 1995; Houselay & Milligan, 1997; Divecha & Irvine, 1995). Catalytic activities involved in cytokine signalling are located at diverse subcellular locations including within the receptor complex; inducibly or constitutively associated with the receptor complex, associated with the receptor complex via "adaptor" proteins, attached to the cytosolic side of the plasma membrane by an anchor moiety; associated with the cytoskeleton, free in the cytosol, in complexed form in the cytosol; or compartmentalised in the nucleus or other organelles (Alberts et al., 1994). Interactions between these activities are often mediated by conserved, modular protein-protein interaction domains (Cohen et al., 1995), such as the src homology 2 (SH2) and 3 (SH3) domains (Kuriyan & Cowburn, 1993). This spatially-grouped hierarchy of regulated catalytic activities forms sequential, branching and interconnected signalling cascades providing both the potential for adjustable amplification or attenuation of cytokine signals and a flexible means of propagating cytokine signals to different subcellular sites.

Perhaps the best understood of these cascades of catalytic activity involved in cytokine signalling are those involving protein kinases and phosphatases (Hunter, 1995). Cytokine receptor complexes can be divided into those that initiate signalling with protein kinase activities intrinsic to the receptor subunits ("receptor tyrosine kinases" and "receptor serine / threonine kinases") and those that must recruit cytoplasmic protein kinases ("non-receptor tyrosine kinases" and "non-receptor serine / threonine kinases") in order to initiate signalling. To date, most cytokine receptor signalling subunits have proven to be receptor tyrosine kinases, the only receptors known to use associated protein kinases to initiate signalling have been members of the haematopoietin receptor family and, at present, only TGFβ receptor subunits are known to have intrinsic serine / threonine kinase activity (Lin et al., 1993). It should, however, be noted that

receptor tyrosine kinases often also associate with non-receptor protein kinases and that both tyrosine- and serine/threonine-kinases and/or a variety of other activities may associate with any given receptor (Ulrich & Schlessinger 1990).

Coupling to catalytic cascades downstream of the cytokine receptor complexes is a major influence in determining the cellular effects of cytokine action. However, there is some evidence that different cellular effects of cytokine action do not necessarily result from the activation of distinct signal transduction cascades since sustained versus transitory activations of the mitogen-associated protein (MAP) kinase pathway elicit different cellular responses in PC12 pheocytoma cells (Marshall, 1995). Transitory activation stimulated PC12 proliferation as chromafin cells, while differentiation into a cell type resembling sympathetic neurons required sustained MAP kinase activation. Evidence for direct interactions between contemporaneous or rapidly consecutive signal transduction processes has recently come to light, a phenomenon sometimes referred to as "receptor cross-talk". For example, interferon β signalling interrupts IL-6 signal transduction in IL-6 dependent myeloma cells (Berger & Hawley, 1997).

1.2.6 Redundancy and Pleiotropy in Cytokine Action

Redundancy and pleiotropy are phenomena now commonly associated with cytokine action. Redundancy describes the ability of related cytokines to fulfil the same biological roles while pleiotropy describes the ability of individual cytokines to fulfil multiple biological roles. Cytokine redundancy appears to result mainly from the shared utilisation of signalling subunits in receptor complexes and signal transduction pathways among related cytokines. Pleiotropy results from the coupling of different signal transduction pathways to receptor complexes for the same cytokine in different cell types.

The most compelling evidence for cytokine redundancy comes from studies of mice made deficient in particular cytokines through gene-targeting procedures. In many cases the phenotypes of such animals have been relatively subtle when compared with the phenotype predicted on the basis of expression mapping and extrapolation from *in vitro* systems (Kishimoto, 1994). In these cases, related cytokines utilising the same signalling subunits in their receptor complexes could, either individually or in combination, assume roles fulfilled by the absent cytokine and thus compensate at least partially for its absence. For example, in mice

homozygous for null mutations in the genes for LIF (*Lif* $^{-/-}$) and CNTF (*Cntf* $^{-/-}$), an earlier and more severe degeneration of motorneurons in adult life occurs than that which is observed in either *Cntf* $^{-/-}$ or *Lif* $^{-/-}$ mice (Sendtner et al., 1996). These observations could be at least partially explained by utilisation of the gp190 and gp130 signalling subunits in the receptor complexes for both cytokines. Redundancy of cytokine action does not seem to be absolute. The related cytokines LIF, CNTF, oncostatin M and IL-6 have only a partial ability to mimic the effects of each other (Piquet-Pellorce et al., 1994) and effects specific to particular cytokines have been described in knockout animals (Stewart et al., 1992; Masu et al., 1994). A lack of redundancy in the action of some cytokines has also been observed. For one example, erythropoietin knockout mice die from acute anaemia early in embryogenesis due to the failure of erythropoiesis (Wu et al., 1995). The biological reason(s) for the widespread redundancy of cytokine action remains obscure.

Pleiotropic effects are exhibited by many cytokines in *in vitro* systems but the biological relevance of these effects is often unclear due to the sharing of receptor subunits by multiple cytokines. A cytokine exhibiting pleiotropic activity *in vitro* may not necessarily be active, or even present, in the equivalent *in vivo* context. Of more interest are the cases where cytokines have been shown to exhibit pleiotropic effects *in vivo* . One such case is that of IL-6 which was shown, by targeted inactivation of the gene in mice, to be involved in the immune, inflammatory and acute-phase responses, in the maintenance and regeneration of haematopoietic cell populations, and in the regulation of osteoclast turnover (Kopf et al., 1995, Poli et al., 1994).

Modulation of the constitution of receptor complexes can also generate cytokine pleiotropy: PDGF receptor complexes consisting of $\beta\beta$ homodimers or $\alpha\beta$ heterodimers, but not $\alpha\alpha$ homodimers, can induce chemotaxis and motile behaviour (Tiesman & Hart, 1993). Pleiotropic cytokine activity can also result from the induced or inhibited expression of other signalling molecules (including cytokines). For example,: the "pro-inflammatory" cytokine interleukin-1 induces the expression of a great many genes in various tissues including acute phase proteins, prostaglandins, peptide and non-peptide hormones, and cytokines (Dinarello, 1992).

Cytokine redundancy and pleiotropy are thus complex phenomena which add further levels of sophistication to cytokine action and debunk the notion, now historical but still reflected in the nomenclature, that cytokines can be neatly classified on the basis of function.

1.2.7 "Networking" of Cytokines in Complex Cellular Responses

In no case has the *in vivo* production of a single cytokine by a cell been demonstrated and, in fact, many cytokines appear to be produced by any given cell (Callard & Gearing, 1993). Furthermore, the sites of production and activity for different cytokines appear to overlap in a complex manner (Kishimoto, 1994). This combinatorial expression of cytokines is thought to provide complex information that can be processed in different ways by cell types in different locations. Cytokines *in vivo* are therefore generally likely to be instructive to cells in combination. These observations and conjectures have lead to the suggestion that cytokines act within "networks" (Arai, 1990) where the cytokine production of diverse cell types is interdependent and forms part of a continuous molecular dialogue between cells within the cellular community.

Perhaps the best elaborated examples of cytokine networks are those involving the CD4+ "helper" T lymphocyte population (Paul & Seder, 1994; Romagnani, 1994). The fundamental basis of these cytokine networks is the intimate signalling relationship formed between individual helper T cells and antigen-presenting cells. The nature of the signals exchanged by the antigen-presenting cell and the helper T cell in the form of cytokines and cell-associated ligands (some of which appear to be related to cytokines), directs the development of naive T cells into two functionally distinct subsets. "T_{H1}" helper T cells produce IFNγ and TNFβ which stimulate the killing of intracellular pathogens by macrophages and are thus important to cellular immunity, while "T_{H2}" helper T cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 which stimulate the B lymphocyte immunoglobulin class-switching and antibody production important in humoral immunity. Interestingly, initial production of either set of cytokines suppresses the subsequent production of the other set during a given immune response. For instance, exogenous IL-4 and IL-10 block the production of IFNγ (and hence TNFβ) during infections by the intracellular pathogen *Leishmania major* (Liew et al., 1989). Such effects may be important in limiting the tissue damaging effects of inflammatory immune

responses. Naive T cells differentiate progressively into T_{H1} or T_{H2} cells in response to sequential "priming" stimuli. For T_{H1} cells production of IL-12 by macrophages and IFNγ by natural killer cells, in addition to autocrine IL-2 production, seem to be important in this process. For T_{H2} cells production of autocrine IL-2 and IL-4, possibly by a subset of helper T cells adept at cytokine production, seem to be the main influences early in this process (Paul & Seder, 1994).

An interesting insight into cytokine signal transduction was recently gained from the use of gene-targeting approaches to study the T_{H1} / T_{H2} system. Despite the ubiquitous expression of STAT family proteins which bind and shuttle between cytokine receptor complexes and *cis*-acting DNA elements (Ihle et al., 1994), the activation of particular STATs mediated particular cytokine signals. The STAT 6 signalling pathway (utilised by IL-4) and the STAT 4 signalling pathway (utilised by IL-12) were found to be essential for the T_{H1} and T_{H2} responses respectively (Shimoda et al., 1996; Kaplan et al., 1997). There is other evidence for the essential nature and specificity of these signalling processes since the STAT 6 and STAT 4 ablation phenotypes overlap with those for ablations of the IL-4 and IL-12 receptor genes (Kaplan et al., 1997).

These findings emphasise the importance of cytokine signalling and networking during the processes of "T cell help" that are involved in specifying immune responses. Reciprocal cross-regulation of cytokine production involved in this cytokine network is also thought to be features of many other biological processes, including haematopoiesis (Watowich et al., 1996) and epithelial renewal (Martin, 1997), in which cytokine networks are thought to be operative.

1.2.8 Modulation of Cytokine Activity by Compartmentalisation

The role of soluble receptor components in the compartmentalisation of cytokine activity has already been discussed (section 1.2.4.2). In addition to this form of compartmentalisation, many cytokines have also been found to be associated with the extracellular matrix and with the plasma membrane. For example, vascular endothelial growth factor (Park et al., 1993), PDGF (Pollock & Richardson, 1992; Raines et al., 1992) and many members of the FGF family (Klagsbrun, 1990; Chintala et al., 1994) become associated with the extracellular matrix through binding to heparin in the form of heparan-sulphate proteoglycans. Other cytokines such as TGF-β1 and IGF-1 become localised to the extracellular matrix as complexes formed with specific

matrix-associated binding proteins (Parker et al., 1996; Taipale et al., 1994; Arai et al., 1996). In some cases these matrix-bound cytokines are active (Klagsbrun, 1990) while in others they are inactive (Taipale et al., 1994).

Immobilisation of cytokines through binding to the extracellular matrix acts to limit the extent of their activities spatially and might provide a mechanism for the creation of gradients of cytokine concentration that are discontinuous in relation to their site of production (Smith et al., 1992). Matrix-localisation may also allow controlled release and / or rapid mobilisation by proteolytic activity and matrix hydrolysis of active diffusible cytokines for specific purposes such as tissue remodelling and responses to injury.

Membrane association of cytokines is commonly achieved by the synthesis of preproteins containing transmembrane domains. Among other cytokines, this occurs with: epidermal growth factor family members (Derynck et al., 1984; Bell et al., 1986), IL-1α (Dinarello et al., 1991), TNFα (Jue et al., 1990) and stem cell factor (Flannagan et al., 1991). Other mechanisms for localising cytokines to the cell membrane have also been reported. The heparin-binding epidermal growth factor-like growth factor associates with cell surface heparan sulphate proteoglycans (Higashiyama et al., 1995; Nakagawa et al., 1996), while latent TGF-β1 will bind to the non-signalling, membrane-associated mannose-6-phosphate receptor (Kovacina et al., 1989). Besides heparan sulphate proteoglycans, many other non-signalling, carbohydrate-containing cell surface molecules, such as the TGF β Type III beta-glycan receptor (Laiho et al., 1990), seem to act as cytokine recruitment and potentiation factors. In addition, numerous "cytokine like" proteins that appear to have no diffusible equivalent are found on the surface of lymphocytes (Paul & Seder, 1994).

The association of cytokines with cell membranes could to limit them to juxtacrine modes of action, or provide a basis for controlled release by regulated proteolysis into the wider extracellular environment. These features of membrane-associated cytokines suggest that they are likely to be involved in processes requiring intimate, localised intercellular communication such as occurs during cell migration (Donnovan, 1994), inflammatory responses (Dinarello, 1992), and "immunological synapsis" (Paul & Seder, 1994).

1.2.9 Intracellular Roles for Cytokines and their Receptors

In recent years, much evidence suggestive of intracellular roles for cytokines and other extracellular signalling proteins and their receptors has emerged (Prochiantz & Theodore, 1995; Morel, 1994, Jans, 1994; Burwen & Jones , 1987). Table 1.2 provides a list of cytokines and other extracellular signalling proteins that have been detected inside the cell. It also indicates their subcellular localisation, where this is known, and whether or not they possess a nuclear localisation sequence and/or secretory signal peptide (adapted from Prochiantz & Theodore, 1995). From this table it is evident that a great diversity of cytokines are found inside the cell and the nucleus.

The intracellular and / or nuclear localisation of cytokines can be required to elicit specific biological activities. Furthermore, in some cases, the biological activities elicited by the intracellularisation of cytokines can occur independently of binding to their characterised receptors. Emerging ideas about cytokine action suggest that the signalling role of these molecules cannot be entirely explained by receptor-mediated signalling at the cell surface and that at least some of the observed cellular effects of cytokine action are likely to be mediated by distinct intracellular signalling mechanisms.

Considered below is the experimental evidence that has been used to support the idea that some cytokines have intracellular roles. At present, the mechanisms by which cytokines are localised and signal intracellularly largely remain to be characterised, therefore mechanisms *hypothesised* to be involved in intracellular cytokine localisation and action are also discussed.

1.2.9.1 Methods of Cytokine Intracellularisation

Several mechanisms for the intracellularisation of cytokines have been suggested. The main purpose of receptor-mediated endocytosis of cytokine ligands (James & Bradshaw, 1984) was originally postulated to be in de-sensitising cells exposed to high or constant concentrations of a particular ligand. It is now also thought to provide a possible mechanism for internalisation of ligands and their receptors for intracellular action since cytokine-cytokine receptor complexes are not always degraded upon internalisation (Jans, 1994). Thus, the ligated receptor complex might also transduce a signal inside the cell or nucleus after endocytosis (Jans, 1994). A number of ligands, including IL-1, growth hormone, EGF, NGF and PDGF (Curtis et al., 1990; Lobie et

Table 1.2 Secreted Signalling Proteins Found Intracellularly

Cytokine / Growth Factor	Target	SS	NLS
Fibroblast growth factor 1	Nucleus	-	+
Fibroblast growth factor 2	Nucleolus	-	+
Angiotensin II	Chromatin	+	-
Brain-derived neurotrophic factor	N/D	-	+
Epidermal growth factor	Chromatin	+	-
Schwannoma-derived growth factor	DNA	+	+
Gonadotropin	Chromatin	+	-
Gonadotropin-releasing hormone	N/D	+	-
Human chorionic gonadotropin	Nuclear Membrane	+	-
Growth Hormone	Chromatin	+	-
Insulin	Nuclear Membrane	+	-
Interferons (βand γ)	N/D	+	-
Interleukin 1 (α and β)	N/D	-	-
Lutenizing hormone-releasing hormone	Outer Nuclear Membrane	+	-
Nerve growth factor	Chromatin	+	+
Notch	N/D	+	+
Platelet-derived growth factor	Chromatin	+	+
Transforming Growth Factor β4	N/D	-	-
Ciliary neurotrophic factor	N/D	-	-
Cardiotrophin 1	N/D	-	-

N/D = Not Determined

(Adapted from Prochiantz & Theodore, 1995)

et al., 1994; Rakowicz-Szulczynska et al., 1986), in association with their receptor complexes are known to localise to the nucleus following receptor-mediated endocytosis. In some cases (Kimura et al., 1993; Imamura et al., 1990), localisation to the nucleus has been demonstrated to be mediated by an intrinsic nuclear localisation sequence (NLS). In others the presence of an NLS motif suggests that this may be the case. Cytokines might also be localised intranuclearly by NLSs present in receptor subunits or other mechanisms. Smaller cytokines (less than ~20 kD) may also be able to enter the nucleus by passive diffusion through pores in the nuclear membrane (Jans, 1994).

Some cytokines are found intracellularly because they lack a secretory signal sequence and are not secreted via the classical Golgi-mediated pathway (Muesch et al., 1990; Mignatti et al., 1992). Cytokines that are retained intracellularly can be released by stimuli such as those generated by heat shock, inflammation, or wounding via a signal peptide dependent and independent pathways (McNiel et al., 1989; Brown et al., 1994; Jackson et al., 1992; Rubartelli et al., 1990). Cytokines lacking secretory signal sequences as well as those secreted using regulated mechanisms could be available for action inside the cell prior to their secretion. Such cytokines include the IL-6 sub-family members CNTF (Lin et al., 1989) and cardiotropin 1 (Pennica et al., 1995) both of which lack secretory signal sequences and sometimes are not secreted when expressed in heterologous mammalian systems.

Cytokines such as brain-derived neurotrophic factor (Wetmore et al., 1991), FGF 2 (Ferguson et al., 1990) and CNTF (Curtis et al., 1994) can be intracellularised by and, in some cases, transferred between, neurons.

1.2.9.2 Production and Action of Intracellular FGF1 and 2

The two cytokines whose intracellular activities are best understood are FGF 1 and FGF 2. Subcellular compartmentation of FGF 2 is directed by alternate initiation of translation, either at CUG codons upstream or an AUG codon downstream of a functional nuclear localisation sequence (Bugler et al., 1991). The "high molecular weight" proteins translated from the CUGs localise to the nucleus, predominantly in the nucleolus, and are functionally distinct from the "low molecular weight" proteins produced from the ATGs which are found in the cytoplasm and at the cell surface (Florkiewicz et al., 1991; Bikfalvi et al., 1995). Initiation of translation at the CUGs or

the AUG can be differentially regulated, and CUG-initiated translation is up-regulated in response to stressful stimuli and oncogenic transformation (Vagner et al., 1996). Translational initiation at the alternative start sites is controlled by *cis*-acting elements in the mRNA (Prats et al., 1992) and initiation at the CUG start sites occurs as a result the internal entry of ribosomes (Vagner et al., 1995). Nuclear localisation of CUG-initiated forms of FGF 2 has also been shown to be influenced by a methylation-directed post-translational modification of the protein (Pintucci et al., 1996). In contrast, the FGF 1 protein appears to be synthesised in just one form (McKeehan & Crabb, 1987).

Relatively little is known about the intracellular activities of FGF 1 and 2 but many experimental observations suggest that they are distinct in nature from their extracellular activities. Secreted FGF 1 can be internalised by receptor-mediated endocytosis and becomes localised to the nucleus (Wiedlocha et al., 1994). Nuclear localisation of FGF 1 has been shown to be sufficient for stimulation of DNA synthesis but not other FGF 1 activities such as tyrosine phosphorylation and c-fos induction (Immamura et al., 1990). Using a diphtheria toxin-FGF 1 fusion protein in combination with cells expressing diphtheria toxin receptors but not FGF receptors it has been shown that the stimulation of DNA synthesis occurs independently of receptor binding (Wiedlocha et al., 1994). Furthermore, the nuclear activity of FGF 1 and the activity of the ligated FGF 1 receptor complex are both required to elicit the full proliferative response to FGF 1 (Wiedlocha et al., 1996). Thus, extracellular and intracellular signal transduction pathways for FGF 1 appear to be distinct but complementary. In at least some cells, nuclear entry of FGF 2 occurs late in the G_1 phase of the cell cycle and stimulates ribosomal gene transcription in the nucleolus (Bouche et al., 1987; Baldin et al., 1990). Intriguingly, addition of FGF 2 to nuclear extracts from Erlich ascites cells was shown to specifically influence the transcription of the *Pgk* 2 and *Pgk* 1 genes positively and negatively respectively (Nakanishi et al., 1992) Nuclear entry of both FGF-1 and FGF-2 has also been shown to correlate with the onset of mesoderm induction in *Xenopus* embryos (Shiurba et al., 1991). Of particular interest, but as yet unknown significance, is the existence of an FGF receptor-1-like, nuclear membrane-associated FGF 2 binding protein that appears to be involved in nuclear translocation of the cytokine (Kilkenny & Hill, 1996) and also an exclusively intracellular isoform of the FGF receptor-1 that can bind FGF 2 and has intrinsic tyrosine kinase activity (Maher, 1996).

1.2.9.3 Production and Action of Other Intracellular Cytokines

There is also some evidence that other cytokines have intracellular activities. Disruption of a putative NLS in Schwannoma-derived growth factor, a member of the epidermal growth factor family, has also been shown to abrogate its mitogenic activity but not receptor-dependent immediate early gene induction (Kimura, 1993). Furthermore, antisense oligonucleotides, but not neutralising antibodies directed against IL-2 (Hassuneh et al., 1997) and IL-6 (Barut et al., 1993; Kong et al., 1996; Lu & Kerbel, 1993; Roth et al., 1995) have been demonstrated to inhibit the proliferation of a variety of cultured transformed and non-transformed cell types, suggesting that these activities may be intracellular.

Thus, it seems that intracellular cytokine activity may be commonplace. In some cases, the diverse effects of intracellular cytokine action may already be partly elucidated and presumed to be cell surface receptor-mediated phenomena. An initial step in the determination of mechanisms of intracellular cytokine action will be the characterisation of the intracellular molecules that they interact with. It is even possible that cytokines might participate in the formation of transcription factor complexes (Jans, 1994); the ability of FGF 2 to affect the transcription of particular genes in nuclear extracts (Nakanishi et al., 1992) is particularly interesting in this regard.

1.2.10 Modulation of Cytokine Activity by Other Means

A diversity of mechanisms other than compartmentalisation are known to be involved in modulating cytokine action. The IL-1 receptor antagonist (IL-1Ra) (Eisenberg et al., 1990) binds to the Type I IL-1 receptor with an affinity comparable to those of IL-1α and IL-1β but does not transduce any signal (Granowitz et al., 1991). Multiple members of the TGFβ family form inactive heterodimeric complexes with active TGFβs, while many TGFβs exist extracellularly as latent complexes formed from proteolyticly cleaved precursor proteins (Jessell & Melton, 1992). Other cytokines, including IL-1 and OSM, seem to require proteolytic processing before they attain full extracellular activity (Dinarello, 1992; Linsley et al., 1990). Differences between the activities of complexed and monomeric forms of IL-6 have also been reported (May et al., 1991, May et al., 1992). Finally, glycosylation has been proposed to influence cytokine secretion (Delli-

Bovi et al., 1988; Sha et al., 1989), the extracellular half life of cytokines and / or their affinity for receptor subunits (Opdenakker et al., 1995).

1.3 Stem and Progenitor Cell Populations: The Source of Cellular Diversity and Renewal

1.3.1 Properties of Stem and Progenitor Cells

Many of the tissues that arise, differentiate, renew, and repair during any mammals lifetime do so from pools of less differentiated, proliferating cells which are generally termed "progenitor" cells. In some cases there is evidence that these progenitor cells are, in turn, generated from a much smaller population of slowly-dividing cells that remain present throughout the lifetime of the organism and retain the ability to reconstitute tissues and even organ systems in their entirety. Such cells have been dubbed "stem" cells. The term stem cell is, applied to cells in a variety of contexts and there is little consensus on its usage. Stem cells are most simply described as cells that are capable of both differentiation and "extensive" proliferation / self-renewal (Morrison et al., 1997). This capacity to either differentiate or self-renew is described as the ability to "divide asymmetrically" and is considered to be the most fundamental of a stem cell's properties. Many other properties have been ascribed to stem cells, however, including persistence throughout adult life and capacities for mitotic quiescence, multipotentiality, and whole-organ regeneration (Morrison et al., 1997; Hall & Watt, 1989). In general terms, progenitor cells are committed to forming terminally differentiated progeny while stem cells are not. The inclusive definitions of stem and progenitor cell populations provided above will be used in this thesis.

1.3.2 Stem and Progenitor Cells in Adult Mammals

The most rigorously characterised *in vivo* stem cell population is that of the haematopoietic system (Morrison et al., 1995). The existence of haematopoietic progenitor and stem cell populations is most persuasively demonstrated by "clonogenic" assays in which multiple cell types and, in some cases, all the cell types that constitute a functional haematopoietic system are found in single colonies that arise from individual cells repopulating the spleens of

mice whose haematopoietic tissues have been destroyed by irradiation (Till & McCulloch, 1961; Till & McCulloch, 1980). Distinctions between stem and progenitor cell populations in this system are somewhat arbitrary: some thymic T lymphocyte populations cannot be regenerated from adult haematopoietic stem cells and arise from cell populations, considered to be "progenitors", that are set aside during embryogenesis and persist throughout adulthood (Ikuta et al., 1990). In adult mammals, oogonia are committed precursor cells that have begun meiosis and are incapable of self-renewal,. However, a sub population of adult spermatognia termed "Type A_1" spermatogonia produce "transit amplifying" progenitor cell populations that proliferate extensively to maintain the production of spermatozoa on an immense scale (Hecht , 1986). In striking parallel to the reconstitutive abilities of haematopoietic stem cells, preparations of spermatogonia from fertile mice are capable of permanently reinstating spermatogenesis in the testis of infertile mice (Brinster & Avarbock, 1994; Dym, 1994) and can therefore be considered stem cells.

It has proven more difficult to demonstrate convincingly the existence of other stem cell populations. The contention that the central nervous system, despite its lack of any obvious regenerative capacity in adulthood, contains stem and progenitor cell populations is gradually gaining acceptance (Alvarez-Buylla & Lois, 1995; McKay, 1997). It is, however, more widely accepted that epidermal and hair follicle keratinocytes arise from small, distinct stem cell populations which give rise to much larger numbers of unipotential, transit-amplifying progenitor cells (Parkinson, 1993; Lavker et al., 1993). The gut epithelium is also thought to harbour a similar stem and progenitor cell system (Potten & Loefler, 1990). The olfactory neuro-epithelium also shows reconstitutive properties consistent with the existence of stem and progenitor cells in this tissue (Monti Graziadei & Graziadei, 1979). In the liver, "differentiated" cells can re-enter the cell cycle and undergo extensive self-renewal in response to tissue damage (Michalopoulos, 1997) and small numbers of transplanted healthy cells can reconstitute severely damaged livers (Rhim et al., 1994). It has been argued however that this impressive regenerative capacity is partly the result of the proliferation of a small number of multipotent stem cells (Sigal et al., 1992). Some mesenchymal cell populations, particularly those found in the bone marrow stroma (Prockop, 1997), appear to have reconstitutive properties and to be capable of forming multiple

mesenchymal lineages. These latter two examples highlight the somewhat arbitrary nature of the distinction between stem and progenitor cells and differentiated cells.

1.3.3 Stem and Progenitor Cells During Mammalian Embryogenesis

During mammalian embryogenesis a great many stem and progenitor cell systems appear transiently. In the mouse, the first such cells to appear are the inner cell mass cells of the blastocyst. The inner cell mass of embryos regenerates rapidly in response to cytotoxic insult and heterologous aggregates of these cells will form normal adult mice with individual stem cells making random contributions to all adult tissues (Petters & Market, 1980; Gardner & Rossant, 1979). The cells of the neural crest (Stemple & Anderson, 1992) and embryonic haematopoietic tissues (Robb, 1996) are the best characterised of the stem and progenitor cell systems arising after gastrulation but evidence that other similar populations exist is beginning to emerge.

Primordial germ cells (PGCs) are the undifferentiated precursors of spermatogonia and oogonia. In the mouse, PGCs undergo extensive proliferation during their period of migration from the extraembryonic mesoderm, where they are first observed, to the genital ridges, where they are incorporated into the developing gonads (Hogan et al., 1994). To date, *in vitro* culture conditions that support the indefinite proliferation of PGCs without changing their phenotype have not been identified. It is therefore unclear whether PGCs are best described as "committed stem cell precursors" or as transiently-arising stem cells. Although PGCs or their progeny must attain totipotency at some point in their development, it is not clear when this is acquired (Donovan, 1994). They or their progeny do, however, exhibit totipotency in certain contexts such as teratocarcinogenesis (Stevens, 1983) and parthenogenesis (Kaufman, et al., 1977).

1.3.4 Stem and Progenitor Cells *In Vitro*

Stem and progenitor cell populations can, in some cases, be isolated, cultured and manipulated *in vitro.* Perhaps the most impressive examples of stem cell systems *in vitro* are the murine embryonic stem (ES) cell, embryonic germ (EG) cell and embryonal carcinoma (EC) cell systems (Smith et al., 1993; Rossant et al., 1993). ES cells are derived from the inner cell mass of the blastocyst (Evans & Kaufman, 1981; Martin, 1981) while EG cells are derived from cultured pre-gonadal PGCs (Matsui et al, 1992; Resnick et al., 1992). EC cells are phenotypically more

diverse than ES or EG cells and are derived either from spontaneous gonadal tumours in the 129*sv* and LT strains of inbred mice or from tumours created by ectopic grafting of ES cells or embryonic material containing pluripotential stem cells into syngeneic host mice (Stevens, 1983; Rossant., 1993). ES, EG and, in some cases, EC cells when injected into the blastocoelic cavity of blastocyst-stage embryos can contribute to many tissues in the resulting chimaeric adult mice. ES and EG cells have the ability to contribute to all adult cell populations, including the germ cells (Robertson, 1991; Stewart et al., 1994). EC cell lines, while they are sometimes capable of contributing to most other cell populations in chimaerae (Papaioannou et al., 1975), have only very rarely been shown to contribute to the germ cell lineage (Stewart & Mintz, 1982). This general inability of EC cells to enter the germ line may indicate that they have undergone genetic transformations incompatible with gametogenesis (Papaioannou & Rossant, 1983). Murine ES, EG and, to a lesser extent, EC cells are thus developmentally pluripotent and sufficiently similar to the embryonic inner cell mass to become incorporated into it.

There has been limited success in obtaining permanent cultures of self-renewing haematopoietic (Domen & Weissman, 1999) and neuronal (Morrison et al., 1999) stem cells. Presently, only short-term culture of the isolated neuronal and haematopoietic stem cells prior to their *in vivo* transplantation is possible.

Although ES, EG and some EC cells appear to show similarities in their growth characteristics and marker gene expression once established in culture (Donovan, 1994), they do not seem to be entirely identical. For example, EG and ES cells differ in their imprinting of the *Igf2r* gene (Labosky et al., 1994). The murine ES, EG and EC stem cell systems are exceptional in their ability to undergo indefinite self-renewal *in vitro* while retaining the capacity to contribute to the formation of normal tissues *in vivo*.

1.3.5 Stem and Progenitor Cells in Homeostasis and Disease

Inappropriate cellular decision making by stem and progenitor cell populations underlies many disease states (Morrison et al., 1997). To maintain a stem cell-derived tissue in a healthy state, differentiation and self-renewal in specific stem and progenitor cell populations must be precisely regulated. This is achieved, in part, by controlling the degree of asymmetry in stem and progenitor cell division (Morrison, 1997). Cytokines appear to have a widespread involvement in

stem and progenitor cell decision making and this linkage is important to our understanding of the disease state of cancer (Cross & Dexter, 1991). This will be further considered below.

1.4 Leukaemia Inhibitory Factor (LIF): A Cytokine Affecting Decision Making by Stem and Progenitor Cells

1.4.1 The Nature and History of LIF

LIF was first purified and described as "D-factor" (Tomida et al., 1984), an activity which induced the differentiation of M1 murine myeloid leukaemia cells into macrophage-like cells. An activity from Krebs ascites tumour cells which inhibited the proliferation of M1 cells and whose activity appeared to be identical to D-factor, was independently purified and cloned as "leukaemia inhibitory factor" (Gearing et al., 1987; Hilton et al., 1988). Concurrently and subsequently, many investigators working with different systems independently purified activities and isolated cDNA clones that proved to be identical to LIF (summarised in Gough et al., 1992). This convergence of multiple lines of investigation provided the first indication that LIF might be a highly pleiotropic cytokine (Hilton, 1992).

Human LIF (hLIF) was first purified as "human interleukin for Da cells" (HILDA) from allo-reactive T cell clones and was found to act as an eosinophil activator / chemoattractant and exhibited burst-promoting activity in bone marrow cultures (Moreau et al., 1987; Goddard et al., 1988). Cloning of cDNAs encoding this activity revealed that HILDA was the human equivalent of murine LIF (Moreau et al., 1988) and this was confirmed by the independent isolation of hLIF genomic clones by homology-based library screening (Gough et al., 1988; Lowe et al., 1989).

In all species in which it has been characterised, the mature LIF protein is 179 amino acids long, secreted, basic and heavily glycosylated. The mature protein is produced by the proteolytic cleavage of a preprotein containing a 22-23 amino acid N-terminal hydrophobic secretion signal sequence. hLIF purified from the HSB2 T-cell lymphoma cell line has an apparent molecular weight of 43 kD in its glycosylated form and 20 kD upon complete de-glycosylation (O-linked oligosaccharides comprise 2-3 kD of the mature protein's molecular weight, N-linked oligosaccharides make up the remainder) (Gascan et al., 1989). The hLIF

polypeptide has an isoelectric point of pH 8.5 - 9.0, although the post-translational addition of sialic acid residues creates a population of hLIF molecules displaying greater heterogeneity in their isoelectric points (Gascan et al., 1989). The glycosylation state of the LIF protein appears to show heterogeneity among different natural sources of LIF from the same species and also among naturally occurring LIF proteins from different species (Gascan et al, 1989; Culinan et al., 1996; Hilton et al., 1988; Yammamori et al., 1989).

1.4.2 LIF is a Member of the IL-6 Sub-Family of Cytokines

Solution of the three dimensional structure for crystallised mLIF protein (Robinson et al., 1994) confirmed the suggestion, based on structural homologies (Bazan, 1991), that LIF is a four α-helical bundle protein with the helices arranged in an "up-up-down-down" configuration, confirming its assignment to the haematopoietin family of cytokines (Sprang & Bazan, 1993; Boulay & Paul 1993). LIF also shows structural similarities to some other haematopoietin cytokines including IL-6 (Somers et al., 1996), CNTF (MacDonald et al., 1995) and, to a lesser degree, growth hormone (De Vos et al., 1992) and G-CSF (Hill et al., 1993). This confirmed earlier suggestions that LIF could be grouped with IL-6, CNTF and other highly homologous cytokines as a sub-family, distinct from growth hormone and G-CSF and the other members of the haematopoietin family (Bazan, 1991).

Other data also support the classification of LIF as a member of the "IL-6 sub-family" (Hibi et al., 1996; Heinrich et al., 1998). Evolutionary studies comparing the amino acid sequences and genomic organisation of the cytokines and their receptor subunits support this classification of LIF (Shields et al., 1995; Boulay & Paul, 1993). Furthermore, they suggest that LIF and OSM arose late in the evolution of the IL-6 subfamily as a result of gene duplication (Shields et al., 1995). This is supported by the finding that both genes localise to band q12 on human chromosome 22 within 19 kb of one another with their promoters adjacent (Jefferey et al., 1993; Giovanini et al., 1993; Budarf et al., 1989). Members of he IL-6 sub-family all utilise the gp130 receptor subunit in their signalling complexes. This has been suggested to provide the basis of the redundancy seen in IL-6 family cytokine action (Piquet-Pellorce et al., 1994). At present, the IL-6 sub-family consists of: OSM (Zarling et al., 1986; Malik et al., 1990; Bruce, 1992), CNTF (Stockli et al., 1989; Lin et al., 1989), cardiotrophin-1 (Pennica et al., 1995a;

Pennica et al., 1995b), IL-6 (Haegeman et al., 1986; Akira et al., 1993) and IL-11 (Yang et al., 1991; Kawashima & Takiguchi, 1992). However, the possibility that other members of the IL-6 family remain to be characterised has been suggested by, among other evidence, the observation that CNTF is the only known ligand for CNTFR α yet inactivation of the CNTFRα gene results in a more severe phenotype than CNTF inactivation (De Chiara et al., 1995).

The postulated co-evolution of IL-6 sub-family cytokines and their receptor complexes, together with the inter-relatedness of their receptor subunits (Shields et al., 1995; Bazan, 1990), contribute to an explanation of the highly pleiotropic action and the conserved mechanisms of receptor engagement of these cytokines (Grotzinger et al., 1997). Figure 1.1 illustrates what is known and some of what has been suggested about the receptor complexes of IL-6 sub-family cytokines and the sharing of receptor subunits (adapted from Hibi et al., 1996). Worthy of note is the finding that two OSM receptor complexes containing gp130 exist (Mosley et al., 1996). As yet, however, there is no evidence of receptor complex heterogeneity among other members of the IL-6 sub-family. Several other haematopoietin receptor β-chains, including those of the G-CSF receptor, IL-12 receptor (Shields et al., 1995) and the leptin receptor (Tataglia et al., 1995), also appear to share structural homology with the β-chains of the IL-6 subfamily.

Experimental observations of the solution chemistry of IL-6 (Ward et al., 1994) and IL-11 (Nedderman et al., 1996) and their receptor subunits suggest that they assemble their receptor complexes sequentially through multimerisation events to form higher order complexes containing cytokine dimers, along with the receptor subunits, within a hexamer. The *in vivo* occurrence of this process is supported by mutagenesis studies performed by many investigators (summarised in Sommers et al., 1997). However, the constitution of signalling complexes for IL-6 sub-family cytokines is still ontentious (Wells, 1996) and that other models of receptor complexes for this sub-family have been proposed (Grotzinger et al., 1997).

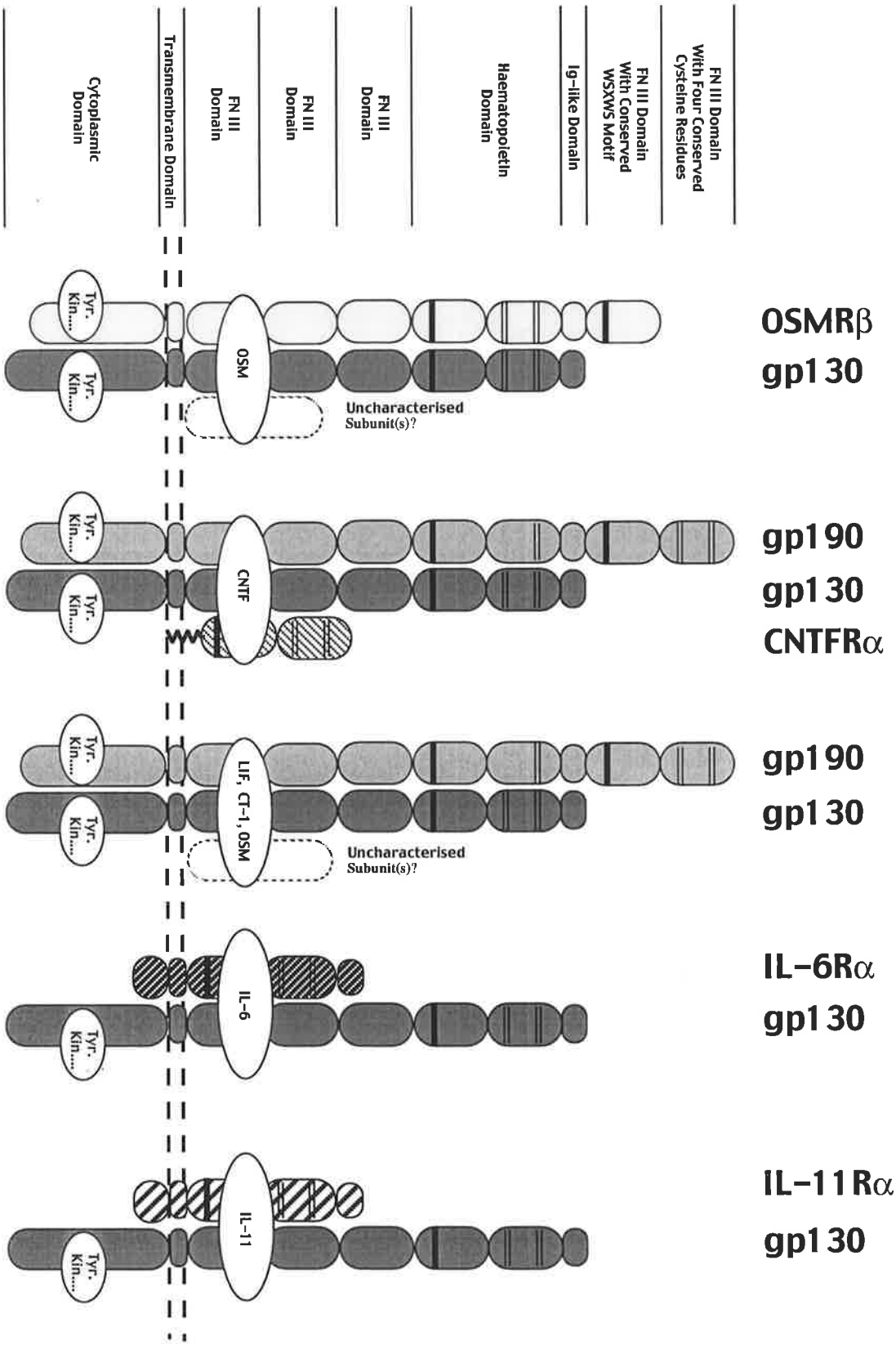
1.4.3 The LIF Receptor and Signal Transduction Pathway

The LIF receptor signalling complex is known to consist of two types of receptor subunit: gp190 (Gearing et al., 1991) and gp130 (Hibi et al., 1990; Gearing et al., 1992). Ligation of gp190 by LIF is thought to trigger the formation of a higher order complex involving gp130 in a manner similar to other IL-6 sub-family cytokines (K.R. Hudson personal communication).

Figure 1.1 Interleukin-6 Sub-Family Cytokine Receptor Complexes

The domain structure of each receptor subunit and the stoichiometry of the receptor complex are indicated. Note that none of the α subunits shown associate with tyrosine kinases or appear to transduce a signal and that the CNTFRα is shown anchored to the plasma membrane by a glycerol-phosphoinositide anchor moiety. The unknown receptor subunits are those suggested by Heyman and co-workers (1996). It is possible that signalling complexes for all these cytokines comprise dimers of the complexes shown here, as appears to be case with IL-6 (Ward et al., 1994) and IL-11 (Nedderman et al., 1996).

(Compiled from: Wells and de Vos, 1996; Hibi et al., 1996; Mosley et al., 1996)


FN III Domain With Four Conserved Cysteine Residues
FN III Domain With Conserved WSXWS Motif
Ig-like Domain
Haematopoietin Domain
FN III Domain
FN III Domain
FN III Domain
Transmembrane Domain
Cytoplasmic Domain
Tyr. Kin.....
Tyr. Kin.....
OSM
Uncharacterised Subunit(s)?
CNTF
LIF, CT-1, OSM
IL-6
IL-11
OSMRβ
gp130
gp190
gp130
CNTFRα
gp190
gp130
IL-6Rα
gp130
IL-11Rα
gp130

Recently, however, the existence of additional 150 kD and 140 kD components in the receptor complexes for LIF and OSM respectively has been reported (Heyman et al., 1996). If these additional components were ubiquitously expressed, it might partially explain why their presence has hitherto gone unnoticed, since high affinity binding of LIF, equivalent to that seen on LIF-binding cells, can be conferred upon multiple non-LIF-binding cell types by transfecting them with gp190 and / or gp130 expression constructs (Gearing et al., 1994). Signal transduction from the LIF receptor complex is discussed below and summarised diagrammatically in Figure 1.2.

Both gp190 and gp130 are members of the haematopoietin family of non-protein-kinase cytokine receptors and they exhibit strong structural homology to one another and to the β-chains of other IL-6 sub-family cytokine receptors (Gearing et al., 1991; Mosley et al., 1997). The extracellular domains of gp190 and gp130 both possess an immunoglobulin-like domain which is followed by two fibronectin III-like (FN-III) domains that, together, are referred to as the haematopoietin domain. The first FN-III domain contains four conserved cysteines while the second FN-III contains the conserved amino acid sequence WSXWS (Grotzinger et al., 1997). Two haematopoietin domains are found in gp190 while gp130 contains only one. This, along with evidence from mutagenesis studies, has lead to the suggestion that the haematopoietin domains form at least three spatially separated sites of interaction between the LIF receptor complex and the LIF molecule (Robinson et al., 1994; Layton et al., 1994a; Layton et al., 1994b), as is the case for growth hormone (de Vos et al., 1992).

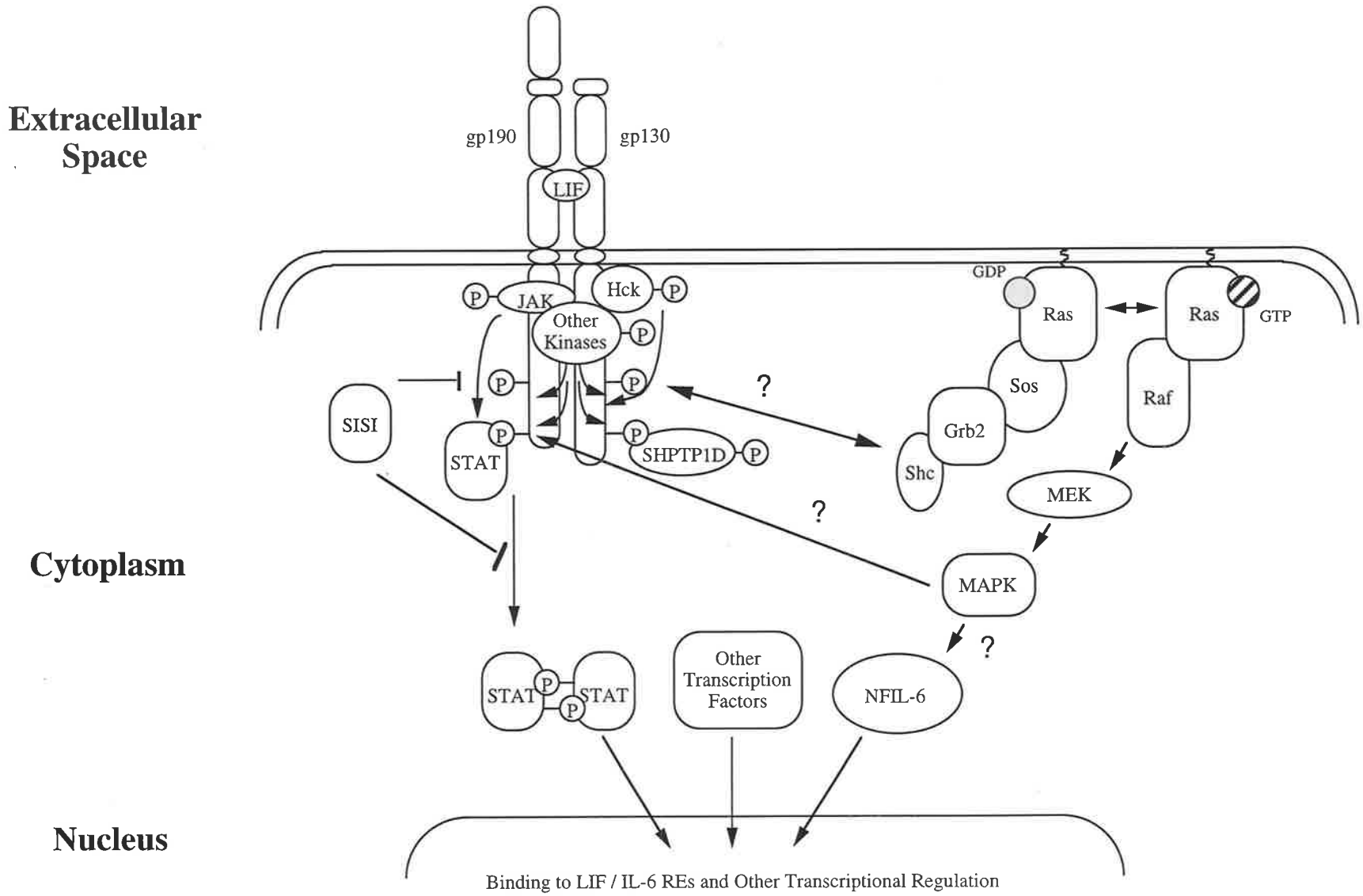
The single transmembrane domains of gp190 and gp130 are followed by cytoplasmic domains consisting of modular, non-catalytic tyrosine-based motifs which bind components of the signal transduction apparatus specifically (Stahl et al., 1994). The creation of receptor subunits bearing mutations and deletions in their cytoplasmic domains has enabled the dissection of signal transduction events that are mediated by specific motifs in gp130-containing receptor complexes (Baumann et al., 1994a; Baumann et al., 1994b). Some enzymatic activities, such as the Janus kinases (JAKs), are constitutively associated with gp130 (Luticiken et al., 1993) and the induction of their activity is one of the first events detected upon receptor complex formation (Stahl et al., 1994). Activation of these kinase activities causes the intracellular domains of gp130 and gp190 to become heavily tyrosine-phosphorylated upon signalling complex formation (Murakami et al, 1991; Ip et al., 1992) and this promotes the recruitment and activation of other

Figure 1.2 Putative Signal Transduction Pathways from the LIF Receptor Complex

Schema summarising some of the events suggested to be involved in signal transduction from the LIF receptor complex to the nucleus.

These pathways represent a generalised situation only. See text (section 1.4.3) for explanation and discussion.

(Adapted from: Hibi et al., 1996)


Extracellular Space
Cytoplasm
Nucleus
gp190
gp130
LIF
JAK
Hck
Other Kinases
P
SISI
STAT
SHPTP1D
?
GDP
Ras
GTP
Sos
Grb2
Shc
Raf
MEK
MAPK
STAT
STAT
Other Transcription Factors
NFIL-6
Binding to LIF / IL-6 REs and Other Transcriptional Regulation

enzymes and adaptor proteins to the complex, some via phospho-tyrosine-binding SH2 domains (Hibi et al., 1996). Given the degree of amino acid sequence conservation in the cytoplasmic domains of gp130 and gp190, it is likely that similar arrays of proteins can associate with their intracellular domains (Gearing et al., 1991; Murakami et al., 1991).

1.4.3.1 Kinases Associated with the LIF Receptor Complex

Of the eight currently recognised families of non-receptor tyrosine kinases (Hardie & Hanks, 1995), ligand-dependent phosphorylation and / or association of members of the JAK (Stahl et al., 1994), Src (Ernst et al., 1994), Tec / Btk (Matsuda et al., 1995a), and Fes (Matsuda et al., 1995b) families with gp130 and / or gp190 has been demonstrated. The ras superfamily of membrane-anchored, G-protein serine / threonine kinases can also be recruited to gp130-containing signalling complexes by SH2 domain-containing adaptor proteins such as SHC (Ernst et al., 1996). JAKs activate a family of proteins known as the signal transducers and activators of transcription (STATs) (Ihle, 1994) and the ras kinases are known to provide one mechanism for the activation of MAP kinases (Thoma et al., 1994). MAP kinases themselves appear to be capable of phosphorylating gp190 (Schiemann et al., 1995), which supports evidence suggesting that JAKs and MAP kinases belong to the same super-family (Winston & Hunter, 1996). However, since all of this work has been performed in different, often cell-free, in vitro systems, mostly using agonists other than LIF, the relevance of these findings to the biological action of LIF remains to be elucidated.

However, stimulation of ES cells with LIF has been shown to immediately induce the activation of JAK 1, JAK 2 and Tyk 2 kinases, the phosphorylation of the SH2-containing adaptor protein SHC, the activation of $p21^{ras}$ guanine nucleotide exchange activity, and an elecrophoretic mobility shift in MAP kinase (Ernst et al., 1996). Furthermore, inhibition of JAK 1 activity in ES cells suggests that activation of both the JAK-STAT and *ras* - MAP kinase signalling pathways is required for full ES cell maintenance activity (Ernst et al., 1996).

Finally, there is some evidence that "cross-talk" occurs between the signal transduction pathways for LIF and other cytokines. For example, insulin stimulation of gp190-transfected rat H-35 hepatoma cells results in phosphorylation of gp190 by MAP kinases at serine 1044 and the attenuation of acute phase protein reporter gene activity (Schiemann et al., 1995).

1.4.3.2 Nuclear Targets of LIF Signalling

Genes that are transcriptionally regulated by LIF *in vivo* are yet to be definitively identified. However, a role for LIF in regulation of proteinase and proteinase inhibitor gene expression during blastocyst implantation has been suggested (Harvey et al., 1995). Also, the abilities of LIF and IL-6 to affect hepatocyte gene expression *in vitro* (Baumann et al., 1992) have been used to identify transcription factor complexes activated by gp130 signalling. Two classes of "LIF / IL-6 response element" have been identified in the promoters of acute phase protein genes and characterised using hepatic cell lines: one binds complexes containing STAT 3 (Hocke et al., 1993) and one binds complexes containing NF-IL6, a member of the C-EBPβ family of transcription factors (Akira et al., 1990). While STAT 3 can be activated, directly and / or indirectly, by JAKs, NFIL-6 can be activated by the ras signalling cascade in hepatocytes (Fey et al., 1995). Subsequently, it was shown that the STAT 3-binding response element could also confer LIF-induced transcriptional activation upon reporter genes in ES and EC cells (Hocke et al., 1995). Additionally, LIF stimulation of ES cells induced a DNA binding activity that appeared to be identical to that characterised in hepatic cells which disappeared upon differentiation of the ES cells by LIF withdrawal (Hocke et al., 1995). Thus, some LIF signalling activities may be shared by cell types which respond differently to LIF.

Transcriptional regulation by LIF has been demonstrated in other contexts: LIF and IL-6 induce the expression of immediate early genes such as c-*fos* and *jun* B in a variety of cultured cells, an effect that is mediated by a complex promoter element which binds, among other things, multiple STAT proteins (Fujitani et al., 1994; Baumann et al., 1992; Yamamori, 1991). This activity is sensitive to, but not abrogated by, inhibitors of both tyrosine- and serine / threonine-kinases, indicating that at least two pathways for STAT activation exist (Nakajima et al., 1993).

Receptor complex formation involving gp130 is also known to suppress apoptosis and stimulate DNA synthesis, effects which are separable by mutagenesis of the cytoplasmic domain of gp130 (Fukada et al., 1996; Baumann et al., 1994b). Although the basis of the proliferative effects is not known, in cardiomyocyte cultures the anti-apoptotic effect is partly due to induction of Bcl XL gene expression by the binding of a complex containing STAT 1 and STAT 3 to an element in its promoter (Fujio et al., 1997). Enforced expression of the SCL / TAL-1 transcription factor can inhibit the LIF-induced differentiation of M1 cells (Tanigawa et al., 1995),

it is therefore possible that this protein may be involved in the regulation of cellular decision making and transcription by LIF.

The mechanisms by which LIF signalling is terminated and the capacity for LIF response is recovered have only recently begun to be elucidated. Recently, a family of at least six SH2-containing proteins, the STAT-induced STAT inhibitors (SISIs), that appear to be capable of binding to and inhibiting JAKs and also competitively inhibiting the binding of STATs to receptor complexes, including gp130, has been identified (Yoshimura et al., 1995; Starr et al., 1997; Naka et al., 1997). It appears that the expression of these proteins may be up-regulated by STAT-responsive promoter elements (Endo et al., 1997). The SH2-containing protein tyrosine phosphatase SH-PTP1D is also recruited to gp130 and phosphorylated in response to receptor complex formation (Hibi et al., 1996) and it seems conceivable that this also has a negative effect on LIF signalling (Frearson & Alexander, 1997). Activated STATs bind to the gp130 promoter and increase gp130 transcript expression, presumably facilitating the regeneration of receptors at the cell surface (O'Brien & Manolagas, 1997).

1.4.4 Modulation and Compartmentalisation of LIF Activity

The murine LIF protein has been found in serum complexed with a truncated form of gp190 that acts as a LIF binding protein (Layton et al., 1992) and also associated with the extracellular matrix (Rathjen et al., 1990a). The rat LIF protein has been found associated with the extracellular matrix as a complex with an immobilised binding protein (Mereau et al., 1993). Murine serum LIF binding protein has an apparent molecular weight of 90 kD and is produced by alternate splicing of the gp190 transcript (Owczarek et al., 1996). It is present at high levels (~1 μg/ml) in healthy mouse serum and was found to antagonise LIF action on cultured cells. It may therefore function to prevent systemic LIF action (Layton et al., 1992). In contrast, the matricial LIF binding protein has an apparent molecular weight of 140 kD and can present LIF to cells in culture in an active form, thus making it probable that LIF becomes localised to the matrix in order to limit the cytokine to a paracrine mode of action (Mereau et al, 1993).

In healthy humans, soluble forms of gp130 are found in serum, also at high levels (~4 μg/ml), and these forms of gp130 could inhibit signalling through membrane-anchored gp130 (Narazaki et al., 1993). It was subsequently reported that 50 and 100 kD soluble forms of gp130

and a 140 kD soluble form of gp190 are present in human plasma and urine (Zhang et al., 1998) but the molecular basis for the production of these species remains unknown. On the basis of studies performed to date (Tomida et al., 1996; Michel et al., 1997), it seems that the molecular mechanisms generating soluble LIF receptor components are not well conserved between species. It is also possible that other, as yet uncharacterised, mechanisms for modulating LIF activity exist, such as differential glycosylation (sections 1.2.10 and 1.4.1).

1.4.5 The LIF Gene and LIF Transcription

The LIF gene as it has been characterised in all species consists of three exons. The first exon encodes the 5' untranslated region, and the amino terminal end of the pre-protein. Exon 2 encodes the LIF hydrophobic leader sequence, the mature protein cleavage site and the first third of the mature protein. Exon 3 encodes the rest of the mature protein and the extensive 3' untranslated region (Stahl et al., 1990). Four TATA or TATA-like elements exist in the 5' region of the LIF gene, with the TATA-box at -31 and the 72 bp upstream acting as the promoter for LIF transcription (Stahl and Gough, 1993). The encoded transcript is approximately 4.2 kb long, with about 3.4 kb of this comprising 3' untranslated region (Stahl et al., 1990).

The sequences of the LIF genes from a number of species have now been described: human (h) and murine (m) LIF (Stahl et al., 1990), porcine (p) and ovine (o) LIF (Wilson et al., 1992), and bovine (b) LIF (Kato et al., 1995). A high degree of cross-species conservation is evident in both the coding regions, particularly in the open reading frame, and putative regulatory regions of the LIF gene (Wilson et al., 1992). The regions of sequence conservation outside the coding sequence correspond to proposed proximal promoter elements, a pyrimidine rich region and the region of the mLIF-M exon in intron 1, and regions in the 3' untranslated region corresponding to instability elements and the 3' end of the message (Stahl et al., 1990; Wilson et al., 1992).This high degree of conservation indicates that important biological roles are fulfilled the LIF protein in all eutherian mammals.

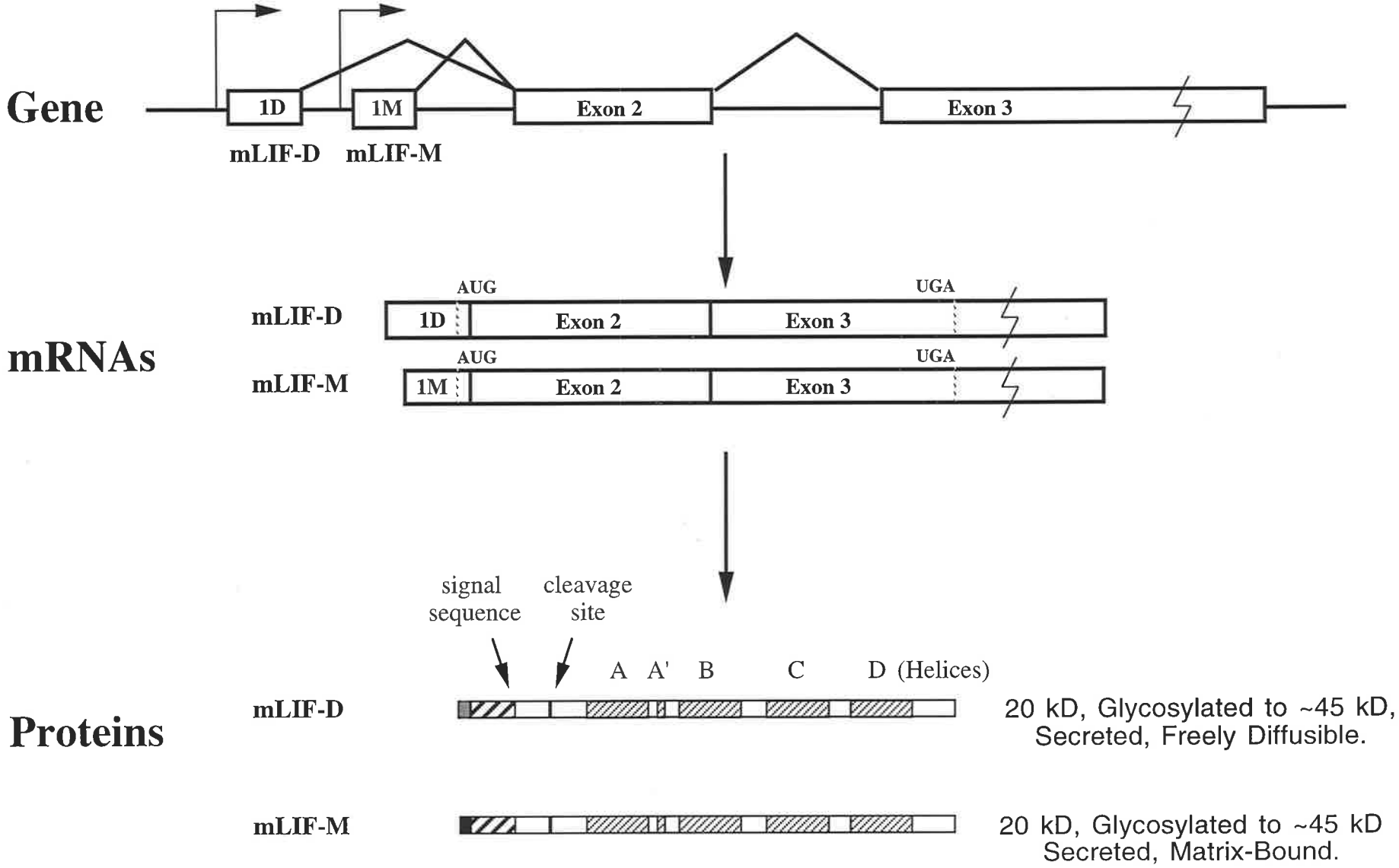
Two different mLIF transcripts have been described (Figure 1.3). These transcripts are thought to arise from alternate promoter usage and contain alternate first exons which are spliced onto common second and third exons of the LIF gene (Rathjen et al., 1990a). In-frame ATG initiation codons in the first exons of these transcripts direct production of precursor proteins that

Figure 1.3 Organisation and Expression of the Murine LIF Gene

Two transcripts, mLIF-D and mLIF-M, with distinct first exons are produced, apparently as a result of alternate promoter usage. These transcripts encode precursor proteins with differences of only 7 and 4 amino acids at their N-termini. The mature mLIF-D and mLIF-M proteins are identical since the signal sequence-containing N-terminus of these proteins is proteolytically removed at a common cleavage site (Rathjen et al., 1990a).

The exonic constitution of the two mLIF transcripts is shown with the longer third exon shown shortened relative to the other exons. The positions of AUG translational initiation codons and UGA translational termination codons that form the boundaries of the mLIF-D and mLIF-M open reading frames are indicated.

The thickly cross-hatched region of the mLIF-D protein contains the core hydrophobic region of its secretion signal sequence that is cleaved at the indicated site to produce the mature protein. The lightly cross-hatched regions contain sequences that form α helices in the crystallised mLIF-D protein (Robinson et al., 1994).


Gene
1D
1M
Exon 2
Exon 3
mLIF-D
mLIF-M
mRNAs
mLIF-D
AUG
UGA
1D
Exon 2
Exon 3
mLIF-M
AUG
UGA
1M
Exon 2
Exon 3
signal sequence
cleavage site
A A' B C D (Helices)
Proteins
mLIF-D
20 kD, Glycosylated to ~45 kD,
Secreted, Freely Diffusible.
mLIF-M
20 kD, Glycosylated to ~45 kD
Secreted, Matrix-Bound.

differ in the first few amino terminal amino acids encoded by the first exons (Figure 1.3). Both preproteins contain secretory signal sequences and upon proteolytic cleavage during secretion, produce extracellular, glycosylated proteins whose 20 kD backbone polypeptides appear to be identical (Rathjen et al., 1990a). The proteins produced from the two transcripts can be differentially localised following overexpression *in vitro*, with one producing a diffusible LIF activity (LIF-D) and the other an activity localised to the extracellular matrix (LIF-M). Since the two mature proteins are identical in sequence, the alternative localisation of the two molecules must be directed by an unknown mechanism utilising determinants in either the different first exons of the messenger RNAs or in the short amino-terminal regions of the precursor proteins.

The two LIF transcripts have been shown to be differentially regulated both in cell culture and in tissues. In Ehrlich ascites cells the diffusible transcript predominates but in Pys-2 cells the matrix transcript is expressed more strongly (Rathjen et al., 1990b) while in the femur the LIF-D transcript predominates, however in the brain the LIF-M transcript is almost exclusively expressed (Robertson et al., 1993). The LIF-M transcript has also been shown to be predominantly expressed in the hypothalamus and the pituitary while expression of LIF-D only is induced in response to endotoxin in culture (Wang et al., 1996). Such differential transcript expression indicates that distinct biological roles are likely to be served by the encoded proteins. This assertion is supported by the observation that chimeric mouse embryos overexpressing LIF-M transcripts underwent developmental arrest prior to primitive streak formation whereas those overexpressing LIF-D transcripts looked essentially normal (Conquet et al., 1992). While there is strong evidence for matricial LIF activities in the mouse and rat (Rathjen et al., 1990a; Mereau et al., 1993), matricial LIF activities in other species have yet to be identified.

Although there is extensive sequence homology in the region of intron 1 corresponding in location to the mLIF-M first exon in all of the characterised LIF genes, the ATG start codon and splice donor site are not conserved (Wilson et al., 1992; Hsu et al., 1994). This indicates that differences exist in the organisation and expression of the LIF gene and protein between rodents and other eutherian mammals.

1.4.6 Expression of LIF and LIF Receptor Subunits

Studies of the distribution of LIF mRNAs indicate that the cytokine is likely to be present in a wide variety of embryonic and adult contexts and also that LIF action may be important in the maintenance of stem and progenitor cell populations. LIF-D and LIF-M transcripts are first expressed very early in mouse embryogenesis, in the trophectoderm of 3.5 day-post-coitum blastocysts and in the trophoblast and ectoplacental cone of 5.5 day post-implantation embryos (Nichols et al., 1996; Conquet & Brulet, 1990). LIF continues to be expressed in the placenta and other extraembryonic tissues between 6.5 and 14.5 days post-coitum (Rathjen et al., 1990b; Robertson et al. 1993; Conquet & Brulet, 1990). Between 16.5 days post-coitum and birth, increasing, differentially regulated, levels of LIF-D and LIF-M transcripts are detected in developing skin, lung, intestine and bone calvaria (Robertson et al., 1993).

In the adult mouse, differential expression of LIF transcripts is detectable in a variety of tissues with increased levels in uterus, intestine, lung, skin, oviduct, ovaries, testes, bone marrow and thyroid (Robertson et al., 1993). Expression of LIF in the human uterus has been localised to the endometrium where it was greatest later in the menstrual cycle (Kojima et al., 1994; Cullinan et al., 1996). *In situ* hybridisation demonstrated that the major site of uterine LIF-D expression in the mouse was the endometrial glands and that the peak in expression coincided with blastocyst implantation (Bhatt et al., 1991). In the adult rat, LIF transcripts have been detected in the brain and the peripheral nerves where it is expressed in areas of developing cholinergic, but not adrenergic, innervation (Yamamori, 1991). Furthermore, LIF transcript expression increases dramatically after injury to adult rat and mouse peripheral nerves (Curis et al., 1994; Banner and Patterson, 1994; Kurek et al., 1996). Finally, many cultured cell lines are known to express LIF transcripts, including murine embryonic stem cells (Rathjen et al., 1990a) and cells of haematopoietic and tumour origin (Szilvassy et al,. 1996; Kamohara et al., 1997; Gascan et al., 1990). There are few reports describing distribution of the LIF protein but it is clear that the uterine epithelium during ovulation is a major site of LIF protein expression (Bhatt et al., 1991; Cullinan et al., 1996) and there are data suggesting that lung epithelium, skin, and skeletal musle express lower levels of LIF protein (Lofler, 1998).

The expression patterns of the LIF receptor component mRNAs indicate the potential for widespread LIF action in embryonic and adult life. In mice expression of gp190 is detected in ES

and other cultured cells, liver, lung, testes, ovaries, placenta and during foetal liver haematopoiesis. Lower level, but not ubiquitous, expression is seen in many other adult tissues (Tomida et al., 1994; Owczarek et al., 1996). Similar gp190 expression patterns are seen in human tissues (Cullinan et al., 1996). Expression levels of rat gp190, in contrast to those of rat LIF, were shown to decrease with peripheral nerve injury (Banner and Patterson, 1994). Expression of the murine serum LIF binding protein transcripts was detected in the liver, uterus and placenta and an increase in expression was seen during early pregnancy (Owczarek et al., 1996).

Expression of mouse and human gp130 appears to be ubiquitous, however, expression levels vary in several cultured cell types and tissues (Hibi et al., 1990; Saito et al., 1992; Cullinan et al., 1996). In the peri-implantation mouse embryo, gp190 expression was found to be localised to the inner cell mass, with expression also present in the uterine epithelium and endometrial glands at 4.5 days post-coitum and in the decidua at 5.5 days post-coitum (Nichols et al., 1996). Apart from some low level expression in the trophectoderm, areas of gp130 expression in the mouse peri-implantation embryo overlap with gp190 expression (Nichols et al., 1996). During early human development, gp190 is expressed by blastocysts and gp130 is expressed by morulae and blastocysts (Sharkey et al., 1995). Thus, since the expression of gp130 appears to be ubiquitous, the sites of LIF are likely to be determined by differential gp190 expression. However, due to the utilisation of these receptor components by other IL-6 sub-family cytokines, the relevance of these data for *in vivo* LIF function is unclear.

1.4.7 LIF as a Stem Cell Maintenance Factor *In Vitro* and *In Vivo*

The maintenance of inner cell mass-derived murine embryonic stem cells in a pluripotential state requires LIF (Williams et al., 1988; Smith et al., 1998) or other gp130 agonists (Piquet-Pellorce et al., 1994). Subsequently, it was found that LIF was expressed by ES cells and that its expression levels increased upon their differentiation (Rathjen et al., 1990b). LIF expression in differentiated cells could also be demonstrated to be regulated by cytokines which are produced by ES cells (Rathjen et al., 1990b). These and other observations lead to the suggestion that LIF might be involved in the maintenance of stem cell populations by a feedback mechanism involving the paracrine action of LIF and other cytokines (Rathjen et al., 1990b; Smith et al., 1992). In support of this notion, the use of a LIF / β-galactosidase dicistronic gene-

targeting construct has demonstrated that such stem cell "niches" are in fact surrounded by areas of higher level LIF transcript expression (Mountford et al., 1994). Co-culture of isolated primitive murine haematopoietic progenitor cells and stromal cells has demonstrated a similar phenomenon whereby addition of exogenous LIF upregulated the expression of cytokines by stromal cells that promoted the long-term self-renewal of the progenitors (Szilvassy et al., 1996). *In vitro,* LIF can also act as a growth and / or survival factor for murine primordial germ cells (De Felici et al., 1991; Matsui et al., 1991) which express functional LIF receptors (Cheng et al., 1994). LIF is also necessary for the long-term culture of primordial germ cell-derived embryonic germ cells (Matsui et al., 1992; Resnick et al., 1992), at least in part because it suppresses their death by apoptosis (Pesce et al., 1993). It was also shown that olfactory neuronal progenitors isolated from 14.5 day-post-coitum mouse embryos proliferated in response to LIF but not CNTF or IL-6 (Satoh & Yoshida, 1997) and that LIF supported the long-term survival of cultured rat embryonic motor neurons (Martinou et al., 1992). The ability of LIF to support renewal of multiple stem and progenitor cell populations *in vitro* has focussed attention on a potential general role for LIF in the maintenance of stem and progenitor cells, particularly pluripotential cells, *in vivo* (Smith et al., 1993).

Many of the tissues in which LIF is expressed at higher levels in the adult, such as the adult femur, thymus, gonads, skin, and intestine, have resident stem and progenitor cell populations (Robertson et al., 1993). Indeed, the studies of *Lif* $^{-/-}$ mice carried out so far give some support to the idea that LIF is involved in the maintenance of stem and progenitor cell populations *in vivo*. While all types of haematopoietic stem and progenitor cells appear to be present in *Lif* $^{-/-}$ mice, their numbers are reduced and the degree of their reduction varies between the splenic, thymic, and bone marrow compartments (Escary et al., 1993). Because no qualitative effects of LIF gene ablation on haematopoietic differentiation were evident, it was suggested that LIF's primary function was in support of stem cell self-renewal (Escary et al., 1993).

1.4.8 Analysis of LIF Function

Exogenous addition of LIF protein *in vitro* or *in vivo* and gain-of-function genetic analyses provide only suggestive, not definitive, evidence of potential *in vivo* LIF functions. Studies utilising gene-targeting technology in mice have confirmed the pleiotropic and redundant

nature of LIF action *in vivo*. Mice with both their LIF genes ablated are viable but their body weight is reduced by 25-35% (Stewart et al., 1992). These studies have revealed roles for the LIF protein in blastocyst implantation (Stewart et al, 1992), thymic T cell activation and the generation of splenogenic myeloid progenitors (Escary et al, 1993), the response of sympathetic neurons to injury (Rao et al., 1993), the recovery of skeletal muscle from crush injury (Kurek et al., 1997), a redundant role in the support of motor neuron function (Sendtner et al, 1996; Li et al., 1995), and induction of the hypothalamo-pituitary-adrenal stress response by interleukin-1 (Auernhammer et al., 1998; Chesnokova et al., 1998). The phenotype of LIF knockout mice is thus very complex and el ucidation of the cellular and molecular basis of these effects will require a considerable effort in the future.

Due to the partially redundant (Piquet-Pellorce et al., 1994) and perhaps combinatorial action of IL-6 sub-family cytokines, studies of mice in which the genes for gp190 and gp130 have been ablated may indicate other potential *in vivo* roles for LIF. Breeding to produce mice with multiple IL-6 sub-family cytokine and / or receptor subunit genes ablated should continue to provide insights into which of these roles are significantly fulfilled by LIF (see for example Sendtner et al., 1996 and Li et al., 1995). Disruption of both genes for gp190 produces mice with a body weight reduced by 20% that die within 24 hours of their birth due to a combination of defects in placentation / foetal nutrition and liver metabolism as well as a severe reduction in motor neuron numbers which seems to prevent proper suckling and locomotion (Ware et al., 1995; Li et al., 1995). While proliferation in the germ cell and haematopoietic lineages seems to be unaffected, osteoblast and astrocyte numbers were found to be significantly reduced in *gp190* $^{-/-}$ mice (Ware et al., 1995). Inactivation of both gp130 genes results in the death of mice late, rather than early, in embryogenesis, between 16.5 days post-coitum and term, due to defects in cardiomyocyte and haematopoietic stem and progenitor cell proliferation which result in poor heart development and anaemia (Yoshida et al., 1996). Primordial germ cell numbers in *gp130* $^{-/-}$ mice are also reported to be reduced by up to 60% (Koshimizu et al., 1996).

1.4.9 Other Potential Roles for LIF

The finding that exogenous LIF acts as a trophic factor for embryos in culture (Lavranos et al., 1995) is intriguing considering the observed reduction in body weight of *Lif* $^{-/-}$ mice

(Stewart et al., 1992) , the poor nutrition during foetal life and reduced body weight of *gp190* $^{-/-}$ neonates (Ware et al., 1995; Li et al., 1995) and the continuous expression of LIF transcripts by the extra-embryonic tissues and placenta during mouse embryogenesis (Robertson et al., 1993). Together, these obsevations suggest that LIF might be involved in promoting foetal growth during embryonic development.

It is also possible that LIF may perform other roles in embryogenesis that are not strictly essential. LIF-D transcripts are expressed at particularly high levels by the oviduct and uterine epithelium during ovulation (Robertson et al., 1993; Bhatt et al., 1991). It would thus be expected that the pre-implantation embryo would be exposed to continuously high concentrations of LIF. The ability of both exogenous LIF (Shen & Leder, 1992) and a LIF transgene (Conquet et al., 1992) to prevent the gastrulation of mouse embryos is interesting since, when this is taken together with the expression data, it may suggest a role for LIF in preventing gastrulation prior to implantation or in retarding early embryonic development to allow recovery from insult or injury. This is consistent the finding that LIF expression serves to delay formation of the primitive ectoderm and retard its differentiation (Rathjen et al., 1999; Sanderson, 1997; J. Rathjen, unpublished results)

In culture, LIF is secreted by retinal Muller cells and blocking this activity with neutralising antibodies to LIF appears to have an inhibitory effect on the development of rod photoreceptors (Neophytou et al., 1997). These findings may be developmentally relevant.

The observation that exogenous LIF could promote the cholinergic differentiation of developing sympathetic neurons lead to the suggestion that LIF might function as a target-derived neuronal differentiation factor (Murphy et al., 1991; Patterson & Nawa, 1993). This hypothesis was also consistent with the expression of the LIF transcript by a subset of sympathetic neurons (Yammamori et al., 1991). Although cholinergic innervation of the pancreas could be promoted by expression of a LIF transgene from the insulin promoter in mice (Bamber et al., 1994), sympathetic nerve innervation in *Lif* $^{-/-}$ mice appeared to be normal (Rao et al., 1993). Thus, while it is possible that LIF plays a role in this process, does not appear to be an essential one.

LIF is expressed in the adult pituitary (Wang et al., 1996) and exogenous LIF affects the differentiation and proliferation of cultured pituitary cells, therefore mice expressing a LIF transgene under the control of the growth hormone promoter were used to study pituitary

development (Akita et al., 1997). Differentiation of the pituitary epithelium into functional cells was severely perturbed in these transgenic animals, a phenotype resembling cystic Rathke's disease in humans. Perturbations of epithelial and lymphoid differentiation processes were also seen in the thymi of mice carrying a LIF transgene expressed in T cells (Shen et al., 1994).

Finally, there is also some experimental evidence that LIF may act as mediator of acute and chronic inflammatory responses and may be involved in the pathogenesis of diseases such as arthritis (Lotz et al., 1992), sepsis (Jansen et al., 1996) and cachexia (Alexander et al., 1994), generally as a result of overproduction induced by pro-inflammatory cytokines such as TNFα.

1.4.10 Possible Intracellular Roles for LIF

Intracellularisation and retrograde transport of radio-labelled LIF and CNTF by sensory and motor neurons has been reported (Cutis et al., 1994; Ure & Campenot, 1994; Hendry et al., 1992). Results from studies where unlabelled LIF and CNTF were used to compete with radio-labelled LIF and CNTF for uptake by neurons were consistent with a receptor-mediated mechanism of uptake involving gp190 (Curtis et al., 1994). Furthermore, intracellularised LIF remained largely intact (Ure & Campenot, 1994) and the rate of LIF retrograde transport in peripheral nerves increased in response to crush injury (Curtis et al., 1994). Taken together, these observations suggest that the intracellularisation of LIF, at least by peripheral neurons, may be biologically relevant.

Hep3B hepatocarcinoma cells, which express hLIF transcripts, when co-transfected with a gp190 expression construct and a reporter construct for LIF activity, exhibit basal activation of the reporter gene. Moreover, this activation is resistant to inhibition by neutralising antibodies directed against hLIF (Baumann et al., 1993). It was suggested that this effect was consistent with either a receptor-mediated intracellular activity for hLIF, or ligand-independent activation of the transfected receptor (Baumann et al., 1993). The parallel observation that a number of IL-6 activities are resistant to inhibition by neutralising antibodies but not IL-6 antisense oligonucleotides, (section 1.2.8) lends some support to the former possibility. Thus, several lines of evidence provide indirect support for intracellular LIF activity.

1.4.11 The Biological Function of LIF: Problems and Possibilities

Comparison of LIF's potential biological roles with its roles established by gene ablation analysis, indicate many questions about LIF function remain unanswered. For example, expression mapping has indicated widespread expression of LIF and its receptor components in tissues not obviously affected in *Lif* $^{-/-}$ mice (Nicholls et al, 1996; Robertson et al, 1993). The correspondence of some of these sites of expression to cell populations responsive to exogenous LIF *in vitro* point to additional roles for LIF which have not yet been uncovered by genetic analysis, possibly because they are masked by partial or complete functional redundancy of the IL-6 sub-family cytokines (Piquet-Pellorce et al, 1994). Analysis of the phenotype of LIF and LIF receptor component knockout mice has revealed many subtle cellular effects and the penetrance of these varies with the genetic background of the mice used (L.M. Whyatt and A.G. Smith, unpublished observations). Thus, further analyses aimed at discovering these subtle, yet potentially important, aspects of LIF function are warranted.

In this regard, the potential involvement of LIF in pluripotential cell maintenance is of interest. Although separate ablation of the genes for LIF, gp190, and gp130 appears to have no effect on pluripotential cells in early embryogenesis, no examinations of peri-implantation embryos carrying any of these gene disruptions have been described. It is thus possible that early embryonic development in these mice is, in fact, quite aberrant yet sufficiently sound for the continuation of foetal life. Full elucidation of LIF function and signalling will require further refinements of the techniques of genetic analysis.

1.5 The Molecular and Cellular Basis of Cancer

1.5.1 Defective Cellular Decision Making Underlies Cancer

Cancers occur as a result of cells gaining the ability to or being allowed to proliferate cell-autonomously, an event referred to as transformation. If unchecked, this will result in a burgeoning tumour cell population undermining the cellular community and, eventually, in the organism's death. Implicit in the processes of cellular transformation and tumourigenesis are a partial or complete escape from, or failure of, the normal controls exerted upon a particular cell's

proliferation. Thus, defective cellular decision making underlies all cancers. Cancer can result from a variety of erroneous cellular decisions that result in inappropriate proliferation including migration from a location non-permissive to a location permissive for proliferation, failure of apoptosis, failure of differentiation, or inappropriate differentiation that permits proliferation (Green et al., 1996). When cellular decision making processes, such as those involving cell cycle progression and surveillance of the genome for damage, are normally coupled, that is controlled in an interdependent manner, cancer may ensue from a simple uncoupling of these processes which allows them to occur independently.

1.5.2 Oncogenes and Tumour Suppressor Genes

A large body of data suggestive of a fundamental genetic basis to cancer has now amassed. To the genes originally conceptualised as (viral) oncogenes have been added genes termed (cellular) proto-oncogenes and tumour suppressor genes (Bishop, 1995). Oncogenes are normal cellular genes which have been mutated and / or whose expression has been amplified in a manner or form which can contribute to the cancerous phenotype of a cell. Thus, cellular transformation events involving oncogenes are gain of function mutations and are generally considered to be dominant. The term proto-oncogene describes any normal cellular gene from which an oncogene may be derived. Well over one hundred oncogenes, involved in almost every conceivable facet of cellular decision making, have now been identified (Bishop, 1995).

The existence of tumour suppressor genes was first suggested by the observations that a predisposition to certain cancers can be inherited and that transformed cells fused with normal cells would sometimes lose their transformed phenotype (Bishop, 1995). Cellular transformation events involving tumour suppressor genes are loss of function mutations and are generally considered to be recessive. Many tumour suppressor genes encoding proteins with key negative regulatory functions in the cell have been identified (Lanfrancone et al. 1994).

1.5.3 Cancer Progression and the Accumulation of Genetic Change

Cellular transformation and tumour progression involve the accumulation of mutations in multiple proto-oncogenes and tumour suppressor genes. In colorectal carcinomas seven key mutations commonly occur at specific points in the progression of the tumour, these include

oncogenes and tumour suppressor genes commonly associated with cancers in general and those associated with colorectal carcinomas in particular (Fearon & Vogelstein, 1990). Although certain mutations will commonly occur in a given type of cancer due to their influence upon important cellular decision making processes in the cell population from which the tumour is derived, the tumour cells accumulate mutations in a haphazard manner with natural selection favouring the growth of cells with advantageous genotypes. This micro-evolutionary process results in increasing de-regulation of cellular decision making in tumour cells with time, making them increasingly mutable. Thus, an understanding of normal cellular decision making processes is fundamental to our ability to distinguish mutations that are genuinely tumour-promoting from those that are not, and to the inference of genetic cause-effect relationships in tumourigenesis.

1.5.4 Roles for Cytokines and Growth Factors in Cancer

Since defective cellular decision making underlies cancer and cytokine action can significantly influence cellular decision making in multifarious ways, the genes encoding cytokines are potentially proto-oncogenes and tumour suppressor genes, depending upon whether their net effect is to promote or suppress proliferation (Cross & Dexter, 1991). A finding which emphasises the potentially oncogenic nature of cytokines is that genes encoding cytokines and, apparently more commonly, their receptors have been transduced by tumour-promoting retroviruses (Callard & Gearing, 1993). For example, the v-*sis* oncogene appears to be derived from the PDGF B chain gene (Bejcek et al., 1992) while the v-*kit* oncogene appears to be derived from the stem cell factor receptor (Chabot et al., 1988). Further to this, many tumour-derived cell populations *in vitro* exhibit growth that is independent of cytokines that are required by their parent cell populations for growth. In some cases, this "factor-independent" growth is achieved through autocrine cytokine production. The inhibition of such autocrine activities will often result in growth inhibition and, sometimes, apoptosis. (For examples see Ethier, 1995; Collins, 1993; Green et al., 1996.)

There is some evidence to suggest that aberrations in cytokine expression are capable of promoting the oncogenic transformation of cells. Epigenetic changes affecting the expression and imprinting of the *Igf 2* gene have been correlated with a predisposition to Wilms tumours (Okamoto et al., 1997) and can promote the malignant hyper proliferation of pancreatic β-cell

tumours (Christofori et al., 1995). TGF α overexpression confers a selective advantage upon cultured breast carcinoma cells (Smith et al., 1995), indicating that the acquisition of genetic changes resulting in aberrant cytokine expression may also have a role later in the transformation process. Subversion, or even reversal, of the effects of existing cytokine activities on cellular decision making has also been found to be a result of the genetic changes that accumulate in some transformed cells. Examples of these phenomena include escape from negative regulatory effects of TGF β on cell proliferation in the gut epithelium which commonly occurs in colorectal carcinogenesis and the ability of some colorectal tumour cell populations to proliferate, rather than quiesce, in response to TGF β (Hague et al., 1993).

The potential of intracellular cytokine activities to contribute to oncogenic transformation by acting as cell-autonomous growth stimulators for tumour cells is intriguing. There is some evidence for the existence of such activities. Several different tumour cell lines are growth inhibited by IL-6 antisense oligonucleotides but not anti IL-6 neutralising antibodies (Barut et al., 1993; Kong et al., 1996) and, at least in one case (Lu & Kerbel, 1993), an inhibitory effect of IL-6 on proliferation seems to have been reversed in the later stages of tumourigenesis.

At present, the mechanisms by which aberrations in cytokine expression and subversions of cytokine-mediated cellular decision making contribute to transformation remain largely uncharacterised and, due to the pleiotropic nature of cytokine action and the haphazard nature of oncogenic transformation, they seem likely to vary widely among different tumour cell populations.

1.6 The Potential Involvement of LIF in Cancers, Particularly Germ Cell Tumours

1.6.1 Potential Roles for LIF in Cancer

A role for elevated LIF expression and / or LIF-stimulated proliferation in a diversity of cancers has been suggested. These include breast carcinoma (Estrov et al., 1995; Crichton et al., 1996; Kellokumpu-Lehtonen et al., 1996), kidney and prostrate cancers (Kellokumpu-Lehtonen et al., 1996), medullublastoma (Liu et al., 1996), melanoma (Paglia et al., 1995), meningioma

(Pergolizzi et al., 1994), oesophogeal cancer (Oka et al., 1995), and osteosarcoma (Marusic et al., 1993). In several rat colon carcinoma cell lines, augmentation or attenuation of LIF transcript expression has been shown to increase and decrease their tumourigenicity respectively (Burg et al., 1996). Broad surveys of LIF transcript and protein expression in human carcinoma cell lines determined that 12 out of 17 cell lines were secreting enough hLIF activity to be assayed (Gascan et al., 1990) and 30 out of 30 cell lines were expressing hLIF transcripts detectable by Northern blotting (Kamohara et al., 1996). Autocrine signalling by the LIF-related cytokines, oncostatin M and IL-6, have also been shown to induce the proliferation of tumour cell lines of varied origin including lung carcinomas (Horn et al., 1990), myelomas (Gu et al., 1996; Kawano et al., 1992), and Kaposi's sarcoma (Miles et al., 1990; Miles et al., 1992; Nair et al., 1992). In addition, the human IL-6 gene is now known to have been incorporated into the genome of the Kaposi's sarcoma-associated herpes virus (Molden et al., 1997), although the oncogenic role, if any, of this has not been determined. IL-6 also appears to be an intracellular autocrine growth stimulator for cell lines derived from melanomas, choriocarcinomas and leukaemias (section 1.5.4). These observations are indicative of potential roles for LIF and other members of the IL-6 sub-family of cytokines in a wide variety of cancers.

1.6.2 Germ Cell Tumours are Stem Cell Cancers

Germ cell tumours (GCTs) are so called because their cellular origin is thought to be from within the germ line. Their occurrence is rare in the general populace (incidence of 7 cases per 100 000 people), but they are particularly common in males aged between 15 and 55 years (Oliver, 1990) and their incidence is increasing (Clemmensen, 1981). Systematic study of GCTs has been confined to humans and mice, although they do occur in other species, and their aetiology and cellular origins remain contentious and somewhat obscure. In part this may be because these vary with the age of onset of the germ cell tumour (Jorgenson et al., 1995), between the human disease and the existing mouse models of it (Pera et al., 1990), and, perhaps, with the type of germ cell tumour (Pera et al., 1987). In the case of adult-onset GCTs in humans, a model, supported by cytogenetic evidence, suggesting a universal gonadal origin for GCTs whereby they result from the progressive transformation of meiotic prophase 1 spermatocytes (and perhaps oocytes) has been proposed (Chaganti et et al., 1994).

The histological classification and putative developmental interrelationships of the various germ cell tumour types are illustrated in Figure 1.4. Diverse tumours can be produced by the transformation of germ line cells, presumably as a consequence of the stem cell properties of the precursor cells. The malignancy of many types of germ cell tumours is sustained by niches of differentiating stem cells which sometimes exhibit the ability to form cell types derived from all three primary germ layers (pluripotentiality). This is supported by the observation that transplantable xenograft tumours showing pluripotential differentiation can be derived from purified human embryonal carcinoma stem cell populations (Pera et al., 1989). However, the stem cells of some germ cell tumours show a restricted potency, for example those of choriocarcinomas seem to form exclusively trophoblast-derived lineages, while yolk sac carcinomas seem to form only parietal and visceral endoderm-derived lineages (Andrews et al., 1987). Some germ cell tumour cells are incapable of differentiation and are described as being nullipotent. Germ cell tumours which differentiate toward the (eventual) elimination of stem cells are therefore benign and are termed "teratomas" while those which preserve a permanent stem cell population are therefore malignant and are termed "teratocarcinomas".

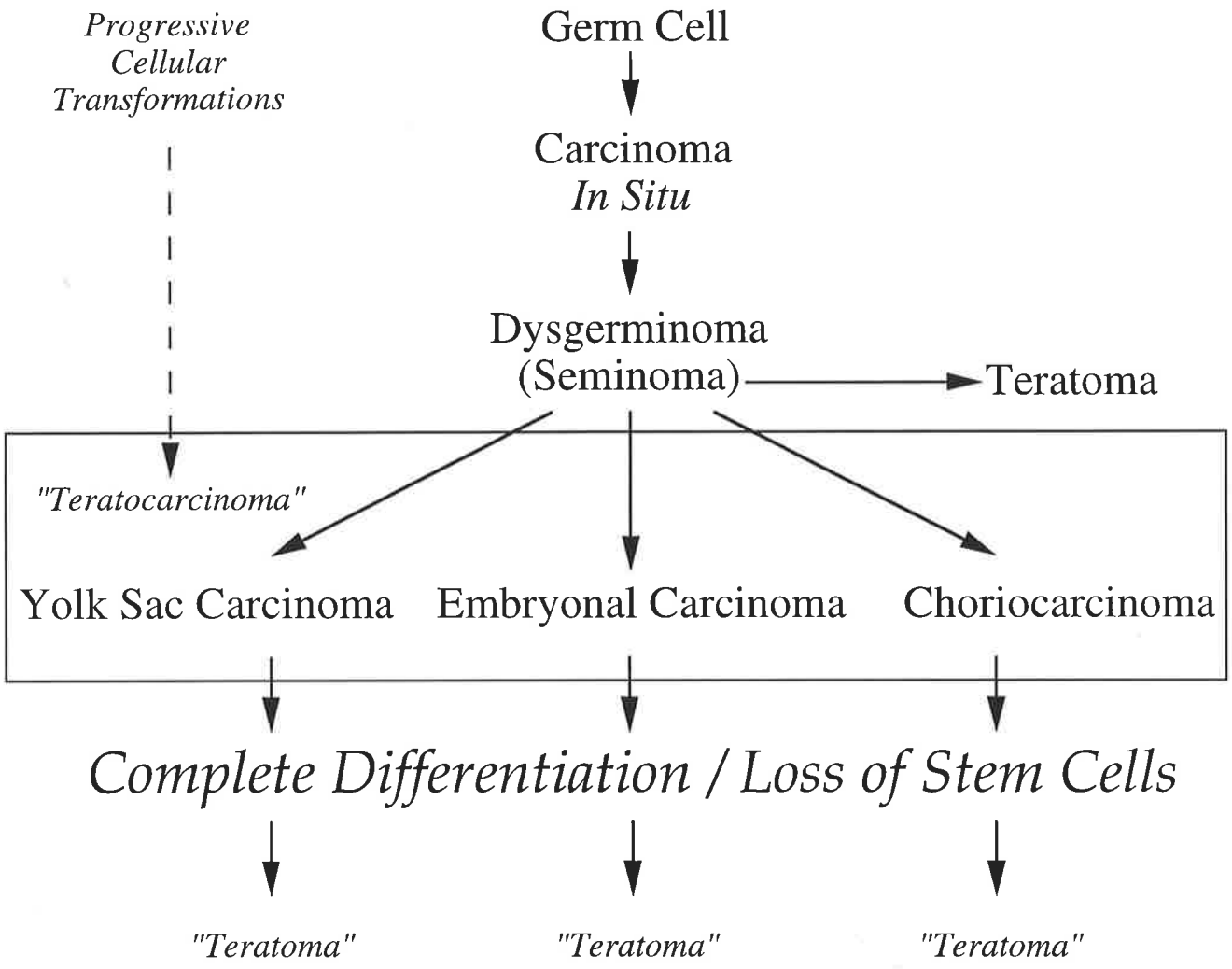
1.6.3 Experimental Models of the Germ Cell Tumours

When cultured *in vitro*, the stem cell populations from the various human germ cell tumours seem to retain the distinctive cell-surface antigen expression and many of the other characteristics of the original tumour stem cells from which they were derived (Andrews et al., 1996; Andrews et al., 1987) and they therefore provide a valuable resource for study (Roach et al., 1993). In mice, spontaneous germ cell-derived tumours resembling embryonal carcinomas form only in the inbred 129*sv* and LT strains (Stevens, 1983), although ectopic grafts either of whole pre-gonadal embryos or male genital ridges from most strains can produce tumours (Damjanov et al., 1987). Surprisingly, production of tumours by the ectopic grafts of embryonic material has not been successful in species other than mice (Pera et al., 1990). Stem cells, termed embryonal carcinoma (EC) cells, from such induced tumours and those occurring spontaneously in 129*sv* and LT mice can also be isolated into culture (Rudnicki et al., 1987). The existence of these genetic components to murine germ cell tumourigenesis (Damjanov et al., 1983) and the very different pathologies of murine and human teratocarcinomas (Pera, 1990) make some forms of

Figure 1.4 Histopathological Classification of Human Germ Cell Tumours

The putative developmental / pathological interrelationships of these tumours and their system of classification are indicated.

(Adapted from: Andrews, 1988)


Progressive
Cellular
Transformations
Germ Cell
Carcinoma
In Situ
Dysgerminoma
(Seminoma)
Teratoma
"Teratocarcinoma"
Yolk Sac Carcinoma
Embryonal Carcinoma
Choriocarcinoma
Complete Differentiation / Loss of Stem Cells
"Teratoma"
"Teratoma"
"Teratoma"

comparison between the disease states in the two species dubious. Certainly, *in vitro* isolates of human germ cell tumours are morphologically and phenotypically distinct from murine EC cells (Pera et al., 1987). Thus, there are strong arguments for using the human EC cell system as a means of gaining insights into human germ cell tumourigenesis.

The potential of *in vitro* murine and human teratocarcinoma stem cell systems to contribute to our understanding of mammalian embryogenesis has long been recognised (Martin, 1980; Roach et al., 1993) and this potential is now slowly beginning to be realised (see for example Roach et al., 1994). However, it is important to note that the generation of germ cell tumours and their subsequent differentiation may involve processes, perhaps including trans-differentiation, occurring pathologically but not during embryogenesis.

1.6.4 Potential Roles for LIF in Germ Cell Tumours

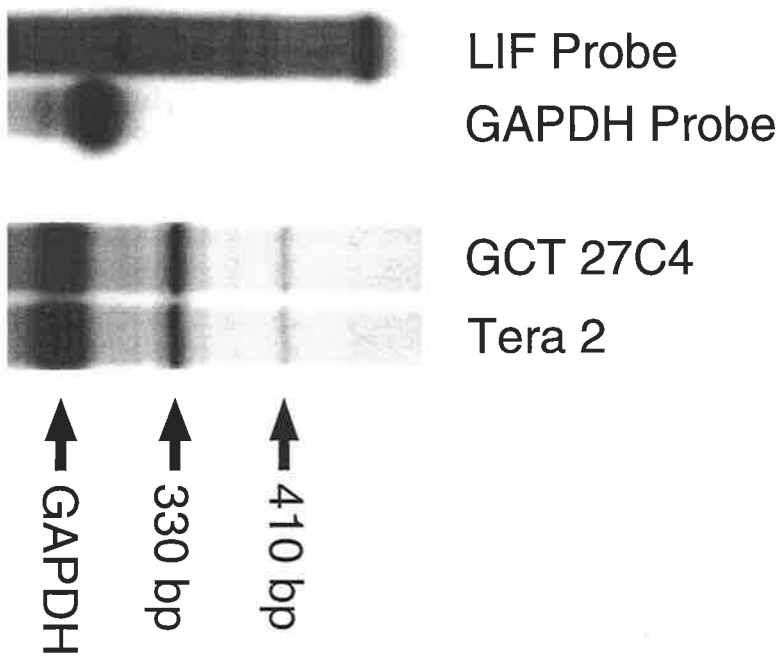
As discussed above (section 1.4.6), a great number of *in vitro* studies have implicated LIF in the maintenance of pluripotential cells, including primordial germ cells, in a proliferating, undifferentiated state. It is also worth re-iterating that studies aimed at determining the *in vivo* functions of LIF may not have uncovered all of its potential roles in pluripotential cell maintenance (section 1.4.7). Cell-autonomous LIF expression therefore has the potential to contribute to the oncogenic transformation of primordial germ cells. The observation that many cell lines derived from murine embryonal carcinomas show hyperploidies and rearrangements of chromosome 11 (which carries the mLIF gene) lends support to this proposition (Kola et al., 1990). It is also worth citing again the evidence for a widespread involvement of LIF and other IL-6 sub-family cytokines in cancers (section 1.6.1), and for the involvement of intracellular signalling by IL-6 in the cell-autonomous growth of many tumours, including choriocarcinomas (Kong et al., 1996), (section 1.5.4) as being supportive of a potential role for LIF in the cell-autonomous growth of germ cell tumours.

Studies of LIF transcript expression by human embryonal carcinoma cells using the ribonuclease protection assay (Figure 1.5) indicated that in the independently-derived GCT 27 and Tera 2 cell lines, both derived from malignant testicular tumours (Fogh et al., 1976; Pera et al., 1989), the predominant hLIF transcript was not the previously reported hLIF-D (Moreau et al., 1988). Moreover, the predominant hLIF transcript diverged in sequence at it's 5' end in the

Figure 1.5 **The Predominant hLIF Transcript in GCT 27 and Tera 2 Human Embryonal Carcinoma Cells is Not the Characterised hLIF-D Transcript**

Ribonuclease protection assay of 15 μg of cytoplasmic RNA from GCT 27C4 and Tera 2 EC cells using hLIF-D- and GAPDH-specific riboprobes. Protected species of approximately 410, 330, and 250 bp corresponding to the hLIF-D transcript, an uncharacterised hLIF transcript, and the hGAPDH transcript, respectively, are indicated.

(Courtesy of Prof. P.D. Rathjen)


LIF Probe
GAPDH Probe
GCT 27C4
Tera 2
GAPDH
330 bp
410 bp

region of the exon 1 / exon 2 boundary. This raised the possibility that expression of a novel or aberrant LIF transcript might play a role in the cell-autonomous growth of embryonal carcinoma stem cells.

1.7 Aims and Approaches

The initial aims of this project were to identify and characterise the novel hLIF transcript(s) predominating in the GCT 27 and Tera 2 human embryonal carcinoma cell lines. Its broad aims were to elaborate potential roles for hLIF in cell-autonomous growth processes and to achieve a better understanding of the regulation of hLIF activity and its effects at the cellular level.

Molecular cloning and expression analysis were utilised to isolate two new LIF transcripts from GCT 27 C4 EC cells, and their origin was determined by analysis of the hLIF genomic sequence. Studies of the distribution of the novel hLIF transcripts indicated that they were regulated independently in a developmentally- and tissue-specific manner. Further analysis by cross-species comparisons of LIF genomic sequences indicated that there was a complex, conserved organisation of the LIF gene in eutherian mammals. Moreover, mapping of exon usage in the mouse oncostatin M gene, which is tightly linked to and homologous to the mLIF gene, by ribonuclease protection assay also indicated unappreciated complexities in its organisation and expression. The functional relevance of the LIF genomic organisation was analysed by overexpression studies using the novel hLIF-M and hLIF-T cDNAs. This identified novel LIF proteins, some with an alternate intracellular localisation, and implied the possibility of a previously unidentified intracellular function(s) for the LIF gene and a novel mechanism(s) of action for these novel LIF protein. Chemical inhibition of glycosylation and secretion, and mutational analysis were used to elaborate the nature and origins of each the novel LIF proteins translated from the LIF-M and hLIF-T transcripts. These studies also suggested potential mechanisms of action and functions for intracellular LIF proteins in normal and tumourigenic contexts. This work provides further insight into the structure and function of the LIF gene and possible mechanisms of action of the alternate LIF proteins it encodes. It also forms part of the emerging body of evidence suggestive of distinct extracellular and intracellular activities for cytokines and proposes additional mechanisms for their production and regulation.

Chapter 2 :

Materials and Methods

2.1 Abbreviations

A	Amperes
APS	Ammonium Persulphate
BCIG	5-bromo-4-chloro-3-indoyl-β-galactopyranoside
β-Me	β-Mercaptoethanol
bp	base pair
BES	N,N-bis(2-hydroxyethyl)-2-aminoethane-sulphonic acid
BSA	Bovine Serum Albumin
Ci	Curie
CIP	Calf Intestinal Alkaline Phosphatase
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide
DOC	deoxycholate
DTT	Dithiothreitol
DTE	Dithioerythritol
E.coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic Acid
EGTA	Ethylene Glycol-bis(β-aminoethyl ether) N,N,N',N', Tetra Acetic Acid
EtBr	Ethidium Bromide
F	Farad
FBS	Foetal bovine serum
FITC	Fluoroscein Isothiocyanate
g	gram
HEPES	N-2-hydroxyethyl piperazine-N-ethane sulphonic acid
IPTG	Isopropyl-β–D-Thiogalactopyranoside
l	litre
M	Molar

m	milli (10^{-3})
μ	micro (10^{-6})
n	nano (10^{-9})
min.	minute(s)
MOPS	3-(N-morpholino)propane sulphonic acid
MQ H_2O	Reverse osmosis filtered water passed through a Milli-Q™ ion-exchange matrix
NP-40	Nonidet P-40
OD_{nnn}	Optical Density at nnn nanometres
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl Sulfonyl Chloride
RA	Retinoic Acid
rATP	Adenosine Ribonucleotide Triphosphate
RNA	Ribonucleic Acid
RNAse	Ribonuclease
RNAsIn	Human Placental Ribonuclese Inhibitor
rUTP	Uridine Ribonucleotide Triphosphate
SDS	Sodium Dodecyl Sulphate
TEMED	N,N,N',N'-Tetramethyl-Ethenediamine
U	Units
V	Volts
W	Watts

2.2 Materials

2.2.1 General Reagents

Acrylamide	Biorad
Agarose	Sigma
Ammonium sulphate	Sigma
Ampicillin	Sigma
APS	BDH chemicals
Bacto-agar	Difco
Bacto-tryptone	Difco
B-Me	Sigma
BSA	Sigma
Bis-acrylamide	Biorad
Bradford reagent concentrate	Biorad
Caprylic (octanoic) acid	Sigma
Coomassie brilliant blue	Sigma
Deoxyribonucleotide triphosphate solutions	Boehringer Manheim
DMF	BDH Chemicals
DTT	Scimar
DTE	Sigma
Freund's Adjuvant	Gibco-BRL
Glutathione-sepharose 4B	Pharmacia
Hoechst 3528 (bis-benzamide)	Sigma
IPTG	Sigma
NP-40	BDH chemicals
PMSF	Sigma
Protein A-Sepharose CL-4B	Pharmacia
rATP (powder)	Sigma
Ribonucleotide triphosphate solutions	Boehringer Manheim
Reduced glutathione	Sigma

Salmon sperm DNA	Sigma
SDS	Sigma
Sepharose G-50	Pharmacia
Sepharose CL-6B	Pharmacia
Sequagel 6	National Diagnostics
TEMED	Sigma
tRNA (yeast)	Boehringer Manheim
Tween 20	Sigma
Yeast extract	Difco

2.2.2 Tissue Culture Reagents

BES	Calbiochem
BCIG	Scimar
DMEM	Gibco-BRL
ESGRO	Amrad-Pharmacia Biotech
FBS	CSL
Gentamicin	Delta West
Ham's F12	Gibco-BRL
L-glutamine	Sigma
Glutaraldehyde	Pharmacia
Lipofectamine	Gibco-BRL
Methionine/cysteine deficient DMEM	ICN
McCoy's 5a	Gibco-BRL
Monensin	Sigma
Opti-MEM	Gibco-BRL
Trypsin (1:250)	Difco
Tunicamycin	Sigma

2.2.3 Enzymes

Avian Myeloblastosis Virus (AMV) Reverse Transcriptase	Molecular Genetic Resources
Calf intestinal phosphatase (CIP)	Boehringer Mannheim
DNAse I	Boehringer Mannheim
Klenow Fragment of DNA Polymerse I	Pharmacia
Proteinase K	Boehringer Mannheim
Restriction Endonucleases	Pharmacia & New England Biolabs
RNAse A	Sigma
RNAsIn	Bresatec
RNAse T1	Boehringer Mannheim
T3 RNA Polymerase	Boehringer Mannheim
T7 RNA Polymerase	Boehringer Mannheim
T4 DNA ligase	Pharmacia
Taq Polymerase	Bresatec
Terminal Transferase	Bresatec
Thrombin (human)	Sigma

2.2.4 Antibodies

Anti Digoxigenin-Fluoroscein-Fab Fragments (raised in sheep)	Boehringer Manheim
Anti GST-hLIF (raised in rabbit)	Ms. Regan Forest
Anti GST-mLIF (raised in rabbit)	Ms. Tricia Pelton
M2 anti-FLAG (mouse monoclonal)	Kodak IBI
Anti Rabbit-FITC (raised in sheep)	Silenus
Anti Mouse-FITC (raised in sheep)	Silenus

2.2.5 Radiochemicals

[alpha-^{32}P]dATP	Bresatec
[alpha-^{33}P]dATP	Bresatec

[alpha-^{32}P]rUTP	Bresatec
Tran-^{35}S Label	ICN

2.2.6 Molecular BiologyKits

AmpliFINDER RACE Kit	Clontech
Marathon cDNA Amplification Kit	Clontech
Advantage KlenTaq cDNA Polymerase Mix	Clontech
BresaClean Kit	Bresatec
Megaprime Probe Synthesis Kit	Amersham
T7 Sequencing Kit	Pharmacia

2.2.7 General Buffers and Solutions

Acrylamide Gel Elution Buffer	10 mM Tris-HCl pH 7.5, 1mM EDTA, 0.1% SDS (autoclaved)
30% Acrylamide Mix	30 gm acrylamide, 0.8 gm bis acrylamide in 100 ml H_20
5 x Blunt-end Ligation Buffer	250 mM Tris-HCl pH7.5, 25% PEG 6000, 50 mM $MgCl_2$, 5mM DTT, 5mM rATP. (stored at -20°C)
10x CIP Buffer	0.5 M Tris, 1 mM EDTA, pH 8.5. (stored at 4°C)
Coomassie Destain	45% methanol, 45% distilled water, 10% glacial acetic acid
Coomassie Stain	45% methanol, 45% water, 10% glacial acetic acid with 2.5 gm / l coomassie brilliant blue
Denhardt's Solution	0.1% (w/v) ficoll, 0.1 (w/v) polyvinylpyrolidine, 0.1% (w/v) BSA. (stored -20°C)
Formamide Loading Buffer	95% (w/v) deionised formamide, 0.02% bromophenol blue, 0.02% xylene cyanol. (stored -20°C)
10 x Gel Loading Buffer	50% glycerol, 0.1% SDS, 0.05% bromophenol blue, 0.05% xylene cyanol
GET Buffer	50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH8.0 (autoclaved)

10 x Ligation Buffer	660mM Tris-HCl pH7.5, 50 mM $MgCl_2$, 10mM DTT, 10mM rATP. (stored -20°C)
NP-40 Lysis Buffer	1% NP-40, 50 mM Tris pH8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF
PBS	136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2P0_4, 8 mM Na_2HPO_4 pH7.4 (autoclaved)
PBST	PBS, 0.05% Tween 20
10 x PCR Buffer	100mM Tris-HCL, 500mM KCl, 20 mM $MgCl_2$, 0.01% Gelatin. (stored -20°C)
2x SDS Gel Loading Buffer	125 mM Tris-HCl pH6.8, 4% SDS, 20% glycerol, 0.1% bromophenol blue (0.8 M B-Me added immediately prior to use).
SDS-PAGE Running Buffer	25 mM Tris-Glycine, 0.1% SDS
TAE	40mM Tris-acetate pH 8.2, 20mM NaAc, 1mM Na_2EDTA
TBE	90 mM Tris-HCl pH 8.3, 90 mM boric acid, 2.5 mM EDTA
TBS	25 mM Tris-HCl pH 8.0, 150 mM NaCl
TNM	30mM Tris-HCl pH 7.6, 150 mM NaCl, 15mM $MgCl_2$, 0.4% NP-40 (made fresh)
4x Tris-SDS Buffer	1.5 M Tris-HCl pH8.8, 0.4% SDS
TUNES	10mM Tris-HCl pH 8.0, 7M urea, 350 mM NaCl, 1mM EDTA, 2% SDS (autoclaved).
10x Super Duper Buffer	350 mM Tris-Acetate pH7.8, 100mM $Mg(CH_3COO)_2$, 625mM KCH_3COO, 40 mM Spermidine, 5mM dithioerythritol.(stored -20°C)
TENS Lysis Buffer	10 mM Tris-HCl pH8.0, 1 mM EDTA, 0.1M NaOH, 0.5% SDS (autoclaved).
TTBS	TBS, 0.1% Triton X-100

2.2.8 Tissue Culture Buffers and Solutions

2 x BBS	100 mM BES, 560 mM NaCl, 3 mM Na_2HPO_4, pH 6.99 (NaOH) (filter sterilised) (stored 4°C)
β-Galactosidase Stain	0.45 mM $K_2Fe(CN)_6$, 0.4 5mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$. (Add 100 µl BCIG (40 mg / ml) per 10 ml immediately prior to use.) (filter sterilised) (stored 4°C)
β-Me in PBS	100 mM B-Me in PBS (filter sterilised) (stored no more than 2 weeks at 4°C)
Cytomix	120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4 / KH2PO_4, 25mM HEPES, 2mM EGTA, 5mM $MgCl_2$, pH7.6 (KOH) (2mM rATP and 5mM reduced glutathione added immediately prior to use). (filter sterilised) (stored 4°C)
DMEM medium	67.4 g DMEM, 23.8 g HEPES and 6.25 ml Gentamicin (40 mg/ml) dissolved in 5 litres sterile water. pH7.4 (HCl) (filter sterilised) (stored 4°C)
ES DMEM medium	67.4 g DMEM, 18.5 g $NaHCO_3$ and 6.25 ml Gentamicin (40 mg/ml) dissolved in 5 litres sterile water. pH 7.4 (HCl) (filter sterilised) (stored 4°C)
Gentamycin-free DMEM	Identical to DMEM medium except that Gentamycin was omitted.
Ham's F12 medium	10.6 g Ham's F12, 1.18 g NaHCO3 and 1.25 ml Gentamicin (40 mg/ml) dissolved in 1 litre sterile water. pH 7.2 (HCl) (filter sterilised) (stored 4°C)
McCoy's 5a medium	12 g McCoy's 5a, 2.2 g NaHCO3 and 1.25 ml Gentamicin (40 mg/ml) dissolved in 1 litre sterile water. pH 7.6 (HCl) (filter sterilised) (stored 4°C)
PBS	136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2P0_4, 8 mM Na_2HPO_4, pH7.4 (HCl) (autoclaved)

Trypsin EDTA	1% (w/v) trypsin (1:250), 0.5 mM EDTA, 137 mM NaCl, 27mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 (filter sterilised) (stored 4°C)

2.2.9 Oligonucleotides

Primers for pGEM1, pBluescript KS+ and pT7T3 19U Sequencing :

SP6	5' CATACGATTTAGGTGACACTATAG 3'
T7	5' AATACGACTCACTATAG 3'
T3	5' ATTAACCCTCACTAAAG 3'

Primers for pXMT2 Sequencing :

Vic1	5' AACTCTAGACCGGTATG 3'
Vic2	5' GCCTTCACGCTAGGATT 3'

Primers for pGEX20T Sequencing (a kind gift of Dr. G.W. Booker) :

GEX5	5' GGGCTGGCAAGCCACGTTTGGTC 3'
GEX3	5' CCGGGAGCTGCATGTGTCAGAGG 3'

Oligonucleotides Used in hLIF RACE-PCR:

hLIFRT	5' ACGTGGTACTTGCTGCACA 3'
Anchor Oligo	5' PO_4-CACGAATTCACTATCG ATTCTGGAACCTTCAGAGG-NH_3 3'
Anchor Primer	5' CTGGTTCGGCCCACCTCTGAA GGTTCCAGAATCGATAG 3'
hLIF3	5' ATAGGATCCGGCGTTGAGCTTGCTG 3'
hLIF3N	5' TTCTGGTCCCGGGTGATGTT 3'

Primer for hLIF -M RT-PCR :

hLIFM5	5' TAGAATTCTGGAAGCGTGTGGT 3'

Primer for hLIF-T RT-PCR :

hLIFT5B	5' ATGAATTCTGTCACCTTTCACTTTCCT 3'
hLIFT5A	5' ATGGAATTCGACCTTTTGCCT 3'

Primer for hLIF Genomic PCR :

hLIFG3	5' TAGAATTCCAGTGCAGAACCAACA 3'

Primers for hLIF Mutagenesis :

hLIFTK	5' GAAGATCTCAACAACATCATGAAC 3'
hLIF(AUG$^-$)	5' CCTGTCAACGCCACCTGTGCCATACGCCAC
	CCATGTCACAACAACCTCGCGAACCA 3'
hLIF e23	5' ATAGGTACCTGAGTTGTGCCCCTGCT 3'

Primers for mOSM RACE-PCR and RT-PCR:

MOGSP1	5' GCCAGAGTACCAGGACCCAGTAT 3'
MOGSP2	5' ATCCTGAGCATGGCACTGGCCAAT 3'.
MOGSP3	5' TTCCCCGTGAGGTTCGCCTGATT 3'
MOGSP4	5' GCCTCTGAGAGCGACATCCTGTAT 3'.
AP1(Adaptor Primer)	5' CCATCCTAATACGACTCACTATAGGGC 3'

2.2.10 Plasmids

Cloning and expression vectors obtained from commercial sources were:

pBluescript KS +	Stratagene
pCH110	Pharmacia
pGEX20T	Pharmacia
pT7T3 19U	Pharmacia
pXMT2	Genetics Institute
pGEM-T_{EASY}	Promega

Recombinant DNA constructs not obtained from commercial sources were :

hLIF-DFLAG in pXMT2	Ms. Lesley Crocker
hLIF-D in B/S	Prof. Peter Rathjen
hLIF-D in pXMT2	Prof. Peter Rathjen
hLIFR in pXMT2	Prof. John Heath
HpGEM1	Prof. Peter Rathjen
HpGEM1 *Sma*$^-$	Prof. Peter Rathjen
pαGalX	Ms. Lesley Crocker
pBS130BES	Dr. Tetsuya Taga
pCXN2-hBcl2	Dr. Sharad Kumar
pCXN2-CrmA	Dr. Sharad Kumar
pGAPM	Dr. Greg Goodall

Ms. Lesley Crocker and Professor Peter Rathjen are at the Department of Biochemistry, University of Adelaide, Adelaide, Australia. Professor John Heath is at the Department of Biochemistry, University of Birmingham, Birmingham, United Kingdom. Doctor Tetsuya Taga is at the Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan. Doctors Sharad Kumar and Greg Goodall are at the Hanson Centre for Cancer Research, Adelaide, Australia.

2.2.11 Bacterial Strains and Growth Media

E.coli strain DH5α: Was the kind gift of Dr. Helena Richardson and was used as a host for all recombinant DNA manipulations.

Genotype: supE44 Δ*lac* U169 (ϕ80 *lacZ* ΔM15) *hsdR* 17 *recA* 1 *endA* 1 *gyrA* 96 thi-1 *rel* A1

Growth media were prepared in double distilled water and sterilised by autoclaving. Antibiotics a other labile chemicals were added after the media solution had cooled to 50°C.

FTB: 2.4% (w/v) yeast extract, 1.2% (w/v) bactotryptone, 0.4% (w/v) glycerol; and 17 mM KH_2PO_4, 72 mM K_2HPO_4 added after cooling.

Luria (L) broth: 1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, pH 7.0 (adjusted with 1M NaOH).

Psi broth: 2% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) $MgSO_4$, pH 7.6 (adjusted with 1 M KOH).

Solid media agar plates were prepared by supplementing Luria broth with 1.5% Bacto-agar. Ampicillin (100 μg/ml) was added where appropriate for growth of transformed bacteria to maintain selective pressure for recombinant plasmids.

2.2.12 Cell Lines and Growth Media

Cell lines used during the course of this work were obtained from the following sources (original references cited in section 2.4.1) :

293T	Prof. John Heath
Cos 1	American Type Culture Collection
D3	Dr. Lindsay Williams
GCT 27C4	Dr. Martin Pera
HeLa	American Type Culture Collection
HepG2	American Type Culture Collection
MBL5	Dr. Lindsay Williams
Tera 2	American Type Culture Collection

Professor John Heath is at the Department of Biochemistry, University of Birmingham, Birmingham, United Kingdom. Dr. Lindsay Williams is at the Ludwig Institute for Cancer Research, Melbourne, Australia and Dr. Martin Pera is at the Institute of Reproduction and Development, Monash University Medical Centre, Melbourne, Australia.

293T, Cos 1, HeLa and HepG2 cells were maintained in DMEM/FBS media (90% DMEM, 10% FBS, 1mM L-glutamine). GCT 27C4 cells were maintained in DMEM/F12/FBS media (45% DMEM medium, 45% Ham's F12 medium, 10% FBS, 1mM L-glutamine). D3 and MBL5 ES cells were maintained during LIF bioactivity assays in Incomplete ES medium (85% ES DMEM medium, 15% FBS, 1mM L-glutamine, 0.1% 100mM β–Me in PBS). D3 and

MBL5 ES cells were maintained in Complete ES media (99% Incomplete ES cell medium, 1% Cos cell conditioned medium containing 100 U / ml LIF (Smith, 1991)).

Tera 2 cells were maintained in McCoy's/FBS media (90% McCoy's 5a medium, 10% FBS, 1mM L-glatamine).

See also sections *2.2.8 Tissue Culture Buffers and Solutions* and *2.4.1 Maintenance of Cell Cultures*.

2.2.13 DNA and Protein Size Markers

Presatined SDS-PAGE Standards (broad range)	Biorad
pUC19, *Hpa* II DNA size markers	Bresatec
SPP-1, *Eco* RI DNA size markers	Bresatec

2.2.14 Miscellaneous Materials

All Tissue Culture Plasticware	Falcon
Bottle top filters (0.22 µM)	Corning
Chromatography paper (3MM)	Whatmann Ltd.
Centricon 10 Columns	Amicon
Electroporation apparatus ("Gene Pulser")	Biorad
Electroporation cuvettes ("Gene Pulser 4 mm")	Biorad
Microplate reader	Molecular Devices
Mini Protean SDS-PAGE apparatus	Biorad
0.22 µM syringe-end filters	Sartorius
Nitrocellulose	Schleicher and Schuell
Nytran	Schleicher and Schuell
Oakridge tubes (10 ml, 50 ml and 500 ml)	Nalgene
Slide Film (Ektachrome 160T)	Kodak
Storage Phosphor Screens	Molecular Dynamics
X-ray Film (Biomax)	Kodak

2.3 Molecular Methods

2.3.1 Restriction Endonuclease Digestion of DNA

Plasmid DNA was digested in 1 x Super Duper buffer with 3-5 U of enzyme/µg DNA and incubation at the appropriate temperature for 30 min. to 16 hours. Complete digestion of DNA was assayed by agarose gel electrophoresis.

2.3.2 Small-Scale Preparation of Plasmid DNA

3 ml LB containing 100µg/ml ampicillin was inoculated with a single transformant colony and incubated shaking overnight at 37°C. 1.5 ml of this culture was transferred to an Eppendorf tube and spun for 1 min at 2000 x g. The cells were resuspended in 50 - 100µl of remaining supernatant, and lysed by the addition of 300µl TENS lysis buffer and briefly vortexing. 200µl 3M sodium acetate pH 5.2 was added, and again mixed by vortexing. The mixture was then spun at 15000 x g in a benchtop centrifuge for 5 min. and the supernatant transferred to a fresh tube, to which 1 ml of cold (-20°C) ethanol was added. This mixture was vortexed and spun at 15000 x g in a benchtop centrifuge for 3 min. and the pellet washed in 70% ethanol, dried *in vacuo*, and resuspended in 20-22µl TE. Restriction analysis of 2-4µl of the resuspended DNA was performed in a 20 µl digest containg 1 x Super Duper Buffer, 5 U of the appropriate restriction enzyme(s) and 50 µg / ml RNAse A.

2.3.3 Intermediate-Scale Preparation of Plasmid DNA

50 ml L broth ampicillin (100 µg/ml) was inoculated with a single transformant colony and incubated O/N at 37°C with shaking. Cultures were transferred to 50 ml Oakridge tubes and pelleted by centrifugation for 5 min. at 2000 x g, 4°C. Cells were resuspended in 3 ml GET and 6 ml 0.2 M NaOH, 1% SDS was added, mixed gently before incubation on ice for 5 min. To the lysate was added 4.5 ml of 3M KAc, 2M HOAc pH 5.8 with gentle mixing before incubation on ice for 5 min., then mixed vigorously and placed on ice for a further 15 min. Cell debris were pelleted by centrifugation for 10 min.at 11000 x g, 4°C and the supernatant transferred to a new tube. Nucleic acid was precipitated by the addition of 8 ml iso-propanol, followed by centrifugation for 10 min.at 11000 x g, 4°C . The pellet was drained and air dried, resuspended in

400 µl H_2O and transferred to an Eppendorf tube. RNA was removed by the addition of 2 µl RNaseA (10 mg / ml) and incubation at 37°C for 30 min. then 8 µl 10% SDS and 2.5 µl Proteinase K (20 mg/ml) was added followed by incubation at 37°C for 15 min. Plasmid DNA was extracted once with an equal volume of 1:1 phenol:chloroform, once with chloroform and precipitated with 100 µl 7M NH_4Ac and 1 ml ethanol (-20°C, 30 min.). Plasmid DNA was pelleted by centrifugation at 15000 x g for 15 min., dried *in vacuo* and resuspended in 200 µl of sterile water. The DNA was quantitated by measuring the OD_{260} and $OD_{210-320}$ of a 1 / 200 dilution, diluted to 1mg/ml and stored at -20°C..

2.3.4 Large-Scale Preparation of Plasmid DNA

All plasmid DNA used to transfect cultured cells was prepared by the following method.

500 ml FTB ampicillin (100µg/ml) was inoculated from a single transformant colony and incubated at 37°C overnight with shaking. The cells were then pelleted in 500 ml Oakridge tubes by centrifugation for 5 min. at 1500 x g, 4°C and resuspended in 5 ml GET and transferred to a 50 ml Oakridge tube. To lyse the cells, 13 ml 0.2M NaOH, 1% SDS was added with gentle mixing on ice for 10 min. 6.5 ml 3M sodium acetate pH4.6 was then added and incubated on ice for a further 20 min. with occasional gentle mixing. The precipitated material was removed by centrifugation for 10 minutes at 17500 x g, 4°C and the supernatant transferred to a fresh tube. Plasmid DNA was recovered from the supernatant by the addition of 0.6 volumes of isopropanol, followed by centrifugation for 10 minutes at 12000 x g, 4°C. The pelleted DNA was air dried for 5 min. and resuspended in 7 ml TE with 7 gm $CsCl_2$ and 400 µl 10 mg/ml. Debris were removed by further centrifugation for 10 minutes at 1000 x g, 4°C. The supernatant was then transferred to a 10 ml ultracentrifuge tube, overlaid with paraffin and subjected to ultracentrifugation for 20 - 24 hours at 200000 x g, 20°C. The layer of supercoiled plasmid DNA formed was visualised by long wave UV light and harvested into an equal volume of TE/NaCl saturated isopropanol. Six extractions with equal volumes of TE/NaCl saturated isopropanol were carried out to remove traces of EtBr. DNA recovered by ethanol precipitation at 4°C overnight, resuspended and ethanol precipitated for a second time at -20°C for 30 minutes. The purified DNA was resuspended in sterile water, quantitated by measuring the OD_{260} and $OD_{210-320}$ of a 1/200 dilution, diluted to 1mg/ml and stored at -20°C.

2.3.5 Double Stranded DNA Sequencing

Sequencing reactions were carried out using small-scale preparations of plasmid DNA (8µl) and intermediate- or other plasmid DNA (4µg). The DNA was incubated at 37°C for 15 minutes with 200 µg / ml of RNase A in 20 µl and denatured by addition of 5 µl of 1M NaOH,1 mM EDTA and incubated a further 15 minutes at 37°C. Denatured plasmid DNA was purified by centrifugation at 2000 x g on a 0.5 ml sepharose CL-6B spun column.

Sanger dideoxy chain termination sequencing reactions using the Pharmacia T7 polymerase sequencing kit and α^{33}P-dATP were carried out according to the manufacturers' instructions. Sequncing products were resolved on 400 mm x 600 mm Sequagel 6 gels run at 60 W, visualised on Kodak Biomax film and read manually.

Alternatively, 0.5 µg of DNA was sequenced using ABI prism Dyeteminator and BigDye Terminator ready reaction mix according to the manufacturer's instructions and analysed on an ABI PRISM 377 DNA Sequencer.

2.3.6 Purification of DNA Fragments

Linear DNA fragments greater than 500 bp were separated by electrophoresis on agarose gels and purified using a BresaClean kit according to the manufacturer's instructions. Smaller DNA fragments were separated by electrophoresis on agarose or non-denaturing acrylamide, excised under long wavelength UV light and eluted overnight at 37°C in 400 µl Gel Elution Buffer. DNA was recovered by ethanol precipitation in the presence of 1 µl glycogen.

2.3.7 Preparation of Genomic DNA

Genomic DNA was isolated from cell pellets (~5 x 10^7 cells) stored at -80°C. Cells were resuspended in 5 ml Tris/saline (25mM Tris 7.6,75 mM NaCl, 24 mM EDTA pH 8.0) before lysis with 0.5 ml 10% SDS and gentle inversion. Cellular protein was degraded by the addition of 50 µl Proteinase K (20 mg/ml) and incubation at 37°C for 2.5 hours with occasional mixing. Genomic DNA samples were extracted twice with an equal volume of Tris-saturated phenol and twice with an equal volume of chloroform : iso-amyl alcohol (24 : 1) before precipitation with 1/10th volume 2M KCl and 2 volumes ice-cold 100% ethanol. Genomic DNA was harvested by

spooling onto a blunted Pasteur pipette, washed in 70% ethanol, transferred into 1 ml MQ H_2O and resuspended overnight.

2.3.8 Southern Blotting

Restriction endonuclease digests containing 20 μg of genomic DNA (2.2.7) were electrophoresed overnight at 70 V and transferred to Nytran nylon membrane according to the method of Thomas and co-workers (1995).

2.3.9 Synthesis of Oligo-Labelled DNA Probes

Single stranded DNA probes were prepared using a Megaprime labelling kit with 50 μCi $a^{32}P$-dATP according to the manufacturer's instructions. To remove unincorporated label, probe reactions were diluted to 100 μl with MQ H_2O, loaded onto a 1 ml Sephadex G-50 spun column and centrifuged at 1500 x g for 5 minutes.

2.3.10 Hybridisation of Probes to DNA Immobilised on Nitrocellulose or Nylon Filters

Filters were placed in a Hybaid cylinder and prehybridised with 10 mls of 1 M NaCl, 40% deionised formamide, 1% SDS, 10 % PEG, 50 mM Tris-HCl pH 7.4 and 5 X Denhardt's. Filters were prehyridised for a minimum of 4 hours at 42°C in a Hybaid filter hybridisation oven. DNA probes were denatured at 95°C for 3 minutes with 2.5 mg sonicated salmon sperm DNA, snap-cooled on ice and added to the prehybridisation solution. All hybridisations were performed overnight at 42°C. Filters were washed three times for 15 min. at increasing stringency. Stringencies were decreased from 2 x SSC at 65°C to 0.2 x SSC at 65°C to 0.1 x SSC at 65°C unless otherwise stated. Filters were stripped for re-probing by boiling them three times for 10 min. in 0.5% SDS.

2.3.11 Isolation of RNA from Mouse Tissues and GCT Cell Lines

RNA from mouse tissues and GCT cell lines was isolated using the acidified guanidium isothiocyanate method (Chomczynski & Sacchi, 1987). Polyadenylated RNA was prepared from GCT cell lines by standard procedures (Roach et al., 1994).

2.3.12 Isolation of Cytoplasmic RNA from Cultured Cells

Cytoplasmic RNA was isolated using the method of Edwards *et al.* (1985). Cells (~2 x 10^7) were harvested by trypsinisation and stored at -80°C until use. Cell pellets were thoroughly resuspended in 2 ml ice cold and TNM and lysed by vigorous pipetting 10 times (minimising bubbles). After incubation on ice for 5 min., nuclei were pelleted by centrifugation for 5 min. at 1500 x g and the supernatant decanted and mixed thoroughly with 2 ml TUNES. This solution was extracted twice with TE-saturated phenol : chloroform (1:1) and the aqueous layer was transferred to a sterile corex tube. RNA was recovered by ethanol precipitation with centrifugation for 30 minutes at 11000 x g, 4°C. The RNA pellet was resuspended in 450 µl MQ H_2O, and transferred to an eppendorf and again subjected to ethanol precipitation and centrifugation for 15 minutes at 15000 x g. The RNA pellet was resuspended in 200 µl MQ H_2O, quantitated by measuring the OD_{260} and $OD_{210-320}$ of a 1/200 dilution, diluted to 1mg/ml and stored at -80°C.

2.2.13 Isolation of Polyadenylated RNA

Oligo-d(T) cellulose beads were prepared by soaking in 0.1 M NaOH and rinsed thoroughly in MQ H20 before resuspending in pre-wash solution (0.5 M NaCl, 0.4 M Tris-HCl pH 7.5, 10 mM EDTA, 0.5% SDS). 500 µg cytoplasmic RNA was precipitated and resuspended in 100 µl MQ H20 before denaturation at 80°C for 2 min. and snap cooling on ice. 100 µl resuspension solution (1 M NaCl, 0.4 M Tris-HCl pH 7.5, 20 mM EDTA, 1% SDS) was added to the RNA before transferring to 100 µl oligo-dT cellulose beads. After incubation at room temperature for 5 min. the beads were washed twice with 0.5 M NaCl / 10 mM EDTA and the polyadenylated RNA eluted twice in 150 µl MQ H20 at 65°C for 5 min. RNA was precipitated by addition of 650 µl ethanol and 8 µl 4M NaCl, resuspended in MQ H20, and stored at -20°C.

2.3.14 Reverse Transcription

Reverse transcription for RACE-PCR cloning was performed using the AmpliFINDER kit's reagents, the hLIFRT primer and 2.5 µg GCT 27C4 polyadenylated RNA according to the manufacturer's instructions.

Reverse transcriptions for RT-PCR were performed by denaturing 10 µg of cytoplasmic RNA in a 6.5 µl volume at 65°C for 15 minutes before snap cooling on ice. The denatured RNA was added to a reverse transcription reaction containing 50 mM Tris-HCl pH 8.5, 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 1.5 mM dATP, 1.5 mM dCTP, 1.5 mM dGTP, 1.5 mM dTTP, 40 units RNAasin, 500 ng of the hLIFRT primer and 8 units of AMV reverse transcriptase. This mixture was incubated at 42°C for 2 hours, diluted to 500 µl in MQ H_2O and stored in 50 µl aliquots at -80 °C.

2.3.15 hLIF RACE-PCR Cloning

Single-stranded ligation of GCT 27C4 cDNAs to the Anchor Oligonucleotide supplied with the AmpliFINDER kit was carried out according to the manufacturer's instructions. Initial PCR reactions were carried out as 20 µl volumes using the supplied Anchor Primer and the primer hLIF3 in a capillary thermal sequencer. Reactions contained 0.5 µl of cDNA and 20 pmol of each primer in 10 mM Tris-HCL pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin and 1 unit of Taq DNA polymerase. Cycling conditions were 94°C for 2 min, 60°C for 10 sec, 72°C for 2 min; then 94°C for 10 sec, 60°C for 10 sec, 72°C for 2 min for 45 cycles; then 72°C for 5 min. A further round of nested PCR under the same conditions using 1 µl of the original amplification, the 5' Anchor Primer, and the nested primer hLIF3N was required in order to increase the yield of specific products. Products were visualised on 2% agarose gels and their identity confirmed by Southern hybridisation with a HpGEM1 *Pst* I probe.

Cloning of PCR products was achieved without their digestion with restriction endonucleases: regions from the smear of products visualised by ethidium bromide staining were excised from a 2% agarose gel and the products recovered by overnight elution in 400 µl of TE at 37°C followed by ethanol precipitation in the presence of 1µl of glycogen. They were then ligated into *Sma* I-cut pT7T3 19U that had not been phosphatase-treated. Resulting transformants were screened for hybridisation with a labelled HpGEM1 *Pst* I probe using a colony-lift procedure (Sambrook et al, 1989). Inserts from isolated, hybridising colonies were then sequenced.

2.3.16 mOSM RACE-PCR Cloning

RACE-PCR was performed using a Marathon cDNA Amplification Kit and Advantage KlenTaq cDNA Polymerase according to the manufacturer's instructions. 1 μg of polyadenylated mouse bone marrow RNA was prepared (Celano et al., 1993) and reverse transcribed using the supplied oligo-dT primer according to the manufacturer's instructions. Double stranded cDNA ligated to the supplied adaptor was then used in 5' and 3' RACE-PCR reactions. Primers used for 3' RACE were: MOGSP1 (exon 1) and MOGSP2 (exon 2) in combination with the supplied AP1 adaptor primer. Primers used for 5' RACE were: MOGSP3 (exon 2) and MOGSP4 (exon 3) in combination with the supplied AP1 adaptor primer.

PCR reactions were carried in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA). Cycling conditions were 94°C for 2 min, 60°C for 10 sec, 72°C for 2 min; then 94°C for 10 sec, 60°C for 10 sec, 72°C for 2 min for 45 cycles; then 72°C for 5 min. Products were visualised on 2% agarose gels and their identity confirmed by Southern blotting using the *Eco*RI *Bam*HI fragment of pME18S/mOSM as a probe. Hybridising products were excised and gel-purified prior to cloning. PCR products representing an alternate mOSM transcript containing only exons 1 and 3 were cloned into pGEM-T_{EASY} according to the manufacturer's instructions to yield the plasmid $pmOSM13_{RACE}$.

2.3.17 hLIF RT-PCR Cloning

RT-PCR amplifications were performed as described above (2.3.15), except that the PCR reaction contained 2.5 mM $MgCl_2$ and primers specific to the hLIF-M and hLIF-T first exons: hLIFM5 or hLIFT5 in combination with hLIF3. Forty cycles of PCR yielded sufficient product for cloning. Products were able to be cut with *Eco* R1 and *Bam* H1 and ligated into *Eco* R1, *Bam* H1-cut pT773 19U by virtue of recognition sites engineered into the primers. Transformants bearing inserts corresponding in size to the cloned PCR product were sequenced.

2.3.18 mOSM RT-PCR Cloning

RT-PCR reaction and cycling conditions were the same as those used for mOSM RACE-PCR except that the reaction contained the primers MOGSP1 and MOGSP4. PCR products

representing an alternate mOSM transcript containing only exons 1 and 3 were cloned as described above to yield the plasmid pmOSM13$_{RT}$.

2.3.19 PCR for hLIF Mutagenesis

To produce product required for construction of phLIF-MA^{-}X and phLIFTK, PCR was performed using the hM23A primer or the hLIFTK primer in combination with the T7 primer. Amplifications were carried as described above 2.3.15 except that they contained 100 ng of HpGEM1 in place of GCT 27C4 cDNA and PCR cycling conditions were 94°C for 2 min, 55°C for 10 sec, 72°C for 45 sec; then 94°C for 10 sec, 55°C for 10 sec, 72°C for 45 sec for 30 cycles; then 72°C for 5 min.

2.3.20 Riboprobe Transcription Templates

Antisense riboprobes were prepared by transcription of the following linearised partial cDNAs with T7 RNA polymerase: HpGEM1*Sma*$^{-}$, *Hin* dIII (Rathjen, 1990a) (hLIF-D transcripts); phLIF-MS1$_{RACE}$, *Eco* R1 (hLIF-M transcripts); phLIF-TS1$_{RT}$, *Hin* dIII (hLIF-T transcripts); and pGAPM, *Dde* I (human glyceraldehyde phosphate dehydrogenase [GAPDH] transcripts). A riboprobe for hgp190 low affinity hLIF receptor transcripts was prepared by transcription of phGP190$_{267}$, *Hin* dIII with T3 RNA polymerase. phGP190$_{267}$ was prepared by ligating the end-filled 0.27 kb *Xmn* I, *Tth* 111 II fragment of hLIFR in pXMT2 into *Sma* I-cut pT7T3 19U,

An mOSM specific riboprobe transcription template was constructed using the plasmid pME18S/mOSM (A kind gift of Dr. Takahiko Hara, Institute of Molecular and Cellular Biosciences, Tokyo) which contained a cDNA comprising the entire open reading frame of the mOSM cDNA.[4] An *Eco*R1, *Tth*111 I fragment of this plasmid containing 346 bp of the mOSM cDNA was subcloned as an end-filled fragment into *Sma*I cut pT7T3 (Pharmacia, Uppsala, Sweden) yielding the plasmid pmOSM123*ET*. pmOSM123*ET* was linearised with *Xho*I to create a T7 RNA polymerase riboprobe transcription template. A *Sma*I, *Tth*111 I fragment containing 208 bp of the mOSM 13 cDNA was excised from the plasmid pmOSM13$_{RACE}$, end-filled and ligated into *Sma*I cut pBluescript KS+ (Stratagene, La Jolla, CA) yielding the plasmid

pmOSM13*ST*. pmOSM13*ST* was linearised with *Bam*HI to create a T3 RNA polymerase riboprobe transcription template.

2.3.21 Riboprobe Synthesis and Ribonuclease Protection Assay

Riboprobe synthesis and RNAse protection assay were performed using the method of Krieg and Melton (1987) as modified by Rathjen and co-workers (1990a) except that hybridisations were for 3 hours at 50°C. To reduce overexposure of the GAPDH signal relative to that of the other probes, low-specific-activity GAPDH probes were synthesised using 5 µCi alpha-^{32}PdUTP / 15 µl reaction while other probes were synthesised using 150 µCi alpha-^{32}PdUTP / 15 µl reaction, 50,000 counts / min. of hGAP probe and 150,000 counts / min. of all other probes was added to each hybridisation. RNAse digestion products were separated on a Sequagel 6 gel and visualised using phosphorimager analysis.

2.3.22 Construction of hLIF Expression Vectors

The complete open reading frames of hLIF-M and hLIF-T were reconstructed by ligating the 0.39 kb and 0.43 kb *Sal*I, *Sma*I fragments of phLIF-MS1$_{RACE}$ and phLIF-TS1$_{RT}$ respectively into *Sal*I, *Sma*I -cut phLIF-D ("HpGEM1"; Rathjen et al., 1990b) creating phLIF-M and phLIF-T. The hLIF-D in pXMT2 expression construct (phLIF-DX) (Rathjen et al., 1990b) derived from the pC106R (Moreau et al., 1988) hLIF cDNA has been described previously. phLIF-MX and phLIF-TX were created by ligating the 0.40 and 0.44 kb *Kpn*I, *Sma*I fragments of phLIF-MS1$_{RACE}$ and phLIF-TS1$_{RT}$ into *Kpn*I, *Sma*I cut phLIF-DX. FLAG epitope tagged hLIF-D in pXMT2 (phLIF-DFX) was a kind gift of Ms. Lesley Crocker (Bresagen Ltd., Adelaide, Australia). FLAG epitope tagged hLIF-M in pXMT2 (phLIF-MFX) was prepared by ligating the 0.41 kb *Kpn*I, *Sma*I fragment of phLIF-MS1$_{RACE}$ into *Kpn*I, *Sma*I -cut phLIF-DFX. hLIF-M in pXMT2 (phLIF-MX)was prepared by ligating the 0.45 kb *Bgl*II, *Sma*I fragment of phLIF-MFX into *Bgl* II, *Sma*I -cut phLIF-DX. FLAG epitope tagged hLIF-T in pXMT2 (phLIF-TFX) was prepared by ligating the 0.49 kb *Kpn*I, *Sma*I fragment of hLIF-TS1$_{RT}$ into *Kpn*I, *Sma*I -cut phLIF-MFX. hLIF-T in pXMT2 (phLIF-TX) was prepared by ligating the 0.51 kb *Bgl*II, *Sma*I fragment of phLIF-TFX into *Bgl*II, *Sma*I -cut phLIF-DX. Construction of the mLIF-D, mLIF-M and mLIF-T expression vectors has been described previously (Smith et al., 1988; Rathjen et al., 1990a; Haines et al., 1999).

hLIF-D and hLIF-M expression constructs in which the in frame ATG in exon 2 had been mutated to GCG (phLIF-MA$^-$X) were prepared in three steps: phLIF-D*Hinc* $^-$ and phLIF-M*Hinc* $^-$ were prepared by cutting phLIF-D and phLIF-M with *Sal*I then end-filling and re-ligating to remove the polylinker *Hinc*II site while leaving the two *Hinc*II sites in the hLIF cDNA intact. The hLIF (AUG$^-$) , T7 PCR product from a phLIF-D template was then digested with *Hinc*II and ligated into *Hinc*II -cut phLIF-D*Hinc* $^-$ and phLIF-M*Hinc* $^-$, creating phLIF-DA$^-$ *Hinc* $^-$ and phLIF-MA$^-$*Hinc* $^-$. The complete LIF open reading frames in phLIF-DA$^-$*Hinc* $^-$ and phLIF-MA$^-$*Hinc* $^-$ were then excised as a 0.70 kb *Eco*RI fragments, end-filled, and ligated into end-filled *Eco*RI -cut pXMT2 to create phLIF-DA$^-$X and phLIF-MA$^-$X.

The phLIF-T_KX expression construct in which sequences upstream the in frame AUG in exon 2 of the hLIF cDNA were omitted and the sequence around the AUG was engineered to produce homology to the consensus sequence for translation initiation described by (Kozak, 1989) were produced from the PCR product generated by amplification of phLIF-DX template using the hLIFTK and Vic 2 primers. The phLIF e23X expression construct containing only sequences from exon 2 and 3 of the hLIF cDNA were produced from the PCR product generated by amplification of phLIF-DX template using the hLIFe23 and Vic 2 primers. These products were digested with *Kpn*I and ligated into *Kpn*I-cut pXMT2 to create phLIF-T_KX and phLIF e23X respectively.

2.3.23 Production of Polyclonal Anti-hLIF Rabbit Sera

A glutathione-S-transferase-hLIF fusion protein (GST-hLIF) was produced in BL 21 *E. coli* cells using the pGEX-20T vector system as described in Forest (1996). This protein was then used to raise polyclonal anti-hLIF sera in rabbits also as described in Forest (1996).

2.3.24 Purification of Immunoglobulins from Crude Serum

10 ml aliquots of crude serum were adjusted to pH 4.8 using acetic acid and 1 ml of caprylic acid was added dropwise with stirring, the serum was then stirred for 30 minutes at room temperature to precipitate non-IgG serum proteins. The precipitated proteins were pelleted by centrifugation at 10000 x g for 10 min. An equal volume of saturated ammonium sulphate was

added slowly to the supernatant with continuous stirring for ten minutes. Precipitated immunoglobulins were recovered by centrifugation at 10000 x g for 10 min. and resuspended in 5 ml PBS. Excess salt was removed by dialysis against 3 changes of PBS over 2 days.

2.4 Tissue Culture Methods

2.4.1 Maintenance of Cell Cultures

293T cells (Pear et al., 1993) were maintained in DMEM / FBS, grown at 37°C in 5% CO_2 and passaged (no more than 1: 40) by soundly striking the flask several times to disperse the cells at confluence.

Cos 1 cells (Gluzman, 1981) were maintained in DMEM / FBS, grown at 37°C in 5% CO_2 and passaged (no more than 1: 10) by trypsinisation at confluence.

GCT 27C4 cells (Pera et al., 1989) were maintained in DMEM / F12 / FBS, grown at 37°C in 5% CO_2 and passaged (no more than 1 : 40) by trypsinisation at confluence.

D3 and MBL5 ES cells (Williams et al., 1988) were maintained as described in Smith (1991) except that ES cells were grown in Complete ES Cell Medium with 15% FBS under 10% CO_2.

Tera 2 cells (Fogh, 1975 and Fogh, 1976) were maintained in McCoy's / FBS, grown at 37°C in 5% CO_2 and passaged (no more than 1:10) by trypsinisation at confluence.

2.4.2 Transient Transfection of Cos 1 Cells by Electroporation

Electroporation was carried out by a method adapted from van den Hoff *et al* (1992). Cos 1 cells were grown in 175 cm^2 flasks to 80% confluence in DMEM / FBS. Cells were harvested by trypsinisation and washed twice in PBS. Cells were resuspended in cold (4°C) cytomix 6 x 10^6 cells /ml, and 500 µl of this cell suspension was added to electroporation cuvettes containing 10 µl salmon sperm DNA (10 mg/ml) and 10 µg circular plasmid DNA. After mixing and incubation for 10 min. at 4°C, the cells were electroporated at 290 volts, 960 µF in a Biorad Gene Pulser apparatus. After a further 10 min. at 4°C, cells were plated in 100 mm dishes with 10 ml

DMEM / FBS. For immunocytochemical staining, cells were plated into six well trays (3 x 10^5 cells / 35 mm well) containing 22 x 22 mm coverslips with 4 ml DMEM / FBS and incubated at 37°C in 5% CO_2.

2.4.3 Transient Transfection of 293T Cells by $CaPO_4$ Precipitation

Cells were seeded into six well trays (sometimes containing 22 x 22 mm coverslips) 20 hours prior to transfection at a density of 2 x 10^5 cells / 35 mm well in 4 ml DMEM / FBS. Twenty minutes prior to transfection, 2.5 µg of circular plasmid DNA was mixed with 60 µl of 250 mM $CaCl_2$ and 60 µl 2 x BBS (section 2.2.28) added drop-wise followed by immediate vortex mixing. This solution was added drop-wise around each well and gently dispersed. The cells were then incubated at 37°C, 3% CO_2 in the presence of the $CaPO_4$-DNA complexes for 16 hours. The transfection mixture was then removed, the cells washed once in PBS and 4ml of fresh DMEM / FBS added to each well and the cells incubated a further 48 hours at 37°C, 5% CO_2. This procedure was also used for cultures (1 x 10^7 cells) in 100 mm dishes as described by Whyatt and co-workers (1993).

2.4.4 Staining for β-Galactosidase Activity

To determine transfection efficiency Cos 1 and 293T cells were transfected with pCH110, a plasmid encoding β-galactosidase under the control of the SV40 early promoter. β-galactosidase activity was detected 2 days post-transfection. Cells were washed three times in PBS and fixed in 0.2% glutaraldehyde, PBS for 5 minutes. After a further 3 washes in PBS, cells were incubated in β-Galactosidase Stain solution overnight at 37°C.

2.4.5 Immunocytochemical Staining of Cells

Coverslips were removed from petri dishes 48 hours post-transfection (section 2.4.2) and washed three times with PBS. The cells were then permeabilised in methanol for 2 min. and rehydrated in PBS for 15 min. Caprylic acid-purified rabbit sera were diluted 1 / 100 (or 1 / 50 for the M2 anti-FLAG monoclonal antibody) in PBST, 3% BSA, and 50 µl applied to each coverslip then covered with a square of vinyl laboratory film to spread the solution and prevent drying. Coverslips were incubated for 2 hours. and washed three times in

PBST for 5 min. Anti rabbit- (with anti-hLIF sera) or anti mouse- (with M2 anti-FLAG monoclonal antibody) FITC-conjugated secondary antibodies were diluted 1/50 and 100 µl applied evenly to each coverslip and incubated in the dark at room temperature for 45 minutes. The coverslips were then washed once in PBST, incubated for 30 sec. in 1 µg / ml Hoechst 3528 in PBST and washed twice more in PBST. Coverslips were mounted in 80% glycerol and viewed using a Zeiss Axioplan microscope equipped for three channel fluorescence (Zeiss filter sets II, IX and XV) and a photographic record made as soon as practicable.

In the case of 293T cells, careful and gentle treatment of the coverslips during the permeabilisation and washing steps was necessary to avoid dislodging these weakly adherent cells.

2.4.6 ES Cell Assay of hLIF in Conditioned Media from Cos 1 Cells

For Cos 1 cells, media was changed from DMEM / FBS to Incomplete ES Cell Media 16 hours post-transfection and the cells grown a further 48 hours to condition the medium. For 293T cells, the $CaPO_4$-containing transfection media was replaced 16 hours post-transfection with Incomplete ES Media Cell rather than DMEM / FBS and the cells grown a further 48 hours to condition the medium.

Appropriate 4 ml serial dilutions of conditioned media in Incomplete ES Cell Media were prepared for each conditioned media. 500 D3 ES cells / well were seeded in 24 well culture trays with 1 ml / well of each of these dilutions. Controls were Incomplete ES Cell Media without LIF and Incomplete ES Cell Media with 100 U / ml recombinant mLIF. Cells were then grown for 5 days and scored for preservation of rounded ES cell colony morphology by phase-contrast microscopy. After 7-8 days the cells were stained for Alkaline Phosphatase activity using a Sigma kit according to the manufacturer's instructions.

2.4.7 Immunoprecipitation of hLIF Proteins from Transfected Cos 1 Cells

Cos 1 cells transfected by electroporation were cultured for 48 hours post-transfection and washed twice in PBS, starved of methionine and cysteine by incubation with 2 ml of met- / cys-deficient DMEM, 2mM L-glutamine for 60 min., 50µCi of Tran^{35}S-label was then added to this media and cellular proteins were labelled over a period of 3-4 hours. Following labelling, all

immunoprecipitation steps were carried out at 4°C. Labelled cells were washed once with PBS before addition of 1ml NP-40 lysis buffer and incubation for 30 minutes with agitation. Lysate was harvested by scraping and centrifuged at 15000 x g for 15 min. to pellet cell debris.

The supernatant was pre-cleared by incubation with 100 µl of 10% Protein A-sepharose / NP-40 lysis buffer slurry for 1 hour with gentle agitation followed by brief centrifugation at 15000 x g. The supernatant was transferred to a fresh tube and a 1 / 50 dilution of caprylic-acid-purified anti hLIF polyclonal antibody was added and incubated for 2 hours with agitation. 100 µl of 10% Protein A-sepharose / NP-40 lysis buffer slurry, previously blocked by incubation with 2% non-fat milk powder for 2 hours, was added and incubated for 2 hours. Sepharose beads were pelleted by brief centrifugation at 15000 x g and washed three times with 1 ml of lysis buffer. Beads were resuspended in 20 µl of SDS-PAGE loading buffer, boiled at 100°C for 3 min. and electrophoresed on an 18% SDS-PAGE gel (see below) at 200 V. Gels were dried and radio-labelled proteins analysed by phosphorimager analysis. For immunoprecipitation of secreted LIF protein, 1 ml of the IP Labelling mix was retained after the 6 hour incubation then pre-cleared and immunoprecipitated as for cellular proteins.

4% Stacker Gel		18% Resolving Gel	
1 ml	30% Acrylamide Mix	5 ml	30% Acrylamide Mix
1.9 ml	4x Tris-SDS Buffer	2 ml	4x Tris-SDS buffer
4.5 ml	MQ H_2O	1 ml	MQ H_2O
75ml	10% APS	75 ml	10% APS
7.5ml	TEMED	7.5 ml	TEMED

2.4.8 Treatment of Cos 1 Cells with Tunicamycin and Monensin Prior to Immunoprecipitation

To inhibit N-linked glycosylation, tunicamycin (8 µg /ml) was included in the methionine / cysteine-deficient media and during the labelling procedure described above.

To inhibit Golgi-apparatus-mediated secretion, cells were "pulse" labelled and "chased" in the presence of monensin (10 µM). Cells were starved of methionine and cysteine by incubation for 60 min. in met / cys-deficient DMEM, 2mM L-glutamine and pulse labelled for 15 min. by the addition of 100 µCi Tran^{35}S-label. For the chase step, labelling media was replaced

with 2 ml met / cys-deficient DMEM with or without 10 μM monensin and incubated for 3 hours. Cells were then subjected to the lysis and immunoprecipitation procedures described above.

2.4.9 Labelling of Apoptotic Cells Using the TUNEL Procedure

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) was performed according to the method of Kressel and Groscurth (1994), except that digoxigenin-11-2'-deoxy-uridine-5'-triphosphate was used in place of its biotinylated equivalent. Labelled cells were detected using anti-digoxigenin-fluorescein Fab fragments at 50 μg/ml

TUNNEL labelling was performed prior to staining with anti-LIF antibodies and Hoechst 33258 (bis-benzamide). Secondary antibody incubations were performed separately. Immunohistochemistry was otherwise performed as in section 2.4.5.

2.4.10 Apoptosis Assay by Co-transfection

Transfected Cos 1 or 293T cells (2.4.2 or 2.4.3) were plated into six well trays (3 x 10^5 cells / 35 mm well), the bottom of which had been marked with a 2 mm^2 grid. The DNA mixture cells were transfected with consisted of:

70% "Rescue Plasmid", either pXMT2, phBcl-2 RSV or pCXN2-CrmA,

25% "Test Plasmid" either pXMT2 or pαGalX or an hLIF in pXMT2 construct,

and 5% "Marker Plasmid" pCH110 (v/v) (all plasmid solutions at 1mg/ml).

Staining for β-galactosidase activity (2.4.4) was performed three days post-transfection, staining cells were visualised under phase-contrast microscopy and individual cells present in a 2 cm^2 area counted. Changes in cell numbers resulting from co-transfection with phBcl-2 RSV or pCXN2-CrmA were derived for each test plasmid by dividing the number of cells staining in co-transfections with these constructs by the number of cells staining in co-transfections with pXMT2.

2.5 Computer-Based Nucleic Acid and Protein Analysis

Nucleic acid sequence data was generally compiled and analysed using MacDNAsis 3.1. PCR primer design was aided by Oligo 2.0. Putative transcription factor binding sites were identified using the MatInspector internet service.

2.6 Phosphorimager Analysis and Digital Image Processing

Gels and filters were exposed to Storage Phosphor Screens (Molecular Dynamics) and processed using a Molecular Dynamics PhosphorImager running the ImageQuant software package, expression levels were determined using this package. PhosphorImager files were handled as TIFF files in AdobePhotoshop 3.0 and PICT files in Canvas 3.2, and printed using either a Hewlett Packard LaserJet 4MP printer or a Kodak 8650 PS dye sublimation printer.

2.7 Containment Facilities

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

Chapter 3 :

Characterisation of Two Novel hLIF Transcripts: Implications for a Complex, Conserved LIF Gene Structure

3.1 Introduction

As one of the few systems available for *in vitro* studies of human germ cell tumours and multipotential cells, cultured embryonal carcinoma (EC) stem cells were chosen as the most appropriate experimental model for elucidating potential roles for LIF action in the maintenance of these tumours (section 1.6). The identification of what appeared to be a novel hLIF transcript (or transcripts) in two independently-derived multipotential human EC cell lines using the ribonuclease protection assay (Figure 1.5) provided a foundation for these investigations.

Firstly, the possibility that expression of the novel transcript resulted from rearrangements of the *Lif* loci in GCT 27 and Tera 2 human EC cells was examined by comparing the *Lif* loci in these cells with the *Lif* loci in other cultured human tumour cell lines using Southern blot analysis. Concurrently, 5' RACE-PCR-based molecular cloning was employed in order to isolate the novel hLIF transcript(s) expressed by GCT 27C4 EC cells since they been previously established to be divergent in their 5' end sequences (section 1.6.3). Two novel hLIF transcripts were cloned using this approach and their existence in GCT 27C4 cells was validated using RT-PCR cloning and genomic PCR cloning. Examination of the cDNA sequences showed that they contained alternate first exons spliced to the previously characterised second and third exons. Comparison of the sequences of these alternate first exons and the various hLIF genomic sequences reported for other species revealed a highly conserved organisation of the LIF gene in eutherian mammals. It also indicated previously unappreciated level of complexity in the regulation of transcript and protein expression from the LIF gene. Intriguingly, the novel hLIF transcripts were found to encode a conceptual protein of potentially intracellular localisation and hence cell-autonomous function.

3.2 Results

3.2.1 Southern Blot Analysis of the Human *Lif* Locus in EC and Other Cell Lines

To determine whether intra-genic rearrangements of the human *Lif* locus could give rise to the expression of the novel hLIF transcript(s) observed in GCT27/C4 and Tera 2 EC cells, Southern blot analysis was used to compare the *Lif* loci in these cells with the *Lif* loci in diverse human tumour cell lines. An hLIF-D cDNA fragment (HpGEM1, Rathjen et al., 1990b) containing sequence from all three exons of the LIF gene and spanning the 5' divergent region was used to probe four different double restriction endonuclease digests of genomic DNA from GCT 27C4, Tera 2, HeLa and 293T cells (Figure 3.1). The sizes of hybridising restriction fragments seen were those predicted using the published restriction map (Stahl et al, 1990). Thus, no gross re-arrangements of the *Lif* locus could be detected in any of the four cell lines examined. The mechanism directing expression of the novel hLIF transcript(s) in EC cells thus did not depend upon gross intra-genic rearrangement of the *Lif* locus.

3.2.2 5' RACE-PCR Cloning of Two Novel hLIF Transcripts from EC Cells

In order to learn the nature of the novel hLIF transcript (or transcripts) expressed in GCT 27 and Tera 2 EC cells, 5' RACE-PCR cloning (Frohman et al., 1988) of hLIF cDNAs from GCT 27C4 cells was carried out using a commercially available kit. After reverse transcription with the hLIF-specific hLIFRT primer (Figure 3.2) and ligation of the anchor oligonucleotide to the resulting single stranded cDNAs, an initial round of PCR was performed using the Anchor primer and the hLIF3 primer (Figure 3.2). To generate sufficient specific products for molecular cloning a further round of "nested" PCR was performed. One tenth of the original PCR reaction was reamplified using the Anchor primer and hLIF3N primers (Figure 3.2). Second round PCR products visualised by ethidium bromide staining of an agarose gel (Figure 3.3 A) appeared as a continuous smear that hybridised with an hLIF-D cDNA probe when Southern blotted. More efficient hLIF-specific amplification was obtained with the Anchor primer and hLIF3N primer combination as assessed by Southern blotting. PCR products were gel purified and cloned without prior restriction endonuclease digestion into *SmaI*-cut pT7T3 19U that had not been phosphatase treated (Figure 3.3 B). Three classes of products in the size ranges of 200-600 bp,

Figure 3.1 **Genomic Southern Blot Analysis of *Lif* Loci in the Human Embryonal Carcinoma Cell Lines GCT 27C4 and Tera 2 and the Unrelated Human Tumour Cell Lines Hela and 293T.**

Bam H1-*Kpn* I, *Bam* H1-*Eco* RV, *Hin*d III-*Kpn* I, and *Hin*d III-*Bgl* II restriction endonuclease double digests were performed using 20 µg genomic DNA prepared from the GCT 27C4, Tera 2, HeLa and 293T cell lines, electrophoresed on a 1% agarose gel and a Southern blot taken (section 2.x.y). The Blot was then probed using the pC10-6R hLIF cDNA fragment (Moreau et al., 1988) to determine if intragenic rearrangement of the LIF locus had occurred. Final washing stringency was 0.2 x SSC, 0.1% SDS at 65°C. Indicated at left are the sizes of ^{32}P end-labelled *Eco* R1-digested phage SPP1 DNA molecular weight markers.

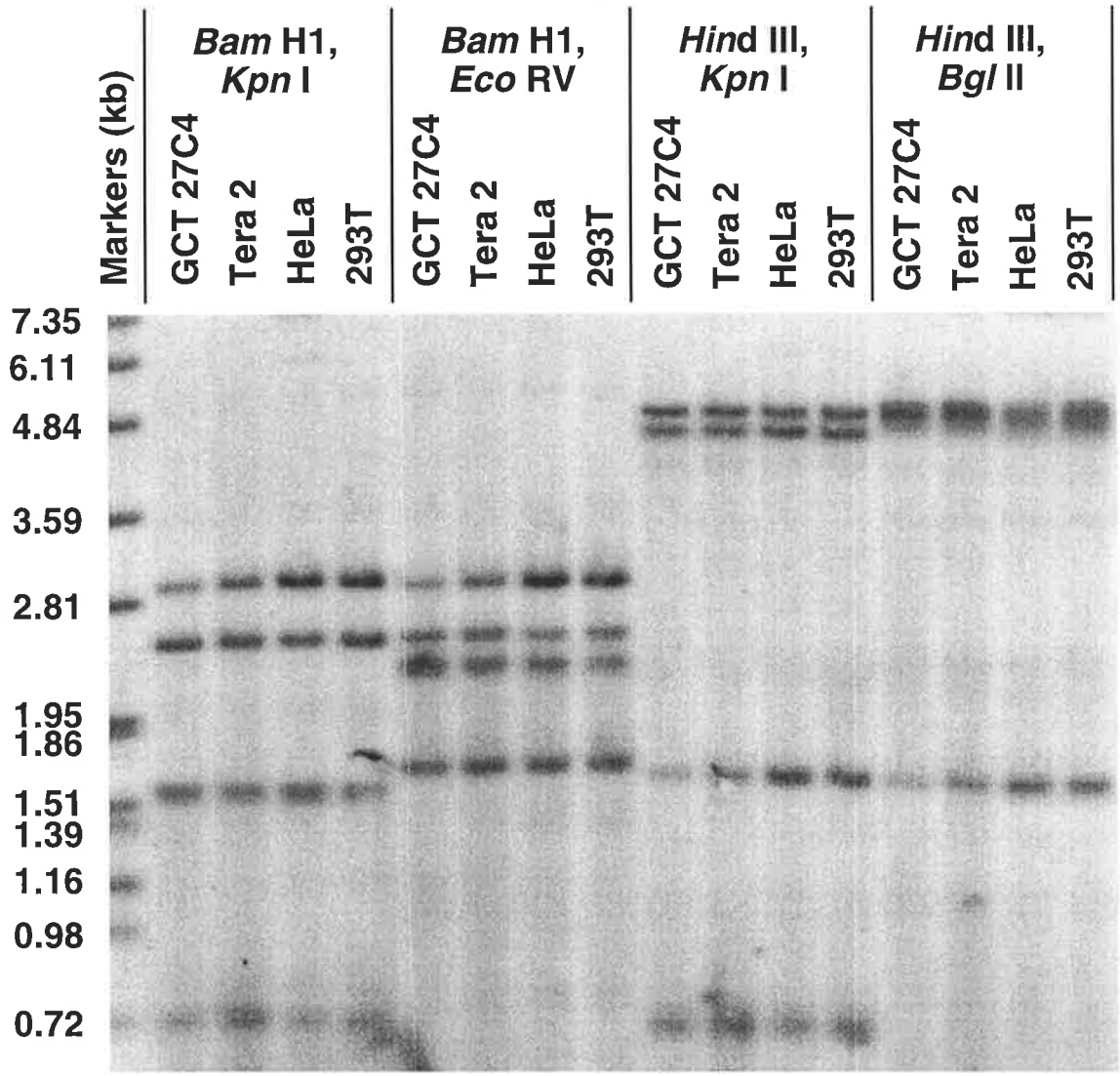
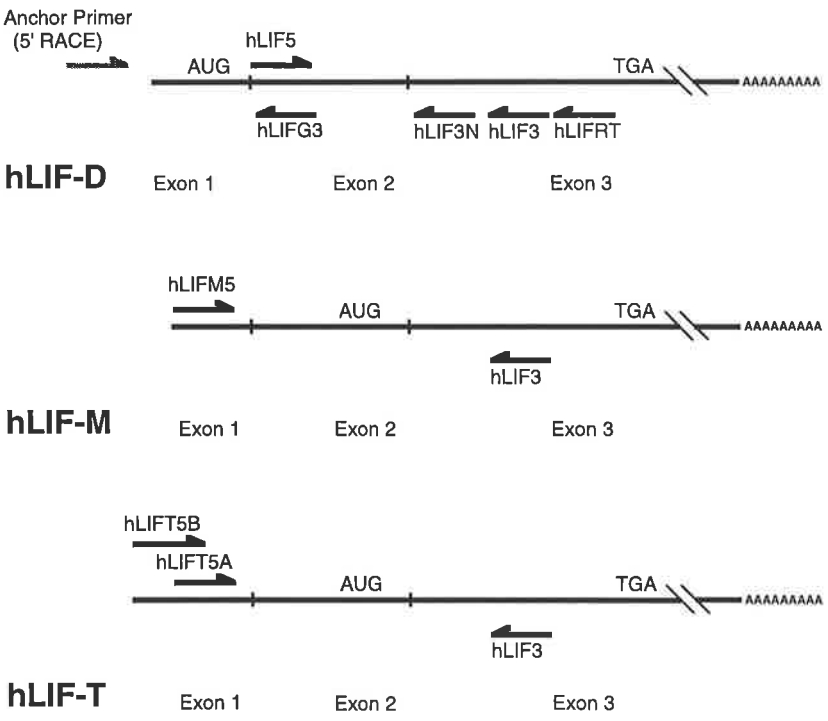

Markers (kb)
Bam H1, Kpn I
Bam H1, Eco RV
Hind III, Kpn I
Hind III, Bgl II
GCT 27C4
Tera 2
HeLa
293T
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72

Figure 3.2 Positioning of Primers Used in PCR Cloning Procedures Described in Chapter 3

(A) Schematic showing positioning and orientation of PCR primers used in isolation of hLIF clones reported in this chapter with reference to the open reading frames contained in the hLIF-D, hLIF-M, and hLIF-T alternate transcripts.

(B) Sequence of hLIF-specific primers shown in *A*. Subscripted numbers indicate the position of the first homologous nucleotide in the primer relative to the hLIF-D cDNA sequence of Moreau and co-workers (1988) or, in the case of hLIF-M- and hLIF-T-specific primers, that given in Figure 3.4. Sequences preceding the subscripted nucleotide numbers are non-homologous and were added to provide convenient sites for restriction endonuclease digestion. > Indicates that the primer is a 5' or "sense" primer. < Indicates that the primer is a 3' or "antisense" primer.

A



B

hLIF5 > $_{76}$CTGCTGTTGGTTCTGCA$_{91}$

hLIFM5 > TAGAAT$_{1}$TCTGGAAGCGTGTGG$_{15}$

hLIFT5A > ATGGAATTC$_{45}$GACCTTTTGCCT$_{57}$

hLIFT5B > ATGAATTC$_{1}$TGTCACCTTTCACTTTCCT$_{17}$

hLIFG3 < $_{80}$ACAACCAAGACGTGACCTT$_{98}$AAGAT

hLIF3N < $_{496}$TTGTAGTGGGCCCTGGTCTT$_{416}$

hLIF3 < $_{446}$GTCGTTCGAGTTGCGG$_{462}$CCTAGGATA

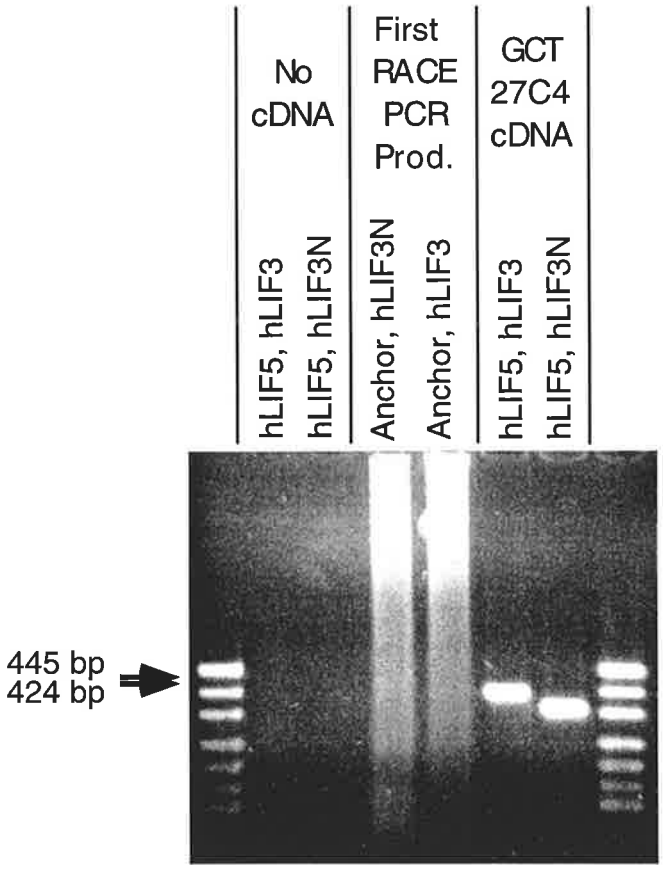
hLIFRT < $_{505}$ACACGTCGTTCATGGTGGA$_{527}$

Figure 3.3 5'RACE-PCR Amplification and Cloning of hLIF cDNAs from GCT 27C4 Cells

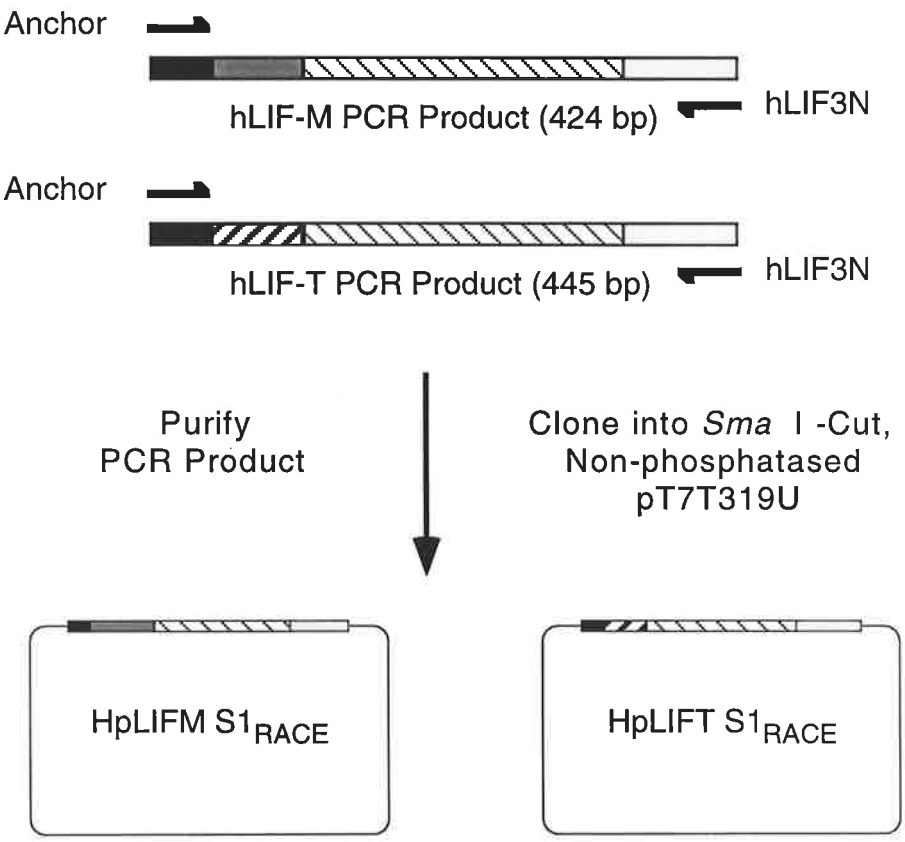
(A) PCR products produced with the indicated primer combinations, visualised on a 3% agarose gel stained with ethidium bromide. Negative control reactions contained no input cDNA. 5'RACE-PCR and cloning were performed as described in section 2.3.5.

(B) Schematic of the strategy used to clone novel hLIF cDNAs from the above PCR products. Three classes of products were gel purified and ligated into *Sma* I-cut pT7T319U that had not been phosphatase treated, and clones containing hLIF sequences were identified for sequencing using a colony-lift procedure. Different shading patterns are used for different exonic regions and the Anchor oligonucleotide, ligated onto the 5' end of the hLIF cDNAs and present in the PCR product, is shown shaded in solid black.

A


No cDNA
First RACE PCR Prod.
GCT 27C4 cDNA
hLIF5, hLIF3
hLIF5, hLIF3N
Anchor, hLIF3N
Anchor, hLIF3
hLIF5, hLIF3
hLIF5, hLIF3N
445 bp
424 bp

B


Anchor
hLIF-M PCR Product (424 bp)
hLIF3N
Anchor
hLIF-T PCR Product (445 bp)
hLIF3N
Purify PCR Product
Clone into *Sma* I -Cut, Non-phosphatased pT7T319U
HpLIFM S1RACE
HpLIFT S1RACE

600-1,000 bp and 1,000-2,000 bp were cloned separately. Resulting transformants were screened for hybridisation to an hLIF-D cDNA probe using a colony-lift procedure (Sambrook et al., 1989). Twenty one independent clones hybridising with the hLIF-D probe were sequenced, 15 from ligation of the 200-600 bp products, 4 from ligation of the 600-1,000 bp products, and 2 from ligation of the 1,000-2,000 bp products.

In addition to PCR products which appeared to be artefactually produced, this revealed four cDNAs representing three distinct hLIF transcripts (all cloned as 200-600 bp products) containing appropriately positioned primer sequences. One cDNA represented the previously reported hLIF-D transcript (Moreau et al., 1988). The other three cDNAs represented two distinct hLIF transcripts that contained the previously reported exon 2 and exon 3 sequences (Stahl et al., 1990) but their sequences diverged upstream of the exon 1 / exon 2 boundary (Figure 3.4 A). Thus, these two transcripts were novel first exon splice variants of the hLIF transcript. Two well separated regions of intron 1 of the human LIF gene (Stahl et al., 1990) that were homologous in sequence to the two novel sequences present in these cDNAs were identified. The homology seen in these genomic regions was complete except that a 9 bp insertion and a 2 bp substitution were present in one cDNA (discussed in sections 3.2.3 and 3.2.6). Furthermore, the novel hLIF first exon sequences also demonstrated significant homology to the mouse LIF-M (Rathjen et al., 1990a) and mouse LIF-T (Haines, 1997; Haines et al., 1999) alternate first exon sequences which are found in the first intron of the mLIF gene (Figure 3.5, section 3.2.5). The mLIF-T transcript is a third mLIF transcript, consisting of an alternate first exon spliced to the common second and third exons of the mLIF gene, that was identified by ribonuclease protection assay of ES cell RNA and was cloned using 5' RACE PCR (Haines et al., 1999) during the same period that the studies reported in this thesis were being carried out. These and subsequent observations suggested that these novel hLIF cDNAs represented transcripts that were the human equivalents of the mLIF-M and mLIF-T transcripts and are hereafter referred to as hLIF-M and hLIF-T (Figure 3.4 A and B).

Like mLIF-T, neither the hLIF-M or the hLIF-T first exon sequences contained an in-frame potential translational initiation codon and hLIF-T had an in-frame UGA translational termination codon immediately upstream of the exon1 / exon 2 boundary (Figure 3.4 A). Thus, the sequences within the hLIF-M and hLIF-T first exons do not appear to encode protein.

Figure 3.4 Sequences of hLIF cDNAs Cloned from GCT 27C4 Cells by 5'RACE-PCR and RT-PCR With Schematics of the hLIF Gene, hLIF Transcripts and Conceptual Proteins

(A) Sequences of the hLIF cDNAs cloned by 5'RACE-PCR from GCT 27C4 cells. The phase of the open reading frame of the hLIF-D cDNA is indicated and the same phase is shown for the hLIF-M and hLIF-T cDNAs. Sequence forming part of the hLIF-D coding region is in **bold**. A 9 bp insertion and a 2 bp substitution present in the hLIF-T cDNA but not in the reported hLIF gene sequence (Stahl et al., 1990), are shown underlined. An in-frame translational termination codon in the hLIF-T first exon is shown boxed. The exon 1 / exon 2 boundary is indicated by a shaded triangle.

(B) Schematic showing the organisation and splicing of the LIF gene. Alternate first exons are transcribed, apparently from independent promoters, and spliced to common second and third exons. Intron sizes are indicated below.

(C) Schematic showing the exonic structure of the transcripts represented by these hLIF cDNAs. Legend is as for Figure 1.4. The lack of an in-frame AUG translational initiation codon in the first exons of hLIF-M and hLIF-T is predicted to force the initiation of translation at the first in-frame AUG in the common second exon.

(D) Schematic of the hLIF-D protein and the conceptual protein encoded by the hLIF-M and hLIF-T transcripts. Legend is as for Figure 1.4. The conceptual protein encoded by the hLIF-M and hLIF-T transcripts would be N-terminally truncated by 22 amino acids relative to the mature hLIF-D protein and lack a conventional secretion signal sequence. It might therefore be localised intracellularly.

A

hLIF-D

GG CAT CTG
AGG TTT CCT CCA AGG CCC TCT GAA GTG CAG
CCC ATA **ATG AAG GTC TTG GCG GCA GGA GTT**

hLIF-M

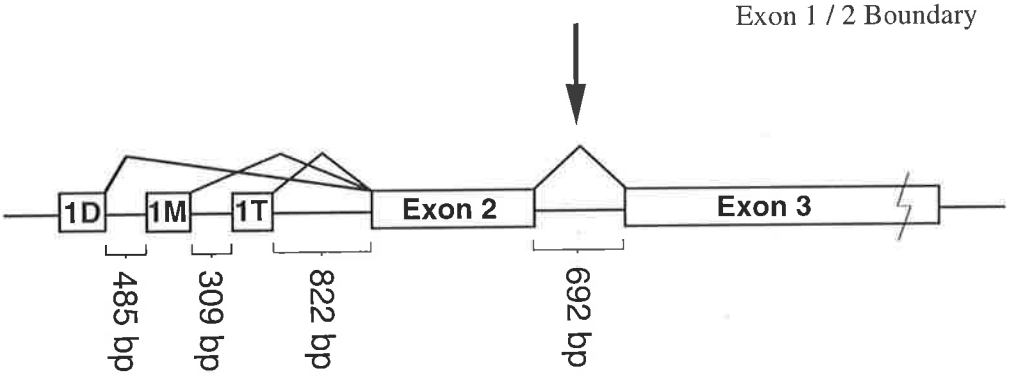
TCT GGA AGC GTG TGG TCT GCG CTA GGA GTT

hLIF-T

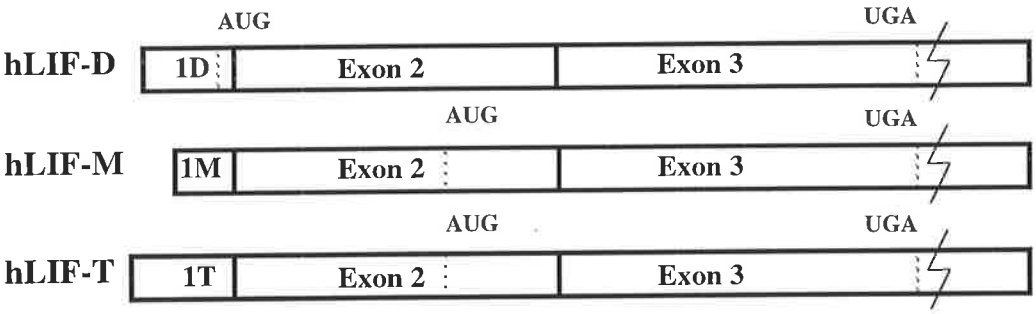
CAC CTT TCA CTT
TCC TTC CTC CCC GCC CAC CCG ACT GCC TAT
GAC CTT TTG CCT TTT CTC TCT CCT GGT GCA
CCA TTT CCT CTC CCT CCC TGA GCC GGA GTT

Exon 1 / 2 Boundary

B



C



D

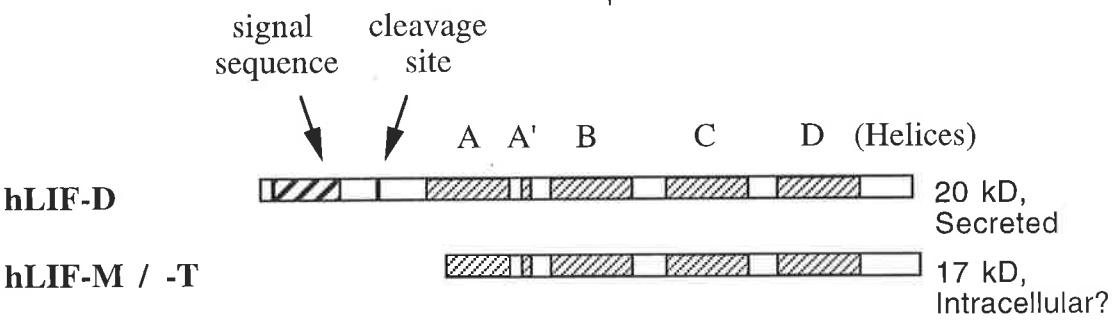


Figure 3.5 Alignment of LIF-M and LIF-T cDNAs Cloned from Murine, Human and Porcine Cells

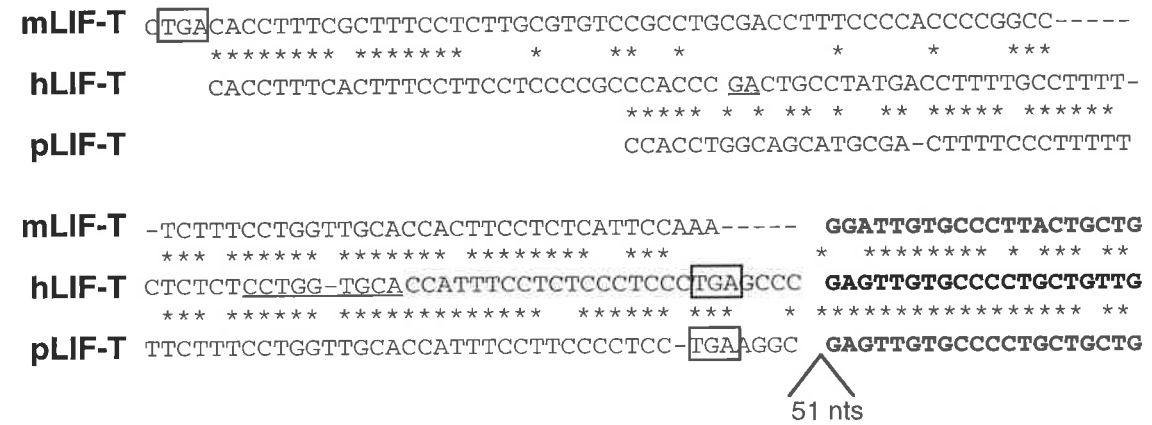
(A) Comparison of nucleotide sequences of cloned first exons of LIF-M cDNAs from murine (m), human (h) and porcine (p) sources. Sequences are aligned at the exon 1 / exon 2 boundary with exon 2 in bold type. Asterisks indicate nucleotide residues conserved between adjacent sequences. An in-frame translational termination codon present in pLIF-M is boxed and an in-frame translational initiation codon present only in mLIF-M is shaded.

(B) Comparison of the nucleotide sequences of the first exons of the cloned LIF-T cDNAs from murine (m), human (h) and porcine (p) sources. Legend is the same as that used in A except that a 9 bp insertion and a 2 bp substitution, not present in the reported human LIF genomic sequence (Stahl et al., 1990), are underlined. 51 nucleotides of extra sequence, present due to the downstream splice donor site utilised in the pLIF-T transcript, are also indicated (not shown but see Figure 3.11).

A


mLIF-M CTAGTCCCTGGAAAGCTGTGATTGGCGCGAGATGAGATGCAG GGATTGTGCCCTTACTGCTG
hLIF-M CTGGAAGCGTGTGGTCTGCGCTAG---------- GAGTTGTGCCCCTGCTGTTG
pLIF-M CTGGAAAGCTGTGATCTGTGCTAG---------- GAGTTGTGCCCCTGCTGCTG

B


mLIF-T CTGACACCTTTCGCTTTCCTCTTGCGTGTCCGCCTGCGACCTTTCCCCACCCCGGCC-----
hLIF-T CACCTTTCACTTTCCTTCCTCCCCGCCCACCC GACTGCCTATGACCTTTTGCCTTTT-
pLIF-T CCACCTGGCAGCATGCGA-CTTTTCCCTTTTT
mLIF-T -TCTTTCCTGGTTGCACCACTTCCTCTCATTCCAAA----- GGATTGTGCCCTTACTGCTG
hLIF-T CTCTCTCCTGG-TGCACCATTTCCTCTCCCTCCCTGAGCCC GAGTTGTGCCCCTGCTGTTG
pLIF-T TTCTTTCCTGGTTGCACCATTTCCTTCCCCTCC-TGAAGGC GAGTTGTGCCCCTGCTGCTG
51 nts

Proteins arising from the hLIF-M and hLIF-T transcripts would be expected to initiate translation downstream of the exon 1 / exon 2 boundary, presumably at the first AUG in the hLIF reading frame (Figure 3.4 C and D). Since hLIF-M and hLIF-T differ only in their apparently untranslated first exons, both would be expected to encode the same conceptual protein. This protein would lack a conventional hydrophobic secretion signal peptide since translation would initiate downstream of the region encoding the signal peptide of hLIF-D (Figure 3.4 C and D). Furthermore, this protein would be N-terminally truncated by 22 amino acids relative to the mature hLIF-D protein.

3.2.3 Validation of the hLIF-M and hLIF-T Transcripts Using RT-PCR Cloning

To confirm the existence of transcripts corresponding to the novel hLIF cDNA clones isolated by 5' RACE-PCR, they were re-isolated *de novo* by RT-PCR using 5' primers specific for the hLIF-M or hLIF-T first exon sequences in combination with the hLIF3 primer (Figure 3.2). The hLIF-M5, hLIF3 primer combination amplified a band of the predicted size (436 bp) for an hLIF-M product, in addition to a smaller band. These bands were clearly visible on ethidium bromide-stained agarose gels and hybridised to an hLIF-D cDNA probe when the gel was Southern blotted. With the hLIFT5A, hLIF3 primer combination, products were only faintly visible as smears on ethidium bromide stained agarose gels, however, a product of the size predicted for an hLIF-T amplification product (469 bp) was found to hybridise to an hLIF-D probe when the gel was Southern blotted (Figure 3.7 A). In conjunction with B. Haines a second 5' primer (hLIFT5B, Figure 3.2) specific for the hLIF-T transcript was used to clone LIF-T cDNAs by RT-PCR amplifications of GCT 27C4 cDNA in combination with the hLIF3 primer (data not shown).

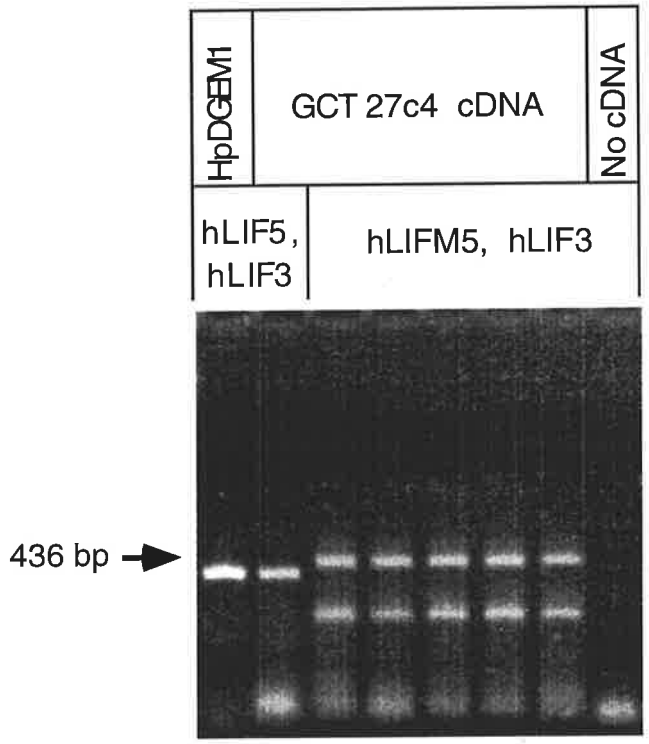
RT-PCR products generated using the hLIFM5, hLIF3 and hLIFT5B, hLIF3 primer combinations were cloned into *Eco* RI-, *Bam* HI-cut pBluescript KS+ using the *Eco* RI and *Bam* HI sites incorporated in the 5' and 3' primers respectively (Figures 3.6 and 3.7). Five independent clones produced from hLIFM5, hLIF3 products were sequenced and found to contain identical amplified sequences to those found in the hLIF-M RACE-PCR cDNA. A clone representing the smaller of the two products generated by the hLIFM5, hLIF3 primer combination (Figure 3.6A) was also sequenced and found to correspond to an LIF cDNA with

Figure 3.6 RT-PCR Amplification and Cloning of hLIF-M cDNAs from GCT 27C4 Cells

(A) PCR products generated by the indicated primer combinations, visualised on a 3% agarose gel stained with ethidium bromide. The reaction from the lane marked HpDGEM1 (hLIF-D cDNA) contained 1 pg of plasmid DNA, this reaction and one containing GCT 27C4 cDNA were performed as positive control reactions using the hLIF5 and hLIF3 primers. The negative control reaction contained no input cDNA.

(B) Schematic of the cloning strategy employed for hLIF-M RT-PCR products. PCR products were cleaved at *Eco* RI and *Bam* HI sites incorporated into the hLIFM5 and hLIF3 primers, gel purified and ligated into *Eco* RI-, *Bam* HI -cut pBluescript KS+. The smaller of the two hLIF-M5, hLIF3 PCR products in *A* was also cloned, sequenced and found to represent an hLIF cDNA with the hLIF-M5 primer in a spurious position in exon 2. Different shading patterns are used to indicate the different exonic regions of the hLIF-M_{RT} cDNA

A


HpDGEM1
GCT 27c4 cDNA
No cDNA
hLIF5, hLIF3
hLIFM5, hLIF3
436 bp

B

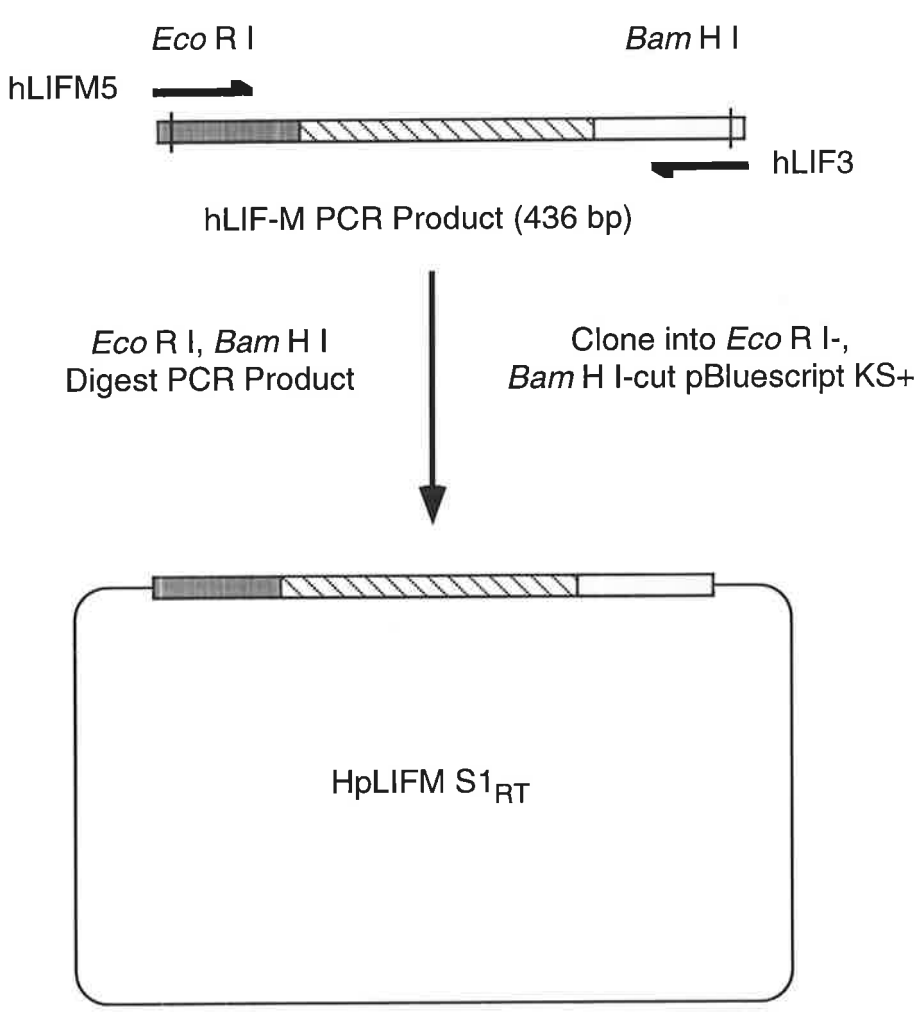
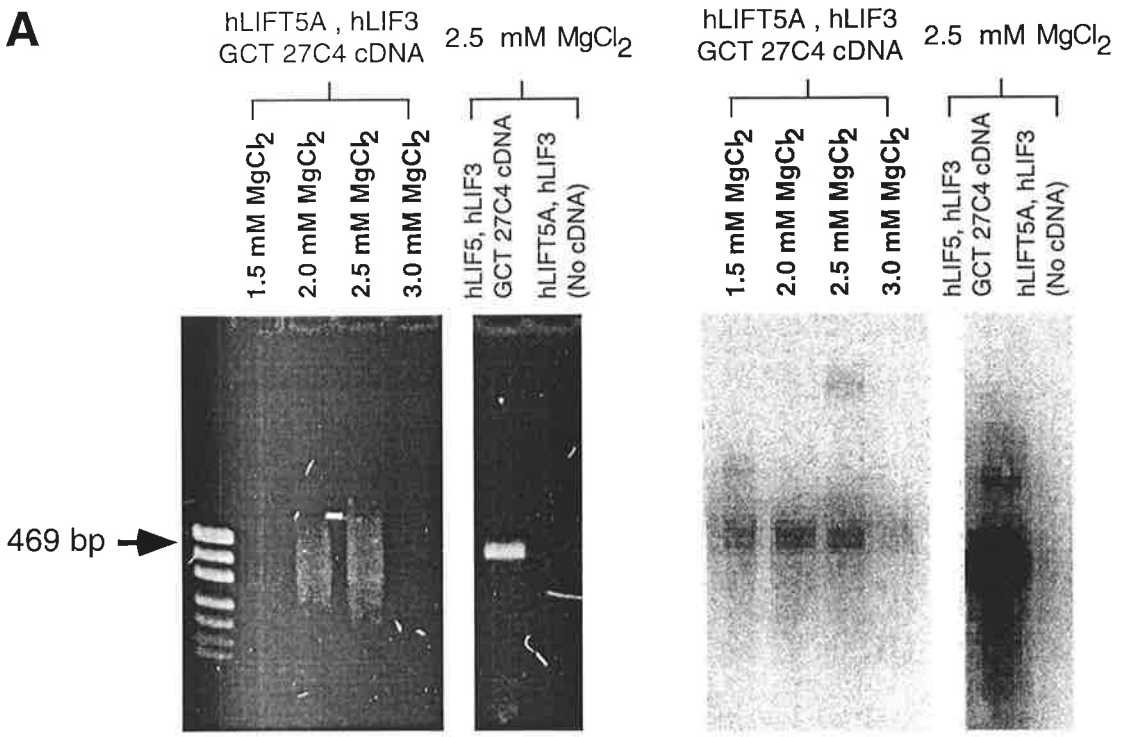
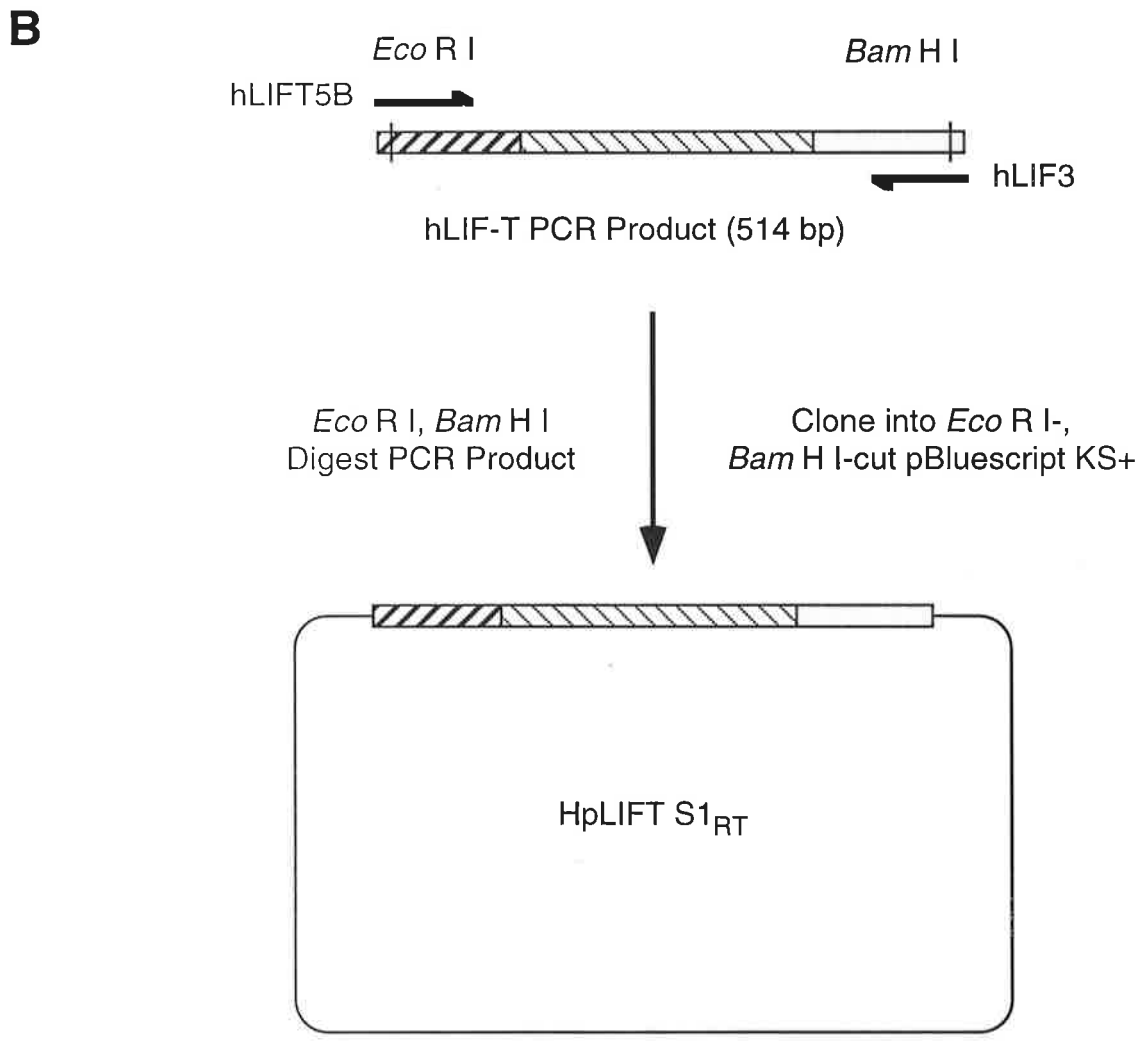

Eco R I
Bam H I
hLIFM5
hLIF3
hLIF-M PCR Product (436 bp)
Eco R I, Bam H I
Digest PCR Product
Clone into Eco R I-,
Bam H I-cut pBluescript KS+
HpLIFM S1RT

Figure 3.7 RT-PCR Amplification and Cloning of hLIF-T cDNAs from GCT 27C4 Cells

(A) PCR products produced with the indicated primer combinations at the four indicated $MgCl_2$ concentrations at an annealing temperature of 60°C. Products were visualised on a 3% agarose gel stained with ethidium bromide and on a Southern blot of the same gel probed with the pC10-6R hLIF-D cDNA probe. The negative control reaction contained no input cDNA.

(B) Schematic of the cloning strategy employed for hLIF-T RT-PCR products The products were cleaved at *Eco* RI and *Bam* HI sites incorporated into the hLIFT5B and hLIF3 primers, gel purified and ligated into *Eco* RI-, *Bam* HI -cut pBluescript KS+. Different shading patterns are used to indicate the different exonic regions of the hLIF-M_{RT} cDNA


A
hLIFT5A , hLIF3
GCT 27C4 cDNA
2.5 mM MgCl2
1.5 mM MgCl2
2.0 mM MgCl2
2.5 mM MgCl2
3.0 mM MgCl2
hLIF5, hLIF3
GCT 27C4 cDNA
hLIFT5A, hLIF3
(No cDNA)
469 bp


B
Eco R I
Bam H I
hLIFT5B
hLIF3
hLIF-T PCR Product (514 bp)
Eco R I, Bam H I
Digest PCR Product
Clone into Eco R I-,
Bam H I-cut pBluescript KS+
HpLIFT S1RT

the hLIF-M5 primer positioned spuriously within exon 2. Three clones representing hLIFT5B, hLIF3 products were found to contain sequence identical to the hLIF-T cDNA isolated by 5'RACE-PCR. This confirmed the divergence in sequence, a 9 bp insertion, of the LIF-T transcript expressed by these cells from the published LIF gene sequence. However, due to the upstream positioning of the hLIFT5B primer (Figure 3.2), they contained an additional 42 bp of exonic sequence at their 5' ends (Figure 3.4 A).

No LIF cDNAs were cloned by PCR that indicated any configuration of exons other than those found in the hLIF-M and hLIF-T 5'RACE-PCR clones. These findings were consistent with previous investigations of hLIF-D transcription (P.D. Rathjen, unpublished observations) and indicated that the newly identified exons served only as first exons. They also suggested that the three LIF transcript classes were likely to arise from separate promoters.

3.2.4 Identification of Porcine LIF-M and LIF-T Transcripts by RT-PCR Cloning

Since conservation of both the sequence and genomic positioning of the human and mouse LIF-M and LIF-T alternate first exons was seen, a wider comparison of the genomic regions containing the LIF-M and LIF-T first exons in every reported LIF gene sequence was undertaken (see section 3.2.5). These observations suggested that LIF-M and LIF-T alternate first exons might exist in the LIF genes of all eutherian mammals. To further investigate this possibility RT-PCR cloning of LIF-M and LIF-T cDNAs from porcine primary embryonic fibroblasts was attempted by P. Rathjen and B. Haines respectively (Haines et al., 1999 and data not shown). Using primers directed against the putative pLIF-M and pLIF-T first exon sequences and the third exon of the porcine LIF gene (Wilson et al., 1992), cDNAs with sequences corresponding to those predicted for the porcine LIF-M and pLIF-T transcripts were cloned (Figure 3.5).

3.2.5 Comparison of the Novel hLIF First Exon Regions With Reported hLIF Genomic Sequences

When the first exons of LIF-M and LIF-T cDNAs cloned from human, murine and porcine cells were compared, significant levels of homology were observed among them (Figure 3.5). This alignment was therefore extended to include regions of the human, murine and porcine

LIF genes immediately upstream and downstream of the LIF-M and LIF-T first exons. Regions from intron 1 of the ovine (Wilson et al.,1992) and bovine (Kato et al., 1995) LIF genes that were conserved in terms of their sequence and position were included in these alignments. These comparisons identified localised regions of sequence conservation within intron 1 of the mammalian LIF gene that encompassed the cloned LIF-M and LIF-T first exons and predicted the existence of others (Figure 3.8).

Conserved sequences containing distinct but overlapping sets of putative transcription factor binding sites could be identified upstream of the LIF-M and LIF-T first exons (Figure 3.8), suggesting that the independent transcription of the LIF-D, LIF-M and LIF-T transcripts has been conserved during evolution of the LIF gene in all eutherian mammals. Similarities in the genomic positioning of the cloned exonic regions of the murine, human and porcine LIF genes (Figure 3.9) support this notion.

Splice donor sites conforming to the consensus described by Mount (Mount, 1992) could be identified in the cloned and predicted LIF-M and LIF-T first exons (Figure 3.8). Multiple consensus splice donor sites that could be utilised in the production of ovine and bovine LIF-T transcripts are present, however, no single probable splice donor site could be predicted because of the lack of positional conservation among first exon splice donor sites utilised in the cloned LIF-T transcripts.

Extensive homology was seen in the region of the LIF-M first exon (Figure 3.8 B) and has been identified by others (Stahl et al., 1990; Wilson et al., 1992). There was substantial sequence identity within the transcribed regions of LIF-M first exons (exon boundaries are as identified by RACE-PCR cloning of hLIF-M and mLIF-M (Rathjen et al., 1990a)) as well as localised high levels of sequence identity upstream of transcribed regions and much lower levels in downstream non-coding regions. Consensus binding sites for a variety of transcription factors were conserved upstream of the cloned and predicted LIF-M first exons in all five LIF genes. Along with other sequences, these may constitute a proximal promoter for LIF-M transcription. This region contained potential binding sites for the transcription factors SP-1, Ets-1, AP-4, δEF1, IRF-2 and SCL/TAL-1, and a barbiturate-inducible element (Figure 3.8 B), but lacked a TATA box. The position of splice donor sites for the hLIF-M and pLIF-M transcripts, and the

Figure 3.8 Interspecies Comparison of the LIF Genes of Eutherian Mammals in the Region of the LIF-T and LIF-M First Exons

(A) Schematic diagram indicating the conserved exonic structure of the LIF gene in eutherian mammals.

(B) Comparison of nucleotide sequences around the LIF-M first exon. m, murine (nucleotides 1275-1524; Stahl et al., 1990); h, human (nucleotides 1091-1299; Stahl et al., 1990); p, porcine (nucleotides 484-728; Wilson et al., 1992); b, bovine (nucleotides 476-734; Kato et al., 1995); o, ovine (nucleotides 773-1028; Wilson et al., 1992).

(C) Comparison of nucleotide sequences around the LIF-T first exon. m, murine (nucleotides 1709-1982; Stahl et al., 1990); h, human (nucleotides 1477-1744; Stahl et al., 1990); p, porcine (nucleotides 899-1174; Wilson et al., 1992); b, bovine (nucleotides 912-1191; Kato et al., 1995); o, ovine (nucleotides 1187-1459; Wilson et al., 1992).

Cloned first exons are in **bold** type and boxes enclosing only four nucleotide residues indicate splice donor sites identified in these clones. Asterisks indicate nucleotide residues conserved between adjacent sequences and boxes enclosing all five sequences indicate blocks of shared sequence identity. Horizontal brackets designate conserved consensus transcription factor binding sites with the "core" residues shaded. Consensus sequences: Barbie, atcaAAAGctggagg (Liang et al., 1995); IRF-2, gAAAg/ct/cGAAAg/ct/c (Tanaka et al., 1993); Sp1, g/tGGGc/aGGg/ag/ac/t (Kadonaga et al., 1986; Letovsky & Dynan, 1989); AP-4, CAGCTG (Unk et al., 1994); Ets-1, a/gccGGAa/tgt/c (Nye et al., 1992); δEF1, a/tt/ctcACCTgan (Selsido et al., 1994); SCL/TAL-1, aaCAGATGgt (Hsu et al., 1994); MZF-1, agtGGGGAngt (Morris et al., 1993); Ik-2, tGGGAa/t (Molnar & Georgopolous,. 1994) (highly conserved "core" residues are in UPPER CASE; variant residues are in lower case with the more frequent residues indicated).

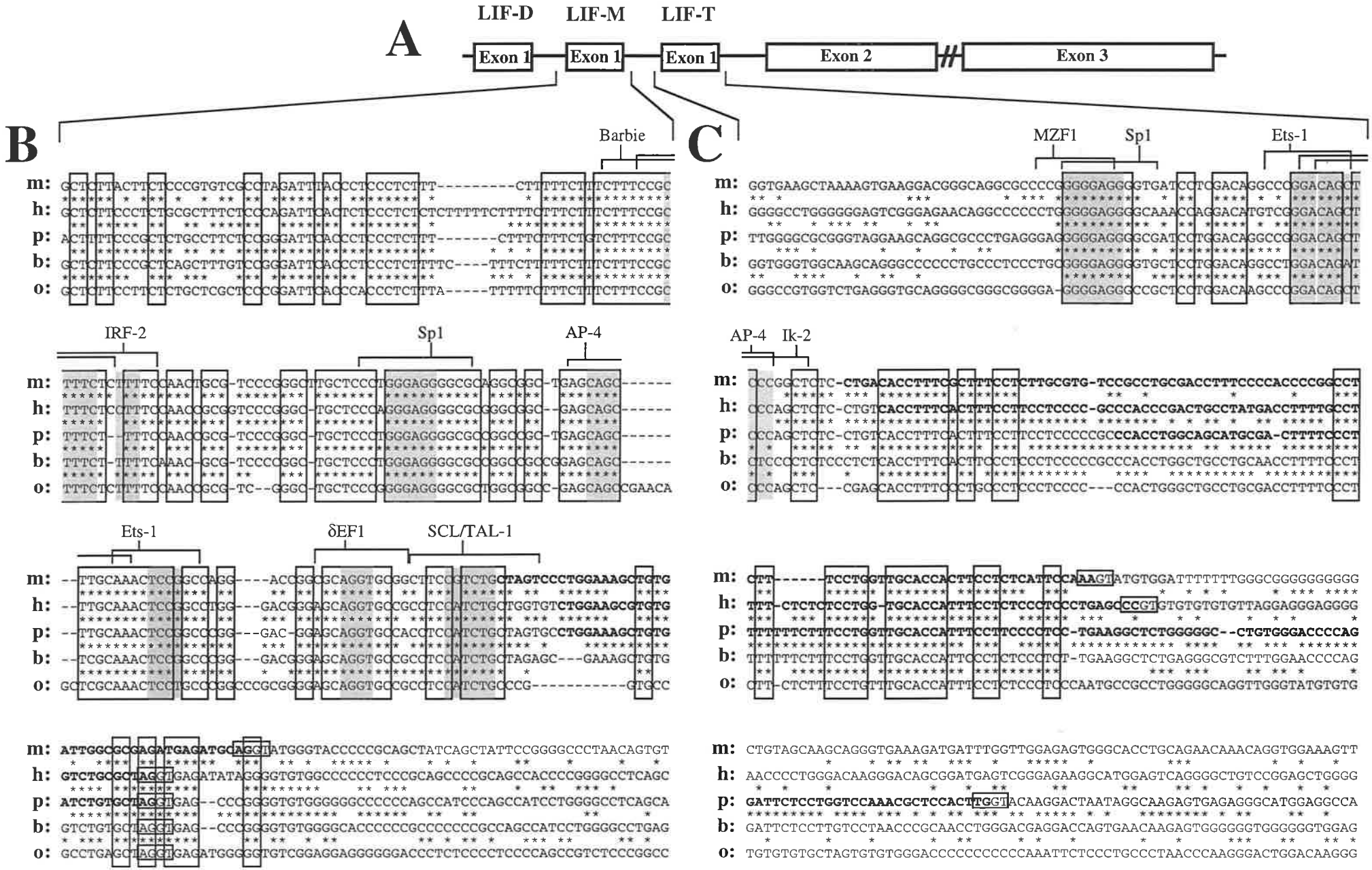
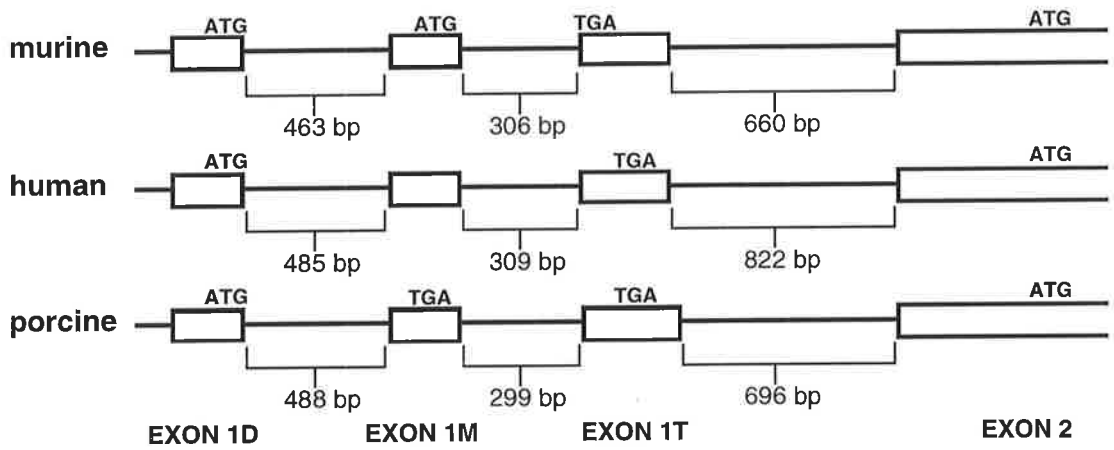

A
LIF-D
LIF-M
LIF-T
Exon 1
Exon 1
Exon 1
Exon 2
Exon 3
B
Barbie
m:
h:
p:
b:
o:
IRF-2
Sp1
AP-4
Ets-1
δEF1
SCL/TAL-1
C
MZF1
Sp1
Ets-1
AP-4
Ik-2

Figure 3.9 Conserved Position of Homologous Alternate First Exon Sequences Within the Murine, Human and Porcine LIF genes

Schematic of the genomic organisation of the alternate first exons and the second exon of the murine (Stahl et al., 1990), human (Stahl et al., 1990) and porcine (Wilson et al., 1992) LIF genes. The positions of the cloned, novel LIF first exons are indicated. In-frame initiation codons and in-frame stop codons are shown. Nucleotide distances between exons are indicated.


murine
ATG
ATG
TGA
ATG
463 bp
306 bp
660 bp
human
ATG
TGA
ATG
485 bp
309 bp
822 bp
porcine
ATG
TGA
TGA
ATG
488 bp
299 bp
696 bp
EXON 1D
EXON 1M
EXON 1T
EXON 2

predicted bLIF-M and oLIF-M transcripts, was well conserved although the mLIF-M splice donor site was located some 11 bp downstream of the other splice donor sites (Figure 3.8 B).

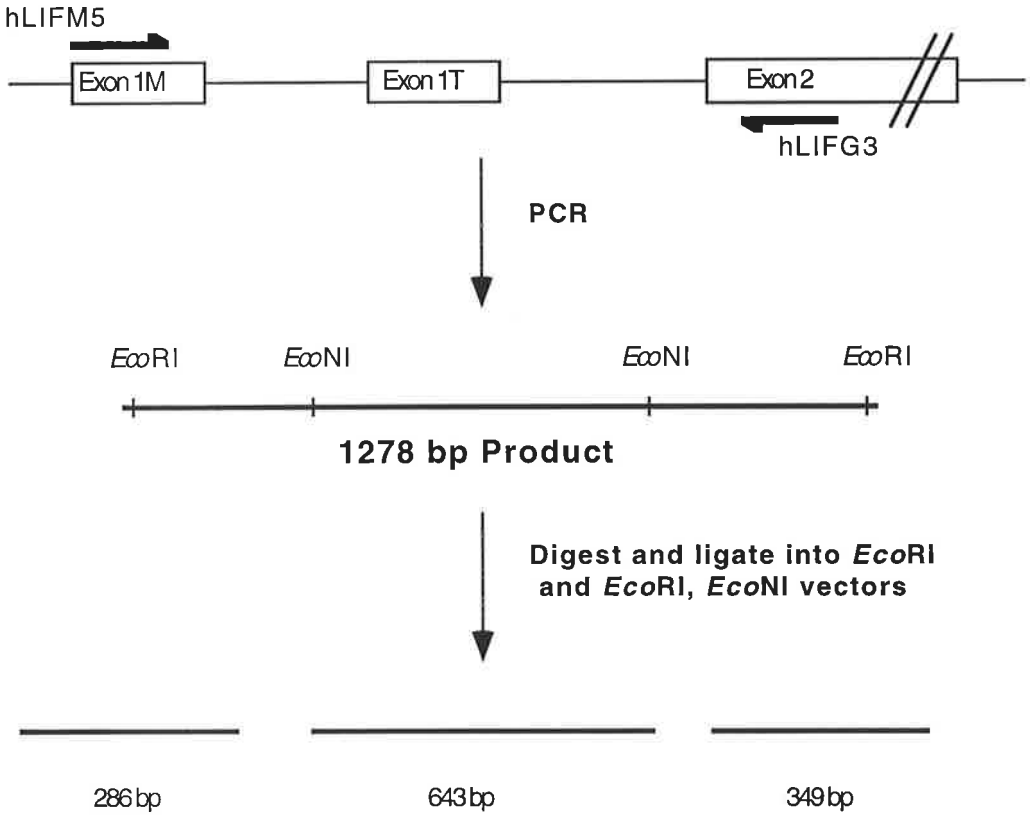
An homologous region of approximately 140 bp, well conserved between all five LIF gene sequences, was identified around the LIF-T first exon (Figure 3.8, C). Again, there was substantial sequence identity within the transcribed regions (exon boundaries are as identified by RACE-PCR cloning of hLIF-T and mLIF-T (Haines et al., 1999) and localised high levels of sequence identity upstream of the transcribed region. These significant levels of conservation contrasted with much lower levels downstream of the transcribed region and upstream of the putative regulatory regions. As with the LIF-M first exon, potential transcription factor binding sites could be identified in the sequences upstream of the cloned and predicted LIF-T first exons of all five LIF genes. Again, these may form part of a proximal promoter for LIF-T transcription. While no TATA box consensus sequences were present, potential binding sites for the transcription factors Sp-1, Ets-1, AP-4, MZF-1 and Ik-2 were present in the sequences from all five species (Figure 3.8, C). Although the transcribed LIF-T sequences showed moderate levels of conservation, the position of the splice donor site utilised varied (Figure 3.8, C). This provides further indication that the LIF-T exon is non-coding in character.

3.2.6 Analysis of the *Lif* locus in GCT 27C4 Cells by Genomic PCR

In identical positions in the hLIF-T cDNAs cloned by RACE-PCR and RT-PCR from GCT 27C4 cells, there was a 2 bp substitution and a 9 bp insertion that did not appear in the published hLIF gene sequence (Figure 3.4 A) (Stahl et al., 1990). When this substitution and insertion were included in the alignments of LIF-T cDNA sequences or the genomic sequences in the region of the LIF-T first exon (as they are in Figures 3.5 and 3.8 C respectively), they were found to increase the homology observed between the human and other cloned LIF-T sequences. In order to clarify the reason(s) for these discrepancies, PCR-based cloning of GCT 27C4 genomic DNA was used to determine the sequence in this region of the hLIF-T first exon. The hLIFM5 and hLIFG3 primers (Figures 3.5 and 3.7) were used to amplify a 1.3 kb product containing the hLIF-T first exon. This product was cloned, subcloned and sequenced (Figure 3.10). The same 2 bp substitution and 9 bp insertion were found to be present in the same position as they were previously observed in the hLIF-T RACE-PCR and RT-PCR products.

Figure 3.10 PCR Amplification and Cloning of the Region Between the hLIF-M First Exon and the Common Second Exon of the hLIF Gene from the Genomic DNA of GCT 27C4 Cells

Strategy used to clone and sequence the 1278 bp hLIFM5- hLIFG3 PCR product which encompassed the region between exon 1M and exon 2 and included exon 1T. The *Eco* RI sites incorporated into the hLIFM5 and hLIFG3 primers allowed the cloning of the product into *Eco* RI-cut pT7T319U. Three subclones of this product were then prepared using two internal *Eco*NI sites. These subclones were sequenced in both directions and sequence in the region of the *Eco*NI sites was verified by sequencing the 1278 bp product from the ends.



Sequencing Subclones

Elsewhere in the genomic PCR product, a small number of other minor substitutions, insertions and deletions could be found. However, since only a single PCR product was sequenced, these would have to be verified by sequencing independent PCR products obtained from GCT 27C4 cells. The sequence of the region surrounding the putative hLIF-M splice donor site, which also formed part of the amplified region of this PCR product, did not differ in sequence from the same region of the previously reported hLIF gene sequence (Stahl et al., 1990).

3.3 Discussion

3.3.1 Two Novel hLIF cDNAs

Three alternate first exon variants of the mouse LIF transcript have been reported: mLIF-D (Gearing et al., 1987), mLIF-M (Rathjen et al., 1990a) and mLIF-T (Haines, 1997; Haines et al., 1999). However, the only human LIF transcript to be reported was hLIF-D, encoding a secreted, freely diffusible protein (Moreau 1988). This thesis reports the cloning of cDNAs from embryonal carcinoma cells representing two novel variants of the hLIF transcript containing alternate first exons. These variant hLIF transcripts appeared to be the human equivalents of the mLIF-M and mLIF-T transcripts. Both the hLIF-M and hLIF-T transcripts appeared to encode an identical, truncated LIF protein that could not be secreted extracellularly by the conventional signal peptide-mediated pathway.

Unlike the mLIF-M first exon, the hLIF-M first exon did not appear to encode any protein. Cross-species comparisons of the regions corresponding to the LIF-M exons of the five reported mammalian LIF genes (Figure 3.8 B) indicated that the mLIF-M first exon was in fact unique in its potential to encode protein as assessed by the presence of an in-frame AUG translational initiation codon (Rathjen et al., 1990a). The positioning of the mLIF-M first exon splice donor site was divergent with respect to the splice donor sites of the other cloned and predicted LIF-M first exons (Figure 3.8 B). In contrast, the positioning of the splice donor sites of the other cloned and predicted LIF-M first exons was precisely conserved (Figure 3.8 B). This provides further indication of the unique nature of the mLIF-M transcript. Furthermore, the

reported sequences of the mLIF gene (Stahl et al., 1990; Hsu et al., 1994) in the region of the mLIF-M first exon splice donor site suggest that unusual RNA processing events would need to occur in order to produce the transcript sequence represented in the cDNA clone. Conventional splicing of donor and acceptor sites present in the reported gene sequences would result in the omission of a guanosine residue that is present in the mLIF gene sequence (Stahl et al., 1990; Hsu et al., 1994). Such an omission would place the AUG in the mLIF-M first exon out of phase with the mLIF reading frame by 1 bp. These peculiarities of the mLIF-M first exon and the lack of evolutionary conservation of the translational initiation site and the splice donor site have been noted by other workers (Wilson et al., 1992; Gough et al., 1992). These workers have concluded that transcripts equivalent to mLIF-M might not exist in other species. This is now known to be incorrect. It will be interesting to determine to what degree, if any, the proteins encoded by other LIF-M transcripts are functionally equivalent to mLIF-M. Certainly, there appear to be many species-specific facets of LIF biology, such as receptor binding properties (Layton et al., 1994a; Layton et al., 1994b) and soluble receptor production (Michel et al., 1997), which appear to be divergent in the mouse.

Sequencing of genomic DNA cloned from GCT 27C4 cells using PCR suggests that the hLIF gene sequence contains polymorphisms within the hLIF-T first exon and also in the surrounding intronic regions. This toleration of sequence variations within the hLIF-T first exon provides further evidence that it does not encode protein. Other workers also report many minor differences, which they also suggest to represent polymorphisms, in the first intron of the mLIF gene (Hsu et al., 1994).

3.3.2 Alternate Transcripts Produced from Other IL-6 Family Cytokine Genes

Other workers have identified alternate splice variants of transcripts for IL-6 family cytokines. A variant hLIF transcript has been reported in a meningioma specimen derived from a single patient, in this transcript the second exon is absent and the hLIF-D first exon is spliced directly to the LIF third exon (Pergolizi & Erster, 1994). The omission of the second exon in this transcript produces a frameshift at the splice junction only six amino acids into the LIF reading frame and results in the production of a truncated conceptual protein bearing no resemblance to LIF. The physiological relevance of these findings therefore remains uncertain. A

variant human CNTF transcript is produced by a point mutation, common among the Japanese, which results in the creation of a new splice acceptor site 4 bp upstream of the one normally utilised in its second exon (Takahashi et al., 1994). This creates a frame shift 38 amino acids into the CNTF protein and results in truncation of the protein. Unlike *CNTF*$^{-/-}$ mice (Masu et al., 1993), humans homozygous for this mutation appear to be free from neurological pathologies. Again, however, this represents an aberration in the normal expression of a cytokine rather than a normal alternate transcriptional process.

A variant hIL-6 transcript that is likely to be physiologically significant has been identified in the peripheral blood mononuclear cells of healthy individuals (Kestler et al., 1995). Expression of this transcript is induced in response to bacterial lipopolysaccharide but not the lectin concanavalin A. Like the hLIF transcript cloned from a meningioma biopsy, the variant hIL-6 transcript also omits the second exon of the gene. However, the frame shift this would be expected to produce is not present in the cloned cDNA, apparently as a result of unconventional splicing processes. Most intriguingly, a 17 kD conceptual protein encoded by the alternate hIL-6 cDNA would have a disrupted signal sequence and lack most of the A helix. Therefore, this protein could not be secreted by the conventional IL-6 secretory pathway and would be expected to interact with IL-6Rα but not gp130. In support of the existence of such a protein, a 17 kD protein was identified in the cell lysates but not the conditioned medium of lipopolysaccharide-stimulated peripheral blood mononuclear cells by Western blotting with anti-IL-6 antibodies. The potential functional similarities between this protein and the conceptual protein encoded by the LIF-M and LIF-T transcripts (see section 3.3.4) are striking.

3.3.3 Alternate LIF Transcripts Appear to be Produced from Independently Regulated Promoters

The conservation of potential transcription factor binding sites immediately upstream of the LIF-M and LIF-T first exons, the observed independent regulation of mLIF-D, mLIF-M and mLIF-T transcription (Rathjen et al., 1990b; Haines et al., 1999), and, particularly, the identification by other workers of functional promoter elements that map in the regions of the LIF-M and LIF-T first exons (Hsu et al., 1994; Kaspar et al., 1993), suggest that production of

the LIF-M and LIF-T transcript is controlled by separate, differentially-regulated promoters rather than through the differential splicing of a single transcript.

In contrast to the hLIF-D proximal promoter, which contains several TATA box consensus sequences (Stahl et al., 1990; Stahl & Gough, 1993), the regions upstream of the LIF-M and LIF-T first exons did not contain TATA boxes. However, these regions showed some features characteristic of promoters lacking TATA boxes, including consensus binding sites for transcription factors implicated in transcriptional initiation at "TATA-less" promoters, hypomethylation and regions relatively enriched for CpG dinucleotides.

Consensus binding sites for the transcription factors Sp-1 and Ets-1 are present in the predicted LIF-M and LIF-T proximal promoters. Sp-1 has been shown to play a crucial role in transcriptional initiation at several TATA-less promoters (Parks & Shenk, 1996; Lu et al., 1993; Faber et al., 1993) and Ets-1 has been shown to interact co-operatively with Sp-1 to initiate transcription from the megakaryocyte-specific αIIb gene TATA-less promoter (Block et al., 1996).

Experimental evidence from other workers also supports our identification of promoters in the LIF gene downstream of that utilised for LIF-D transcription. A conserved CpG island, often found in association with proximal promoters (Gardiner-Garden & Frommer, 1987: Bard, 1986), has been identified in the region previously characterised as the first intron of the mLIF gene (Kaspar et al., 1993). This CpG island encompasses the putative proximal promoter regions for the LIF-M and LIF-T transcripts. Additionally, two clusters of hypo-methylated *Hpa* II restriction sites, indicative of promoter activity, were identified in this region. These mapped precisely to the putative mLIF-M and mLIF-T proximal promoters, between residues 1357 and 1421, and 1707 and 1785 respectively (Figure 3.8 B and C; Stahl et al., 1990). In addition a *Sma* I site, located in the putative hLIF-M proximal promoter has been found to be hypo-methylated in meningioma specimens obtained from some patients (Pergolizi & Erster., 1994).

Transcriptional analysis of the mouse LIF gene led to the identification of two independent sites of transcriptional initiation located between the LIF-D first exon and the common second exon (Hsu et al., 1994). The first site, located between the LIF-D and the LIF-M first exons, is located within the CpG island identified by Kaspar and co-workers (Kaspar et al., 1993) and is presumed to be the proximal promoter of the mLIF-M transcript. Furthermore, a site

of transcriptional initiation was also identified downstream of the mLIF-M first exon. Although this site was not precisely mapped in these studies, it could be the mLIF-T proximal promoter postulated in this thesis.

In addition to Sp-1 and Ets-1, conserved predicted binding sites for the transcription factors AP-4 and Ik-2 were found in the putative promoters for both LIF-M and LIF-T in all five LIF gene sequences. The proposed LIF-M proximal promoter but not the proposed LIF-T proximal promoter contained conserved predicted binding sites for the SCL/TAL-1, IRF-2, δEF1, and Barbiturate-inducible factors. A conserved predicted binding site for MZF1 was identified in the proposed LIF-T proximal promoter but not that of LIF-M.

The "core" binding sequences (shaded in Figure 3.8 B & C) for each of the predicted transcription factor binding sites in the putative LIF-M and LIF-T promoters were among the most strictly conserved sequences in these regions. This suggests that these sites may be functionally relevant. While there is no direct information about the biological relevance of these predicted transcription factor binding sites, it appears that the LIF-D promoter (Stahl et al., 1990; Stahl & Gough, 1993) and the proposed promoters of LIF-M and LIF-T (Figure 3.8 B and C) are likely to be influenced by distinct but overlapping sets of transcription factors.

3.3.5 A Potential Novel LIF Protein Lacking a Secretory Signal Sequence

A key finding reported here is that the hLIF-M and hLIF-T transcripts conceptually encode a 17 kD protein that would be N-terminally truncated by 23 amino acids that is localised intracellularly and closely resembles a reported intracellular IL-6 protein (Kestler et al., 1995). What roles such a LIF protein would fulfil and how it might transduce a signal are, as yet, unknown. Given that its localisation might preclude interaction with cell surface receptors, it is possible that this protein fulfils a biological role that is distinct from that of the extracellular cytokine. It should, however, be emphasised that although this protein could not be secreted via the conventional signal peptide-mediated pathway, other mechanisms for extracellularisation may exist since the related cytokines CNTF (Lin et al., 1989; Stockli et al., 1989) and cardiotrophin 1 (Pennica et al., 1995a) are secreted despite lacking a signal sequence.

Chapter 4 :

Expression of the hLIF-M & hLIF-T Transcripts in Cultured Human Cell Lines

4.1 Introduction

Although re-cloning of the novel hLIF transcripts using a conventional RT-PCR-based procedure partly validated the original clones obtained by RACE-PCR, the existence of these transcripts still needed to be verified by direct analysis of cellular mRNAs. Ribonuclease protection assay was selected as the means to achieve this since its efficacy in detecting the very low expression levels of the alternate mouse LIF transcripts and it s ability to distinguish between them had already been demonstrated (Rathjen et al., 1990a). It was of particular interest to determine which of the novel hLIF transcripts, if any, could be associated with human germ cell tumour cell lines.

Since a LIF gene structure allowing the production of at least three alternate LIF transcripts appeared to be widely conserved among eutherian mammals, it would be expected that expression of hLIF-M and hLIF-T transcripts would not be confined to germ cell tumour cell lines. It was therefore of interest to determine whether other, more diverse, human cell lines expressed the novel hLIF transcripts.

A key inference of the work presented in Chapter 3 was that the three alternate hLIF transcripts arose from three distinct promoters rather than through the differential splicing of a single precursor mRNA. Surveying the expression of the novel hLIF transcripts might therefore provide information about the activities of these promoters. In particular, it needed to be determined whether hLIF-M and hLIF-T expression levels are constitutive or differentially regulated and, if they were differentially regulated among cell populations, whether each transcript could be independently regulated

Here, it is reported that relatively high levels of hLIF-M transcript expression are a common feature of germ cell tumour isolates and that five out of five of the independent embryonal carcinoma (EC) isolates examined predominantly expressed the hLIF-M and hLIF-T transcripts at consistent levels. Widespread expression of the hLIF-M and hLIF-T transcripts, at varible levels, was seen in a variety of other cultured cells. In support of the notion that the alternate hLIF transcripts originate from distinct promoters, it was also found that the expression of each of the alternate hLIF transcripts was independently regulated in the various cell lines examined.

4.2 Results

4.2.1 Preparation of Riboprobe Transcription Templates

The partial hLIF-M and hLIF-T cDNA clones obtained in Chapter 3 were used in the synthesis of riboprobes that were antisense to the 5' ends of the hLIF-M and hLIF-T transcripts. The phLIF-MS1_{RACE} and phLIF-T S1_{RT} plasmids (sections 3.2.2, and 3.2.3) were linearised and transcribed to produce hLIF-M- and hLIF-T-specific antisense riboprobes respectively (Section 2.3.20) (Figure 4.1). phLIF-TS1_{RT} was chosen over phLIF-TS1_{RACE} because of its longer exon 1 sequence. These probes were expected to produce two protected species as a consequence of differential first exon usage in hLIF transcripts (Figure 4.1 A & B). In order to investigate the potential of cells to respond to extracellular LIF, a template was constructed for the generation of riboprobes specific for hLIF receptor subunit gp190 (Gearing et al., 1993) transcripts (section 2.3.20). Antisense riboprobes to human glyceraldehyde phosphate dehydrogenase (GAPDH) transcripts were also produced using the plasmid pGAPM (G. Goodall, unpublished) (section 2.3.20) to act as a control for RNA loading.

4.2.2 Analysis of hLIF-M and hLIF-T Transcript Expression in Germ Cell Tumour Cell Lines

The riboprobes described above were used to protect RNA obtained from nine independent GCT cell lines representing nullipotent embryonal carcinoma (GCT 27C4, GCT 48) multipotent embryonal carcinoma (Tera 2, GCT 35, and GCT 63), primitive endoderm yolk sac carcinoma (GCT 44, GCT 46 and GCT 85), and visceral endoderm yolk sac carcinoma (GCT 72) (Andrews et al., 1996; Pera et al., 1987; Pera et al., 1988; Pera et al., 1989). In assays that used the hLIF-M probe (Figures 4.2 A and 4.3), protected species were observed that migrated at approximately 422 bp, representing digestion products containing the hLIF-M first exon and the common second and third exons of hLIF, and 398 bp, representing digestion products containing the common second and third exons. In assays that used the hLIF-T probe (Figure 4.2 B), two protected species migrated in the region of 497 bp and must represent partial digestion products or utilisation of two distinct sites of transcriptional initiation by the hLIF-T transcript. A species migrating at approximately 397 bp represented digestion products containing the common second

Figure 4.1 Production and Digestion of Riboprobes Used to Detect the Expression of hLIF-M and hLIF-T Transcripts by Ribonuclease Protection Assay

(A) HpLIF-MS1$_{RACE}$ (hLIF-M 5'RACE-PCR partial cDNA) was linearised with *Eco* RI and was transcribed using T7 RNA polymerase, protected species expected were 417 bp (Exon 1M + Exon 2 + Exon 3) and 392 bp (Exon 2 + Exon 3).

(B) HpLIFT S1$_{RT}$ (hLIF-T 5'end RT-PCR product) was linearised with *Bam* HI and transcribed using T3 RNA polymerase, protected species expected were 487 bp (Exon 1T + Exon 2 + Exon 3) and 392 bp (Exon 2 + Exon 3).

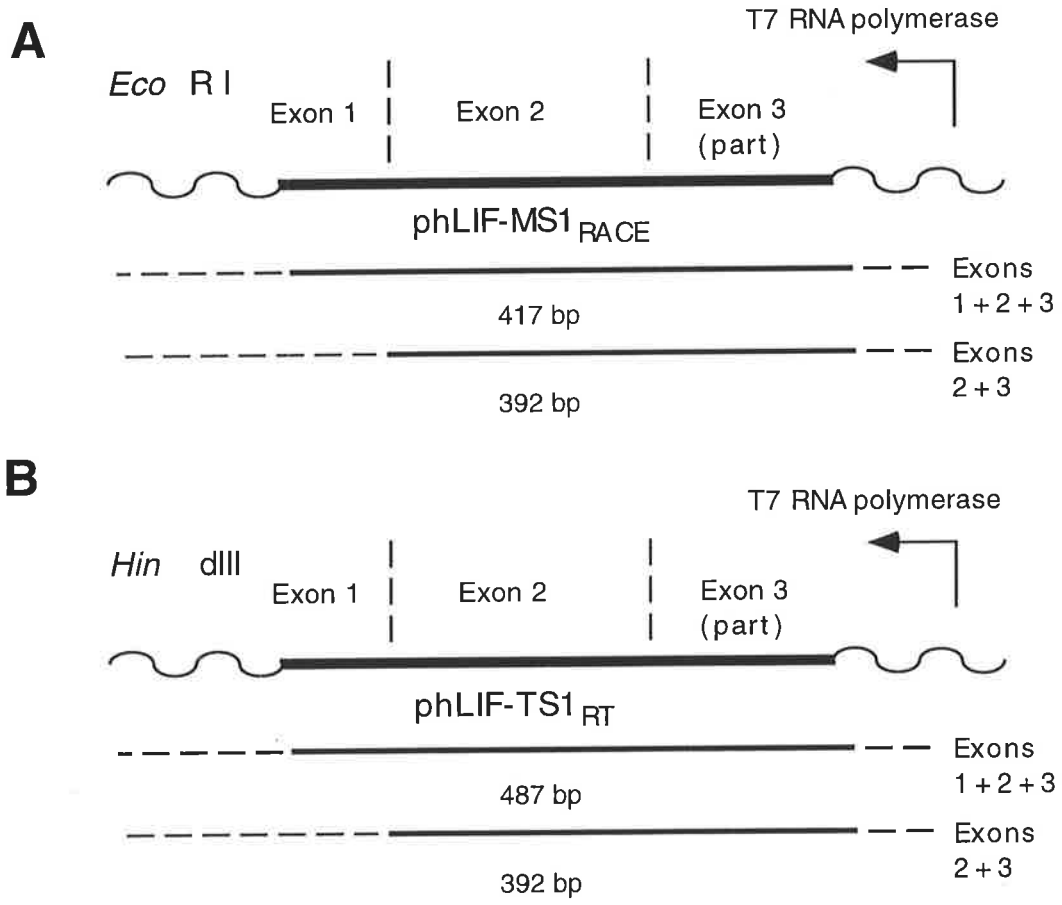

A
T7 RNA polymerase
Eco R I
Exon 1
Exon 2
Exon 3
(part)
phLIF-MS1 RACE
Exons
1 + 2 + 3
417 bp
Exons
2 + 3
392 bp
B
T7 RNA polymerase
Hin dIII
Exon 1
Exon 2
Exon 3
(part)
phLIF-TS1 RT
Exons
1 + 2 + 3
487 bp
Exons
2 + 3
392 bp

Figure 4.2 **Detection of hLIF-M, hLIF-T, and hgp190 Transcript Expression in Human Embryonal Carcinoma Cell Lines by Ribonuclease Protection Assay**

1µg of poladenylated RNA was protected with either :

(A) hLIF-M, hgp190, and hGAPDH riboprobes or

(B) hLIF-T and hGAPDH riboprobes as described in section 2.3.21.

Protected species representing the hLIF-M (Exon 1M + Exon 2 + Exon 3; 417 bp), hLIF-T (Exon 1T + Exon 2 + Exon 3; 487 bp), hgp190 ((267 bp), hGAPDH (133 bp) transcripts and hLIF transcripts diverging at the exon 1 / exon 2 boundary (Exon 2 + Exon 3; 392 bp) are indicated. To control for spurious probe hybridisation and digestion processes, the same procedure was performed using yeast transfer RNA (tRNA) in place of human mRNA.

(Polyadenylated RNA from human embryonal carcinoma cell lines was the kind gift of Dr. M.F. Pera.)

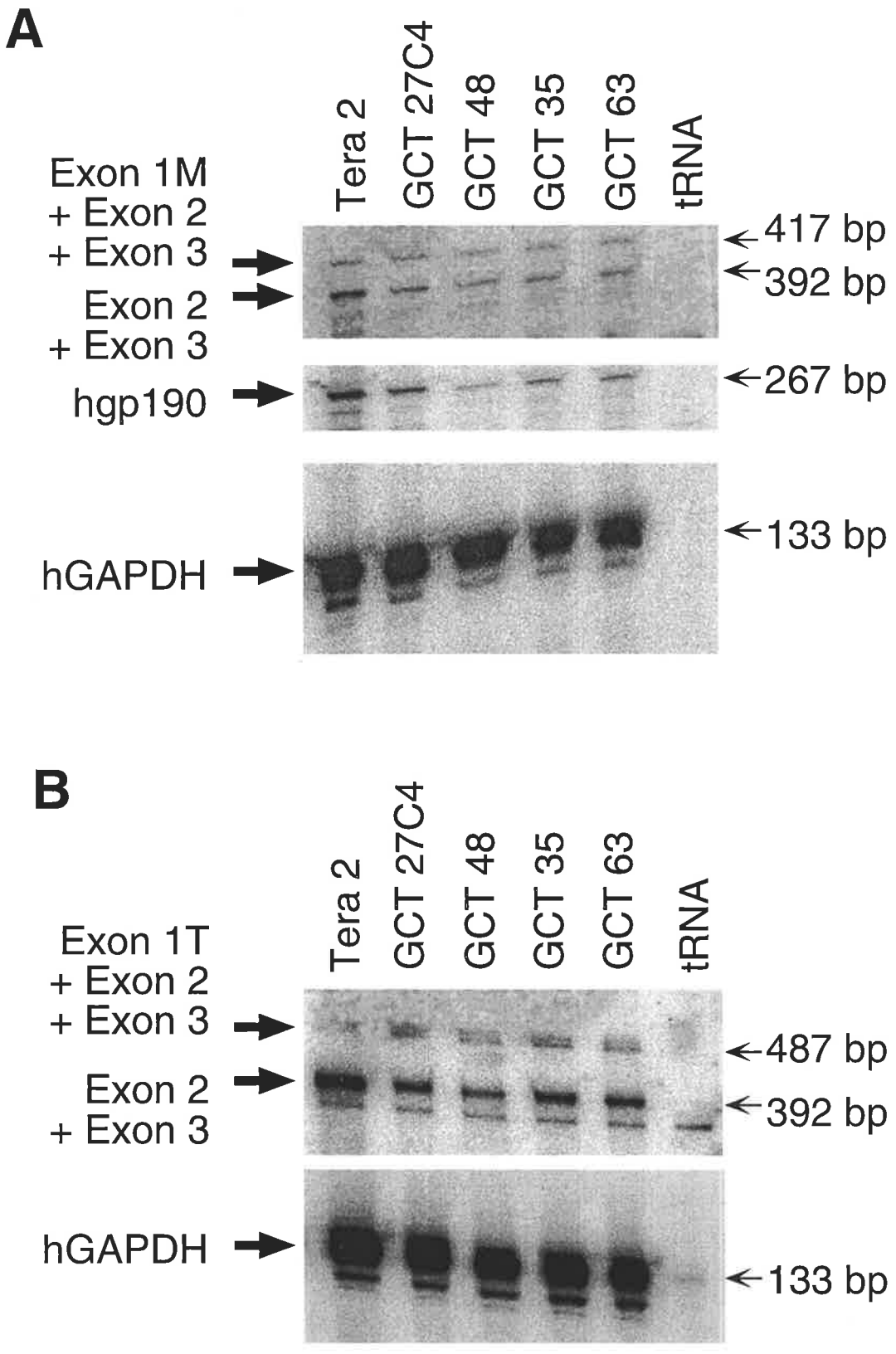
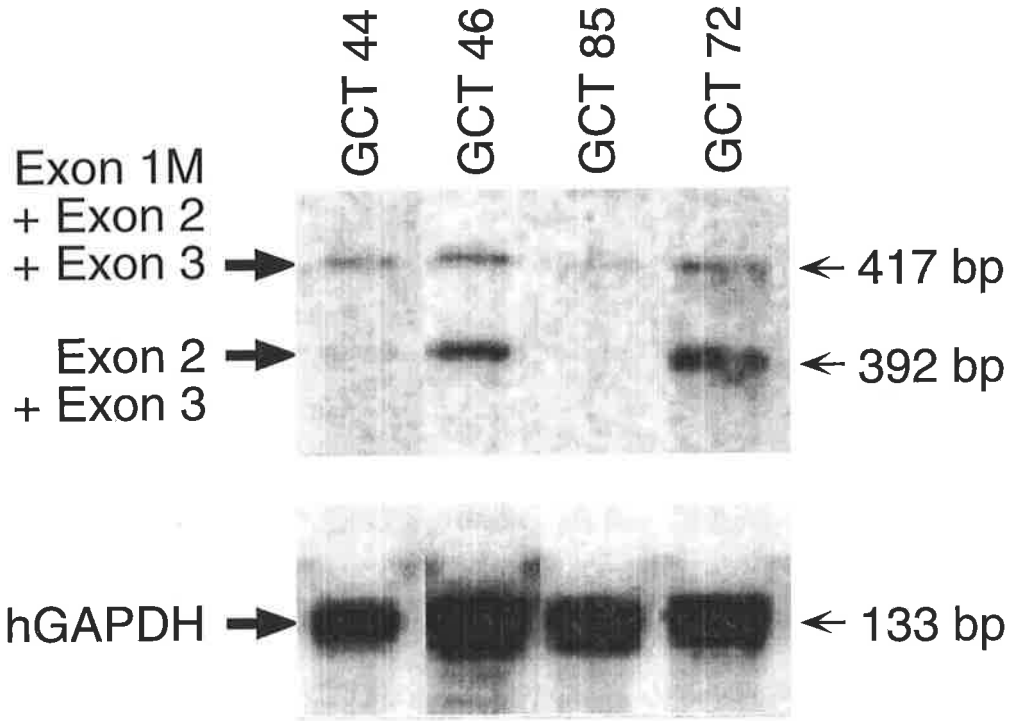

A
Tera 2
GCT 27C4
GCT 48
GCT 35
GCT 63
tRNA
Exon 1M
+ Exon 2
+ Exon 3
Exon 2
+ Exon 3
hgp190
hGAPDH
417 bp
392 bp
267 bp
133 bp
B
Tera 2
GCT 27C4
GCT 48
GCT 35
GCT 63
tRNA
Exon 1T
+ Exon 2
+ Exon 3
Exon 2
+ Exon 3
hGAPDH
487 bp
392 bp
133 bp

Figure 4.3 Detection of hLIF-M Transcript Expression in Yolk Sac Carcinoma Cell Lines

1μg of poladenylated RNA or 20 μg of total RNA was protected with hLIF-M and hGAPDH riboprobes. Protected species representing the hLIF-M (Exon 1M + Exon 2 + Exon 3; 417 bp), hGAPDH (133 bp) transcripts and hLIF transcripts diverging at the exon 1 / exon 2 boundary (Exon 2 + Exon 3; 392 bp) are indicated.

(Polyadenylated RNA from human yolk sac carcinoma cell lines was the kind gift of Dr. M.F. Pera.)


GCT 44
GCT 46
GCT 85
GCT 72
Exon 1M
+ Exon 2
+ Exon 3
Exon 2
+ Exon 3
417 bp
392 bp
hGAPDH
133 bp

and third exons. All bands were demonstrated to be due to the specific hybridisation of these probes to cellular transcripts by inclusion of mock assays containing only yeast tRNA (Figure 4.2) and the use of each probe individually in protections (data not shown).

All five of the embryonal carcinoma cell lines surveyed expressed hLIF transcripts at consistent levels (Figure 4.2). Levels of hLIF-M transcription were constant both in absolute terms and in terms of their levels relative to total LIF transcription (Figure 4.2 A). hLIF-M was the predominant hLIF transcript in all five cell lines, with the possible exception of Tera 2. hLIF-T transcripts were also expressed by all of these cell lines at consistent absolute levels and as a constant proportion of total hLIF transcription (Figure 4.2B). Levels of hLIF-T expression were lower than levels of hLIF-M expression but were comparable to hLIF-D expression (Figure 1.5; data not shown). Together, hLIF-M and hLIF-T thus represent a major proportion of the hLIF transcripts produced by human EC cells.

The four yolk sac carcinoma cell lines surveyed all expressed hLIF transcripts, albeit at highly variable levels (Figure 4.3). hLIF-M transcripts were expressed by all cell lines and in GCT 44 cells accounted for the vast majority of hLIF transcription and in GCT 85 cells were the only hLIF transcripts detected. Two hLIF expression patterns were apparent in these four cell lines. GCT 46 and GCT 72 cells expressed relatively high levels of hLIF transcripts with hLIF-M transcripts present at significant, but not preponderant, levels whereas GCT 44 and GCT 85 cells expressed lower levels of hLIF transcripts than GCT 46 and GCT 72 and hLIF-M was the preponderant LIF transcript. The variable levels of hLIF expression in yolk sac carcinoma cell lines contrast with the constant levels seen in embryonal carcinoma cell lines. The variations seen in the absolute level of hLIF-M expression and in the levels of hLIF-M expression relative to other hLIF transcripts suggested that expression of hLIF-M and, potentially, the other hLIF transcripts is independently regulated.

4.2.3 hLIF Transcript Expression During Retinoic Acid-Induced Differentiation of EC Cells

Significant upregulation of mLIF-D and -M transcript expression occurs in murine embryonic stem cells and embryonal carcinoma cells in response to retinoic acid induced differentiation (Rathjen et al., 1990b). hLIF transcript expression in GCT 27/X1 multipotent

embryonal carcinoma cells was examined during the course of retinoic acid induced differentiation. hLIF-M and hLIF-T specific riboprobes were used to protect RNA from cells harvested 0, 4, 24, 96 hours after exposure to 5 μM 9-*cis* retinoic acid (Figure 4.4 A and B). Despite overt morphological differentiation into a homogeneous cell population resembling rodent extraembryonic endoderm (Pera et al., 1989; Roach et al., 1994), no significant effect on the level of hLIF transcription or the ratios of individual hLIF transcripts to one another was seen in these cells during this time period.

4.2.4 Analysis of hLIF-M and hLIF-T Transcript Expression in Non Germ Cell Tumour Cell Lines

To determine whether the hLIF-M and hLIF-T transcripts were expressed by cultured cell lines other than germ cell tumour isolates, further ribonuclease protection assays using RNA from a variety of sources were carried out (Figures 4.5 and 4.6). Widely varying levels of hLIF transcription and hLIF-M and hLIF-T transcript expression were seen in these cells. Moreover, like the yolk sac carcinoma cell lines, the expression levels of the hLIF-M and hLIF-T transcripts in these cells varied independently of each other and as a proportion of total hLIF transcription. Thus, production of each of the alternate hLIF transcripts was regulated independently in a manner similar to that observed for the alternate mLIF transcripts (Robertson et al., 1993; Haines et al., 1999).

U937 histiocytic lymphoma cells (Sundstrom et al., 1976) expressed very low levels of hLIF transcripts which appeared to be mainly hLIF-M transcripts. HeLa uterine epithelial carcinoma cells (Gey et al., 1952) expressed relatively high levels of hLIF transcripts that were neither hLIF-M nor hLIF-T transcripts and are thus likely to be hLIF-D transcripts. In 293T, a human embryonic kidney cell line (Pear et al., 1993; Graham et al., 1978), the hLIF-M transcript was particularly abundant and lower levels of hLIF-T transcript expression were also seen. Relatively high levels of LIF transcription were also seen in the hepatocarcinoma cell line HepG2 (Aden et al., 1979), since hLIF-M and hLIF-T transcripts were expressed at low levels, the predominant transcript appeared to be hLIF-D. The levels of hLIF transcription in the hepatocarcinoma cell line Hep3B (Aden et al., 1979) were at the threshold of detection (Figure 4.5), making it difficult assess whether these cells expressed hLIF-M and / or hLIF-T transcripts.

Figure 4.4 **Expression of hLIF-M and hLIF-T Transcripts During Differentiation of GCT 27X1 Human Embryonal Carcinoma Cells Induced by all-*trans*-Retinoic Acid**

1μg of poladenylated RNA was protected with either :

(A) hLIF-M and hGAPDH riboprobes or

(B) hLIF-T and hGAPDH riboprobes as described in section 2.3.21.

Protected species representing the hLIF-M (Exon 1M + Exon 2 + Exon 3; 417 bp), hLIF-T (Exon 1T + Exon 2 + Exon 3; 487 bp), hGAPDH (133 bp) transcripts and hLIF transcripts diverging at the exon 1 / exon 2 boundary (Exon 2 + Exon 3; 392 bp) are indicated. To control for spurious probe hybridisation digestion processes, the same procedure was performed using yeast transfer RNA (tRNA) in place of human mRNA.

(Polyadenylated RNA from GCT 27X1 cells differentiated with 5μM all-*trans*-retinoic acid [as described in Roach et al., 1994] was the kind gift of Dr. M.F. Pera.)

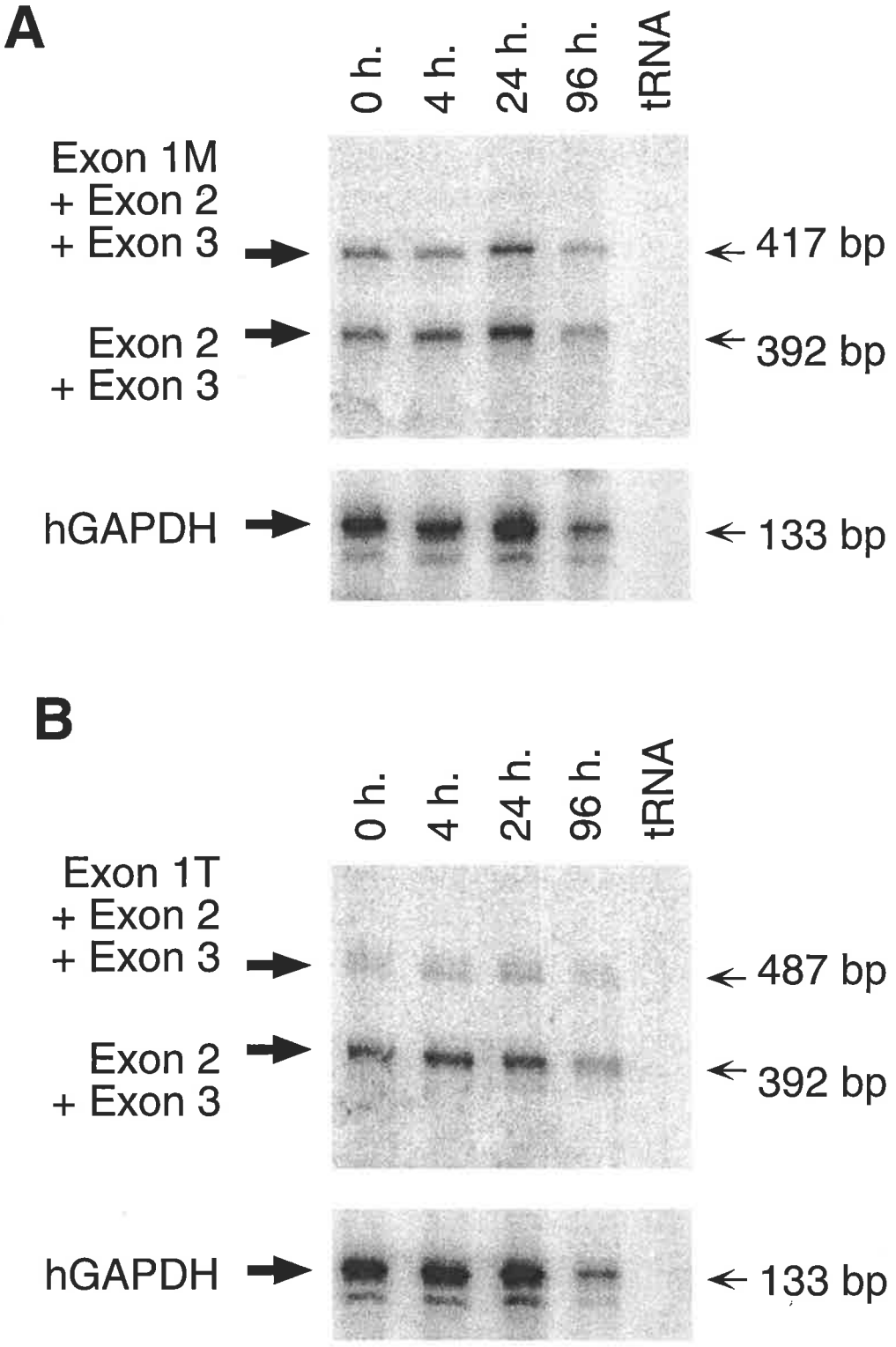

A
0 h.
4 h.
24 h.
96 h.
tRNA
Exon 1M
+ Exon 2
+ Exon 3
417 bp
Exon 2
+ Exon 3
392 bp
hGAPDH
133 bp
B
0 h.
4 h.
24 h.
96 h.
tRNA
Exon 1T
+ Exon 2
+ Exon 3
487 bp
Exon 2
+ Exon 3
392 bp
hGAPDH
133 bp

Figure 4.5 Detection of hLIF-M, hLIF-T, and hgp190 Transcript Expression in Human Cell Lines by Ribonuclease Protection Assay

1μg of poladenylated RNA or 20 μg of total RNA were protected with either :

(A) hLIF-M, hgp190, and hGAPDH riboprobes or

(B) hLIF-T and hGAPDH riboprobes as described in section 2.3.21.

Protected species representing the hLIF-M (Exon 1M + Exon 2 + Exon 3; 417 bp), hLIF-T (Exon 1M + Exon 2 + Exon 3; 487 bp), hgp190 ((267 bp), hGAPDH (133 bp) transcripts and hLIF transcripts diverging at the exon 1 / exon 2 boundary (Exon 2 + Exon 3; 392 bp) are indicated. (Data are from same experiment depicted in Figure 4.2. Refer to this figure for yeast tRNA control).

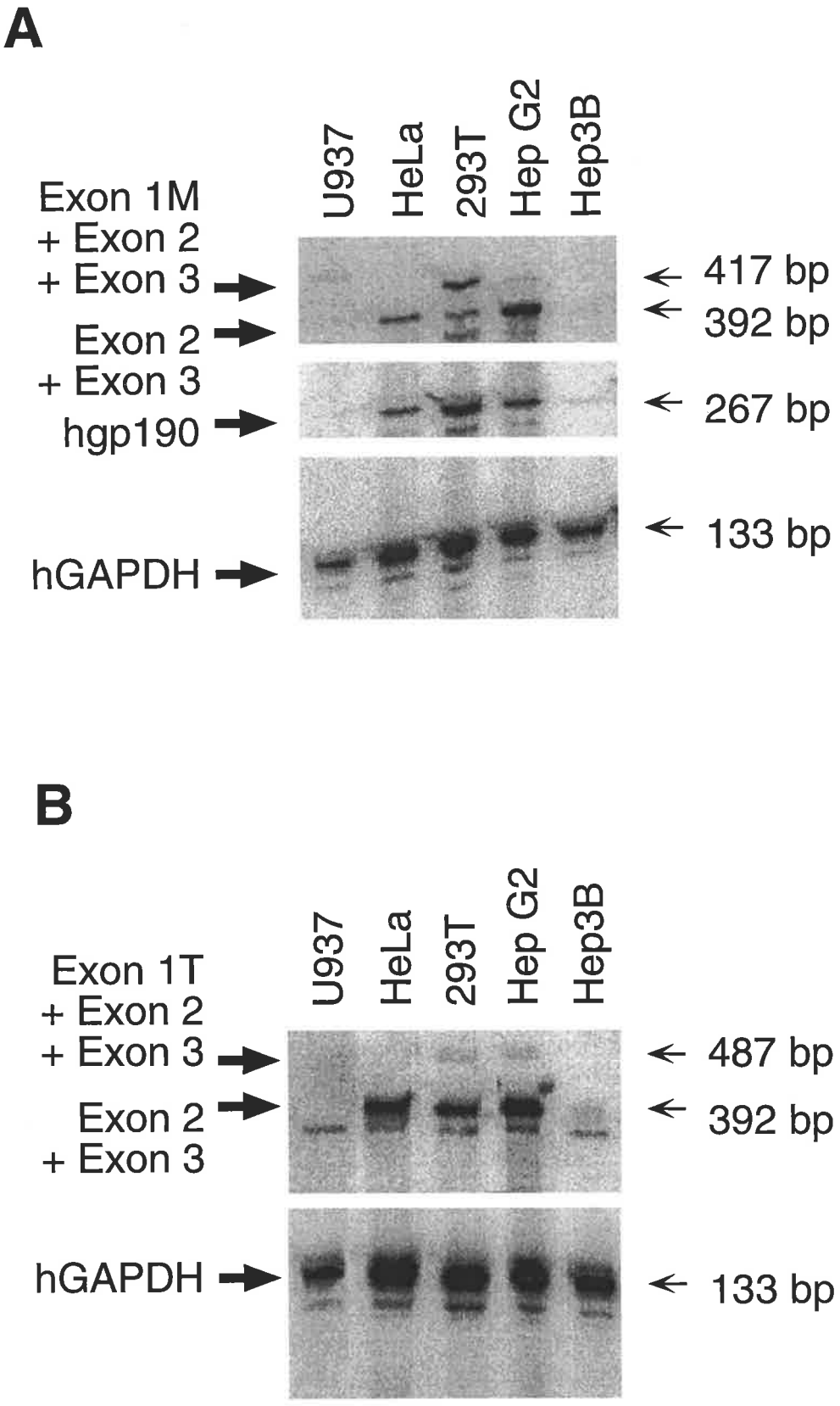
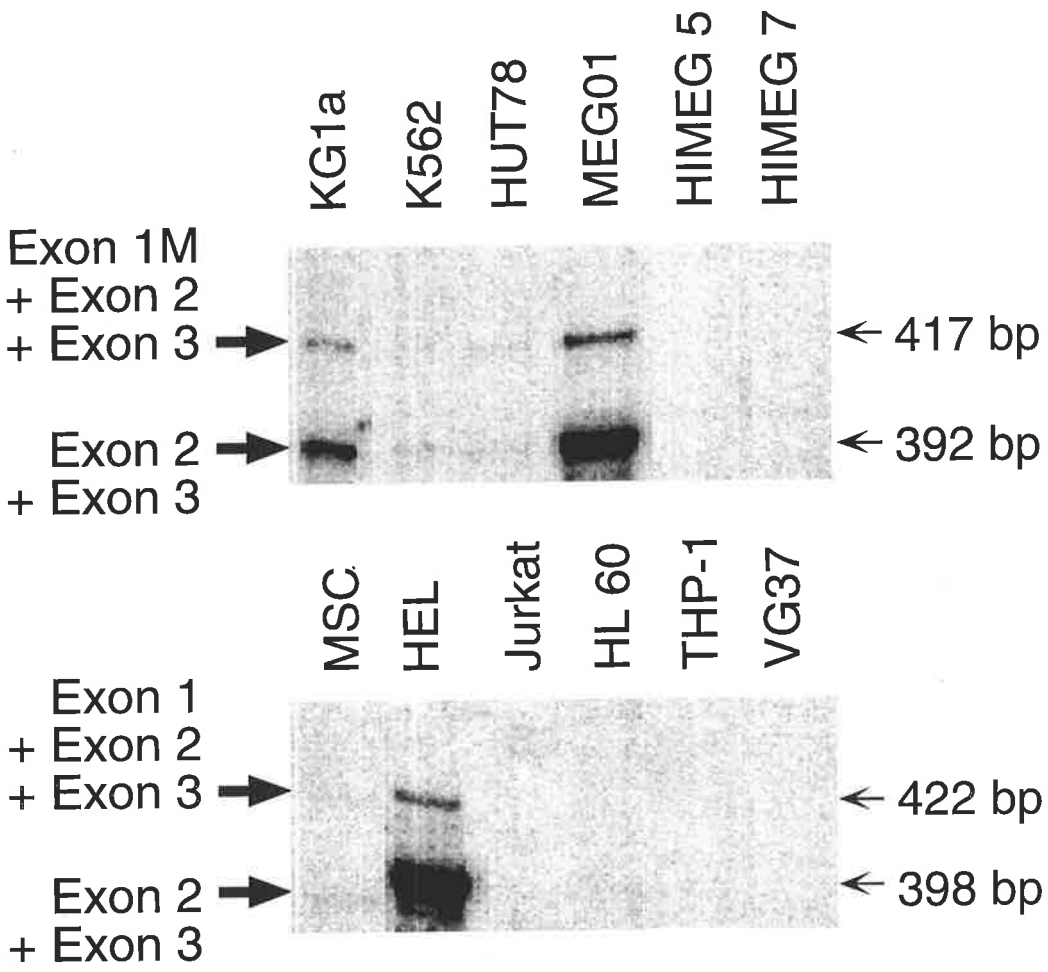

A
U937
HeLa
293T
Hep G2
Hep3B
Exon 1M
+ Exon 2
+ Exon 3
Exon 2
+ Exon 3
hgp190
hGAPDH
417 bp
392 bp
267 bp
133 bp
B
U937
HeLa
293T
Hep G2
Hep3B
Exon 1T
+ Exon 2
+ Exon 3
Exon 2
+ Exon 3
hGAPDH
487 bp
392 bp
133 bp

Figure 4.6 Detection of hLIF-M Transcript Expression in Human Cell Lines of Haematopoietic Origin

10μg of cytoplasmic RNA was protected with an hLIF-M riboprobe as described in section 2.3.21. Protected species representing the transcripts probed for and hLIF transcripts diverging at the exon 1 / exon 2 boundary are indicated. Protected species representing the hLIF-M (Exon 1M + Exon 2 + Exon 3; 417 bp) and hLIF transcripts diverging at the exon 1 / exon 2 boundary (Exon 2 + Exon 3; 392 bp) are indicated.

(Polyadenylated RNA from human haematopoietic cell lines was the kind gift of Dr. R.. D'Andrea)


KG1a
K562
HUT78
MEG01
HIMEG 5
HIMEG 7
Exon 1M
+ Exon 2
+ Exon 3
Exon 2
+ Exon 3
417 bp
392 bp
MSC
HEL
Jurkat
HL 60
THP-1
VG37
Exon 1
+ Exon 2
+ Exon 3
Exon 2
+ Exon 3
422 bp
398 bp

A variety of cell lines derived from tissues and cancers of haematopoietic origin were surveyed for their expression of hLIF-M transcripts (Figure 4.6). Haematopoietic cell lines were chosen because of the evidence implicating LIF in the maintenance of haematopoietic stem and progenitor cell populations (Escary et al., 1993; Szilvassy et al., 1996; section 1.4.7). Among the haematopoietic cell lines, relatively high levels of hLIF transcription and hLIF-M transcript expression were seen in KG1a myelogenous leukaemia (Koeffler et al., 1980), MEG01 megakaryocytic leukaemia (Ogura et al., 1995), and HEL erythroleukaemia (Martin et al., 1982) cells. Low levels of hLIF transcription and hLIF-M transcript expression were detectable in K562 erythroleukaemia (Kozio & Lozio, 1975), HUT78 T cell lymphoma (Gazdar et al., 1981), and marrow stroma (Gronthos et al., 1996). In the cell lines expressing hLIF transcripts at relatively high levels, KG1a, MEG01, and HEL, hLIF-M transcripts comprised a major component of hLIF transcription. No hLIF transcription was detectable in the other cell lines: HIMEG 5 and HIMEG 7 megakaryocytic leukaemia (Ogura et al., 1995), Jurkat T cell lymphoma (Weiss et al., 1984), HL 60 promyelocytic leukaemia (Frischkoff et al., 1977), and THP-1 promonocytic leukaemia (Tsuchiya et al., 1980).

4.2.5 Expression of gp190 LIF Receptor Subunit Transcripts by Cultured Human Cell Lines

Inclusion of a riboprobe specific for hgp190 transcripts in some protections (Figures 4.2 A and 4.5 A) provided an indication as to whether these cells were likely to be capable of mounting a conventional response to extracellular LIF proteins. Furthermore, expression of hgp190 transcripts and hLIF transcripts by a cell line might be indicative of a capacity for autocrine LIF signalling in corresponding tumour cell populations. The riboprobe used was also designed to indicate whether alternate splicing of the hgp190 transcript produced a soluble hLIF binding protein in an analogous manner to the mgp190 transcript (Owczarek et al., 1996).

The five human embryonal carcinoma cell lines examined all expressed hgp190 transcripts at consistent levels (Figure 4.2 A). Thus, consistent, widespread expression of gp190 in human embryonal carcinoma cell lines correlated with a consistent, widespread expression of LIF transcripts in the same cells. Other cultured human cell lines expressing appreciable levels of hgp190 were HeLa, 293T, and HepG2 (Figure 4.5 A). All these cell lines expressed LIF

transcripts at levels proportional to their hgp190 expression, although HeLa cells expressed only hLIF-D and only 293T cells expressed relatively high levels of hLIF-M (Figure 4.5 A). No protected species were observed using the hgp190 riboprobe that could correspond to alternate splice variants of the hgp190 transcript homologous to the transcript encoding soluble mgp190 (Owczarek et al., 1996).

4. 3 Discussion

4.3.1 Levels of hLIF-M and hLIF-T Transcription are Constant in Embryonal Carcinoma Cell Lines and Variable in Other Cell Lines

All five embryonal carcinoma cell lines expressed highly consistent levels of hLIF-M and hLIF-T transcripts despite their independent origins. hLIF-M was a predominant transcript in these cells, with hLIF-T transcripts present at lower levels. The proportions of individual hLIF transcripts produced were therefore remarkably constant. This could reflect the similar cellular origin of these tumours, or the common acquisition of an aberrant gene expression profile that favours tumourigenesis. However, the constancy of hLIF-M and hLIF-T expression levels in the five independent cell lines examined presumably indicates that they arose from a similar pre-cancerous cell type since the acquisition of aberrations in gene expression during tumourigenesis is a random and haphazard process. It might therefore be the case that hLIF-M transcripts are expressed at relatively high levels in the spermatogonia or developmentally-arrested germ cells from which germ cell tumours are thought to arise (Chaganti et al., 1995, Raepert-De Meyts et al., 1998. *APMIS* 106:198-204). Together with existing markers (Sutton et al., 1996; Caricasole et al., 1998), hLIF-M expression might thus be useful in identification of *in vivo* germ cell populations and sub populations.
hLIF-M transcript expression was maintained in all nine germ cell tumour lines examined even when levels of overall hLIF transcription were low (see GCT 44 and GCT 85 in Figure 4.3). This suggests that if expression of hLIF transcripts, and LIF-M in particular, is a feature of the germ cell tumour precursor cells, it may also provide some selective advantage to them later in tumourigenesis. Expression of this transcript might therefore favour the growth and / or maintenance of human germ cell tumour stem cells.

In contrast to the embryonal carcinoma cell lines, the yolk sac carcinoma cell lines surveyed showed variable levels of hLIF transcription and the proportions of individual hLIF transcripts produced also varied widely. This was in keeping with postulated distinct cellular origins and histopathology of these tumours (Pera et al., 1987) and suggests that hLIF transcription plays a less significant, if any, role in their maintenance. Sporadic, variable hLIF-M and hLIF-T expression were also seen in a variety of human cell lines of diverse origin including a panel of haematopoietic cell lines. However, no clear relationships between the cell phenotype and the hLIF transcription profile were evident.

It has been noted previously that the levels of mLIF transcript expression are extremely low (Rathjen et al., 1990a). The levels of expression of the novel hLIF transcripts seen here were correspondingly low. In a survey of mLIF-D and mLIF-M transcript expression in murine tissues and cell lines (Robertson et al., 1993) the detection limit of their RNase protection assay, which was performed under nearly identical conditions to those employed here, was estimated to be of the order of one transcript per cell. Therefore, the low levels of hLIF transcription seen here could represent either extremely low level expression of hLIF in all cells or somewhat higher level of transcription in subpopulations of cells. While levels of LIF transcription could vary dramatically between cell types in tissue systems, a uniform low level of LIF transcription is the more likely possibility in the morphologically homogeneous cell cultures examined here. However, LIF (Escary et al., 1993; Robertson et al., 1993) and other IL-6 family cytokines, including oncostatin M (Voyle and Rathjen, 2000), are expressed at low levels in biological contexts where they have important activities. Thus, the low levels of hLIF transcript expression seen here could still underlie important biological functions for LIF in these cells.

Much higher levels of LIF transcript and protein expression are induced in a variety of specific contexts including implantation of the conceptus (Shen & Leder, 1992; Cullinan et al., 1996), stimulation of monocytes with phorbol esters or endotoxin (Gascan et al., 1990; Grolleau et al., 1991), inflammatory responses (Lotz et al., 1992), sepsis (Jensen et al., 1996), and neural or muscular injury (Banner et al., 1994; Curtis et al., 1994; Kurek et al., 1996). It would therefore be of interest to investigate expression of hLIF transcripts in these contexts and determine whether they are distinguished by production of particular alternate transcripts.

All previous investigations of hLIF transcript expression in human cancers have utilised Northern blotting and RT-PCR-based approaches (see Chapter 1 section 1.6.1). Therefore, none of these studies have produced data allowing the three variant hLIF transcripts to be distinguished. Consequently, it would now be of interest to use the ribonuclease protection assay to assess alternate hLIF transcripts expression during progression of the various forms of human cancer in which a role for hLIF expression has been suggested, particularly through the examination of biopsy material.

4.3.2 Expression of Each Alternate hLIF Transcript is Independently Regulated

Expression studies have indicated that the three mouse LIF transcripts are regulated independently, *in vitro* and *in vivo* (Rathjen et al., 1990a; Rathjen et al., 1990b; Robertson et al., 1993; Haines et al., 1999). Here, it is reported that the production of the three alternate human LIF transcripts is also independently regulated *in vitro*. This was manifest in terms of the absolute levels of each transcript expressed by a given cell type and the relative levels to which each of the three transcripts was expressed by a given cell type. Cell lines, such as GCT 44, GCT 85 and 293T, expressing predominantly LIF-M were apparent as were cell lines, such as HeLa and HepG2, in which hLIF-D appeared to predominate. LIF-T transcription, while not ubiquitous, appeared to be generally lower and LIF-T was not the predominant transcript in any of the cell lines examined. Thus, like mLIF transcription (Robertson et al., 1993; Haines et al., 1999), the transcription of each of the three alternative hLIF transcripts appears to be independently regulated in a cell type-specific manner. This is a key finding since, taken together with the preponderance of hLIF-M and hLIF-T transcripts in many cell types and the evolutionary conservation of the three alternate first exons and their postulated regulatory sequences, it argues that these novel transcripts are likely to be physiologically relevant and fulfil different biological roles from the hLIF-D transcript. The differential regulation of each alternate hLIF transcript observed is also consistent with the notion (section 3.3.3) that they arise as a result of the differential activity of independent promoters. This is also in good agreement with the mapping of transcriptional start sites in regions of the mLIF gene corresponding to the LIF-M and LIF-T first exons (Hsu et al., 1994).

It also seems likely that human cells will express hLIF-M and hLIF-T transcripts *in vivo* since mLIF-M and mLIF-T transcripts are expressed *in vivo* by adult and embryonic tissues (Robertson et al., 1994; Haines et al., 1999) and the pLIF-M and pLIF- T were cloned by RT-PCR from cDNA derived from primary cultures of porcine embryonic fibroblasts (Haines et al., 1999). This, taken together with the evolutionary conservation of the LIF-M and LIF-T exons (Chapter 3), implies that LIF-M and LIF-T are not artefactual transcripts produced by transformed cells in culture but are transcripts normally expressed in a conserved, highly-regulated fashion in the cells of all eutherian mammals.

4.3.3 hLIF Transcription in GCT 27/ X1 cells is Unresponsive to Retinoic Acid Induced Differentiation

There was no effect on hLIF transcription when GCT 27/X1 cells were induced to differentiate by retinoic acid, whereas mLIF transcription in murine embryonal carcinoma and embryonic stem cells is generally upregulated in response to retinoic acid-induced differentiation (Rathjen et al., 1990b). This could reflect differences which have been noted between human and murine embryonal carcinoma cells, including differences in their origins, progression, morphology and gene expression (Pera et al., 1990). Alternatively, it may indicate that hLIF transcription in this particular cell line, but not necessarily other GCT cell lines, is unresponsive to retinoic acid induced differentiation. Indeed, mLIF transcription in some murine embryonal carcinoma cell lines is not responsive to retinoic acid induced differentiation (Rathjen et al., 1990b). Regardless, this result indicates that expression of hLIF transcripts is not always strictly associated with the stem cell state in human embryonal carcinoma cells.

4.3.4 Co-expression of gp190 and LIF Transcripts in Human Cell Lines Suggests Possible Autocrine LIF Action in These Cells

The expression of both hLIF and hgp190 transcripts by all five embryonal carcinoma cell lines as well as by other unrelated cell lines provides an opportunity for autocrine LIF signalling in these cell populations given that expression of hgp130 is ubiquitous (Hibi et al., 1990). The possibility that autocrine LIF action could promote cell-autonomous growth of embryonal carcinoma stem cells merits further investigation, given the observed ability of LIF to maintain

stem cell populations *in vivo* and *in vitro* (sections 1.4.7 and 1.4.8). Intriguingly, cells expressing hLIF-M and hLIF-T transcripts may express intracellular proteins (section 3.3.4). Co-expression of the LIF receptor and intracellular LIF proteins by a cell population would therefore suggest that it is capable of mounting paracrine / autocrine and cell-autonomous / intracellular responses to LIF. Thus, multiple modes of LIF signalling might occur in embryonal carcinoma stem cells and other cell populations.

4.3.5 The Alternate Splicing Processes that Produce a Soluble Form of the mgp190 Protein do not Appear to Occur in the Human gp190 Gene

The riboprobe used to protect the hgp190 transcript was designed to span the gp190 protein's extracellular and transmembrane domains. This region of the mouse gp190 cDNA has been reported to be involved in alternate promoter usage and splicing processes which result in the production of membrane-bound and soluble forms of the protein (Tomida et al., 1994; Owczarek et al., 1996; Chambers et al., 1997). Since the riboprobe used was complementary to the membrane-bound hgp190 transcript, any transcripts encoding a soluble form of hgp190 produced by a similar alternate splicing process would give rise to a smaller protected species. In none of the 11 cell lines examined, was a smaller hgp190-specific protected species observed. These findings are in agreement with those recently reported by other workers who have been unable to find cDNA clones homologous to that of the soluble mgp190 in other species (Tomida et al., 1996; Michel et al., 1997). These workers concluded that this alternate splicing process is peculiar to the mouse gp190 gene, and a consequence of the recent evolutionary insertion of a B2 SINE transposable element.

Chapter 5 :

Novel hLIF Proteins are Encoded by the hLIF-M and hLIF-T Transcripts

5.1 Introduction

The absence of in-frame AUG potential translational initiation codons in the hLIF-M and hLIF-T first exons suggested that translation of these transcripts would initiate at the first in frame AUG in the hLIF reading frame (section 3.3.4). This AUG, the only one present in the hLIF-M and hLIF-T open reading frames, is found in the second exon which is common to all three alternate hLIF transcripts. Thus, the primary translation products encoded by hLIF-M and hLIF-T were predicted to be identical. However, the differential regulation of these transcripts seen in diverse cell lines (Chapter 4) suggested that production of these transcripts served distinct biological functions. This raised the possibility that the proteins produced from hLIF-M and hLIF-T, while they might share a common polypeptide backbone, could differ in other ways such as in their localisation and/or post-translational modification. It was also possible that non-AUG initiated translational mechanisms might produce proteins from these transcripts. In this regard, it was of particular interest to ascertain whether there were similarities between proteins translated from the hLIF-M and mLIF-M transcripts despite the divergent nature of the mLIF-M first exon sequence.

In order to investigate the nature and activity of the protein(s) encoded by the hLIF-M and hLIF-T transcripts, the partial hLIF-M and hLIF-T cDNAs obtained by RACE-PCR were used to construct vectors for expression of these transcripts in mammalian cells. Transient transfection of these vectors into Cos 1 cells demonstrated that the proteins encoded by hLIF-D, hLIF-M and hLIF-T differed in terms of their translational initiation sites, secretion, and post-translational modification. Maintenance of murine ES cells was used as an assay for extracellular LIF bioactivity of proteins encoded by hLIF-D, hLIF-M, and hLIF-T. Each alternate hLIF transcript differed in the level of extracellular LIF activity generated by its overexpression in this system. Furthermore, the differences observed in these activities were reflected in the extracellular levels of the different hLIF proteins encoded by these transcripts as visualised by immunoprecipitation analysis. Translation of both the hLIF-M and hLIF-T transcripts produced LIF proteins from the in frame AUG translation initiation codon in exon 2 that were localised intracellularly, raising the possibility of a cell autonomous action(s) for the LIF protein. These studies revealed hitherto unappreciated complexity in hLIF protein production, modification and localisation.

5.2 Results

5.2.1 Construction of hLIF-M and hLIF-T Expression Vectors and Overexpression of hLIF Proteins in Mammalian Cells

An hLIF-D mammalian expression vector, phLIF-DX, containing the complete open reading frame of the hLIF-D cDNA in the vector pXMT2 was available from co-workers and has been described previously (Rathjen et al., 1990b). The hLIF-M and hLIF-T cDNAs obtained by RACE-PCR and RT-PCR (Chapter 3) were used to reconstruct the complete LIF open reading frames of these transcripts. Briefly, a unique *Sma* I site in the third exon of hLIF allowed insertion of the unique 5' end of the hLIF-M and hLIF-T cDNAs into resected phLIF-DX containing only the shared exon 3 LIF sequences downstream of this site. This completed the hLIF open reading frame to create the vectors phLIF-MX and phLIF-TX (Figure 5.1).

The pXMT2 vector utilised throughout these studies directs high level overexpression of transcripts from the papovavirus SV40 major-late promoter and contains the SV40 origin of replication. Transfection of pXMT2-based vectors into the Cos 1 African Green monkey kidney cell line (Gluzman, 1981), which is competent for SV-40-replication, allowed vector replication resulting in the overexpression of hLIF transcripts and proteins.

5.2.2 Assay of Extracellular LIF Activity Produced by Cos 1 Cells Overexpressing Alternate hLIF and mLIF Transcripts

Conditioned media from Cos 1 cells transfected with hLIF expression vectors were collected 72 hours after transfection. The media were then diluted with ES cell media lacking LIF and assayed for their ability to maintain murine ES cells in an undifferentiated state (Figure 5.2). Since maintenance of mouse ES cells requires gp130 receptor complex assembly and signalling, the activity of extracellular LIF proteins produced from the transcripts expressed from these vectors could be measured.

Media from pXMT2 transfected cells was unable to maintain undifferentiated ES cell colonies (Figure 5.2), indicating that Cos 1 cells produced no LIF activity in this assay. hLIF-D and mLIF-D transfected cells could be diluted 20,000-fold before losing ES cell maintenance activity, reflecting the production of secreted, active LIF proteins at high levels. Media from hLIF-

Figure 5.1 Construction of hLIF and hLIF Epitope-Tagged Expression Vectors

Schema illustrating construction and salient features of the hLIF mammalian expression vectors. phLIF-MX, and phLIF-TX were constructed using the available phLIF-DX vector (section 2.2.10) and the hLIF-M RACE- and hLIF-T RT-PCR clones. A similar strategy was also used in the construction of expression vectors for hLIF proteins with C-terminal FLAG epitope tags. phLIF-MX.FLAG and phLIF-TX.FLAG were constructed using the available phLIF-DX.FLAG vector (section 2.2.10) and phLIF-M and phLIF-T (indicated at last step).

Production of these vectors is described in detail in section 2.3.22.

The suffix "X" indicates the vector used is pXMT2. pXMT2 contains an SV40 origin of replication (open rectangle), an SV40 major late promoter (half arrowhead, thickened line), and the dihydrofolate reductase 3' untranslated region and polyadenylation signal (thickened line). Differential shading is used to indicate alternate first exons of hLIF.

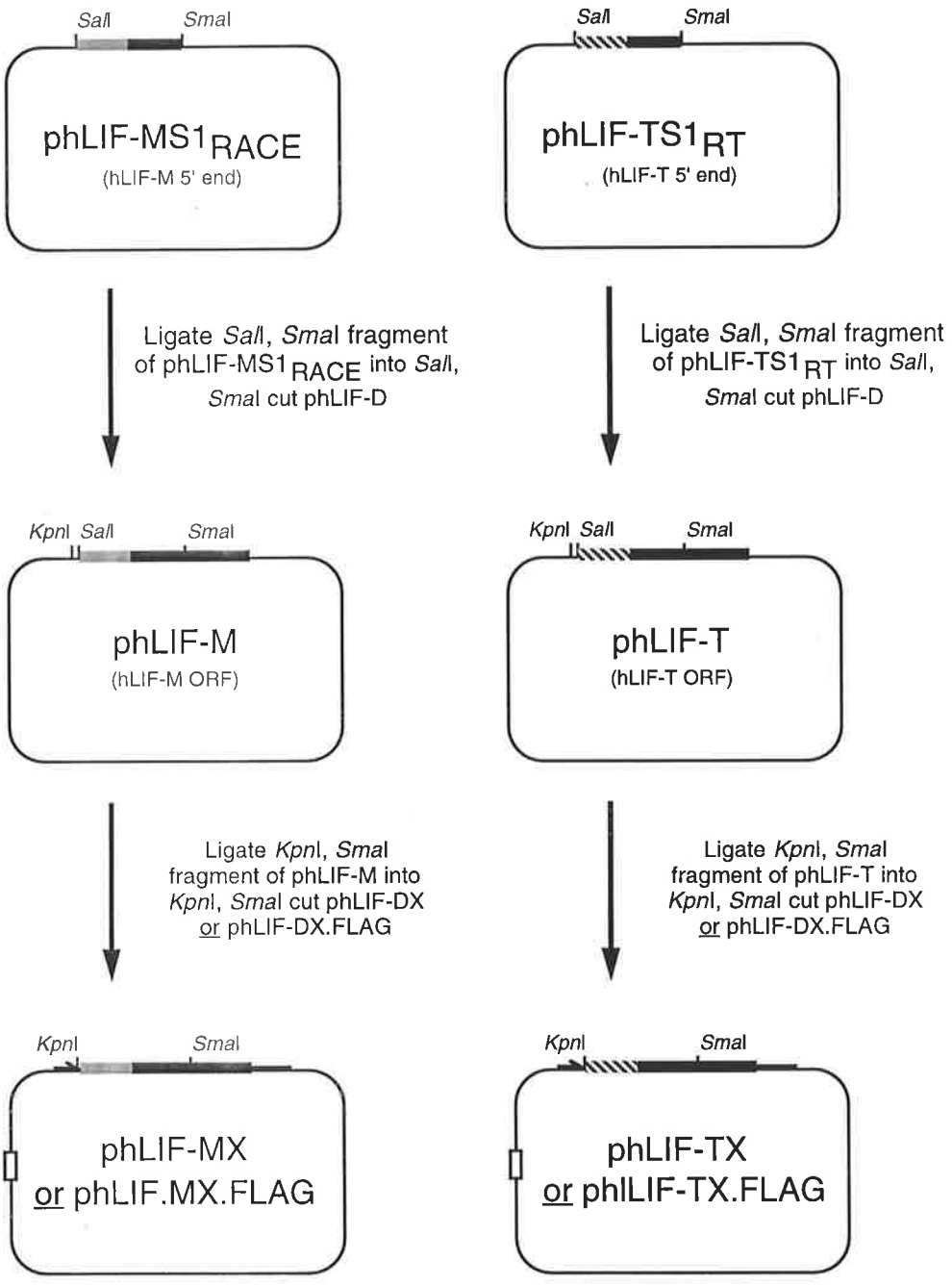

SalI
SmaI
phLIF-MS1RACE
(hLIF-M 5' end)
SalI
SmaI
phLIF-TS1RT
(hLIF-T 5' end)
Ligate SalI, SmaI fragment of phLIF-MS1RACE into SalI, SmaI cut phLIF-D
Ligate SalI, SmaI fragment of phLIF-TS1RT into SalI, SmaI cut phLIF-D
KpnI SalI
SmaI
phLIF-M
(hLIF-M ORF)
KpnI SalI
SmaI
phLIF-T
(hLIF-T ORF)
Ligate KpnI, SmaI fragment of phLIF-M into KpnI, SmaI cut phLIF-DX or phLIF-DX.FLAG
Ligate KpnI, SmaI fragment of phLIF-T into KpnI, SmaI cut phLIF-DX or phLIF-DX.FLAG
KpnI
SmaI
phLIF-MX
or phLIF.MX.FLAG
KpnI
SmaI
phLIF-TX
or phlLIF-TX.FLAG

Figure 5.2 Extracellular LIF Bioactivity in Media Conditioned by Cos 1 Cells Overexpressing Alternate hLIF and mLIF Transcripts

Cos 1 cells were transfected with either parental vector (pXMT2) or the same vector containing cDNAs for hLIF and mLIF alternate transcripts (hLIF-D, hLIF-M , hLIF-T, mLIF-D, mLIF-M, and mLIF-T). Conditioned media from these cells was collected after 72 hours and assayed at dilutions between 1 in 10 and 1 in 20,000 for ability to maintain ES cell colonies in an undifferentiated state. ES cell colonies were scored as undifferentiated on the basis of their dome shaped morphology and staining for alkaline phosphatase activity (section 2.4.6).

(A) The same assay results presented in tabular format showing the actual dilutions performed.

(B) Representative ES cell colonies from LIF activity assay wells showing alkaline phosphatase positive (purple) and negative (dull pink) cells.

(+) Undifferentiated ES cell colonies,

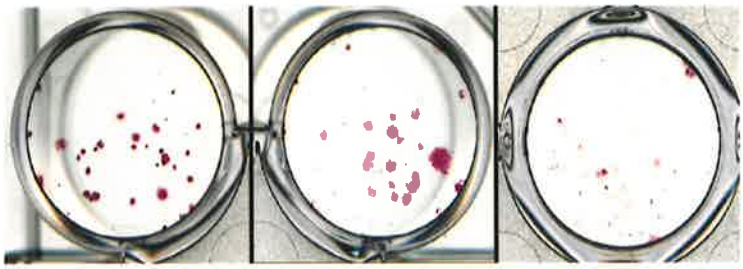
(+/-) Some differentiated ES cell colonies,

(-) Differentiated ES cell colonies.

A

	1/10	1/50	1/100	1/1 000	1/2 000	1/10 000	1/20 000
hLIF-D	+	+	+	+	+	+	+/-
hLIF-M	+	+	+	+	+/-	-	-
hLIF-T	+	+/-	-	-	-	-	-
pXMT2	-	-	-	-	-	-	-
mLIF-D	+	+	+	+	+	+	+/-
mLIF-M	+	+	+	+	+	+/-	-
mLIF-T	+	+/-	-	-	-	-	-

B



\+ +/- -

M- and mLIF-M-transfected cells were less active and could be diluted 2,000- and 10,000-fold respectively before activity was lost. In contrast, media from hLIF-T- and mLIF-T-transfected cells contained very low levels of activity and could not be diluted more than 50-fold before activity was lost.

A consistent trend was seen in the levels of activity produced from human and murine LIF transcripts where activity produced from LIF-D were always higher than LIF-M which were always higher than LIF-T. The high levels of extracellular activity produced from the mouse and human LIF-D transcripts and the mLIF-M transcript were expected since they were known to encode secreted glycoproteins (Gearing et al., 1988; Moreau et al., 1988; Rathjen et al., 1990a). The mLIF-T transcript encodes an intracellular protein (Haines et al., 1999) and the hLIF-T transcript was predicted to (section 3.3.4). The production of low levels of extracellular bioactivity from these transcripts was therefore in good agreement with these observations. However, since the hLIF-M transcript was also predicted to encode an intracellular protein, the high levels of activity produced from this transcript were unexpected. In fact the levels of extracellular activity produced from hLIF-M transcripts were only five fold lower than the levels of activity produced from mLIF-M transcripts, despite their divergent first exon sequences. Further experiments were therefore required to determine whether these differences in the levels of extracellular activity reflected differences in the levels, the specific activities, or the identity of the proteins translated from the alternate LIF transcripts.

5.2.3 Immunoprecipitation of Proteins Encoded by the Alternate hLIF and mLIF Transcripts

Western blotting using polyclonal antisera raised against the mLIF-D protein (Haines et al., 1999) could not be used visualise hLIF proteins overexpressed in Cos 1 cells since none of the four anti-mLIF antisera tested showed sufficient cross-reactivity to denatured cellular hLIF protein. Three rabbit polyclonal antisera were raised against GST-hLIF (Forrest, 1996), however, these antisera were still only weakly reactive against hLIF protein by western blot. A more sensitive technique for the visualisation of mLIF proteins overexpressed in Cos 1 cells based upon the immunoprecipitation of metabolically labelled cellular protein was developed for

visualisation of the proteins encoded by hLIF-M and hLIF-T transcripts in association with Regan Forrest (Forrest, 1996).

Cellular proteins produced by Cos 1 cells transfected (section 2.4.2) with expression constructs for the alternate hLIF and mLIF transcripts were metabolically-labelled using ^{35}S-methionine / cysteine forty eight hours after transfection. Media containing secreted proteins were collected and the cells were washed and lysed. The media and cell lysates were then immunoprecipitated with anti-GST-hLIF or anti-GST-mLIF antibodies (section 2.4.7) as appropriate (Figure 5.3). Cells transfected with the parental vector, pXMT2, were immunoprecipitated using anti-GST-hLIF or anti-GST-mLIF antibodies to indicate the levels of non-specific background proteins precipitated. phLIF-DX transfected cells expressed a series of proteins 20-45 kD in size that were found both in cell lysates and conditioned media, indicating that the hLIF-D transcript encoded secreted glycoproteins based on a 20 kD core polypeptide as previously reported (Gascan et al., 1989). phLIF-MX transfected cells expressed proteins 40-45 kD in size that were present in both cell lysates and conditioned media, indicating that the hLIF-M transcript also directed the production of secreted proteins (Figure 5.3), consistent with extracellular activity detected by ES cell assay. In addition to these 40-45 kD secreted proteins, phLIF-MX transfected cells expressed proteins of 17 and 23 kD that were found exclusively in the cell lysate (Figure 5.3). Proteins that were apparently identical to the 17 and 23 kD proteins encoded by hLIF-M were also immunoprecipitated from hLIF-T transfected cells and found exclusively in cell lysates (Figure 5.3). The lack of extracellular proteins expressed from hLIF-T transcripts was consistent with the very low levels of activity seen in the ES cell bioassay. Thus, the 17 and 23 kD proteins expressed by phLIF-MX and phLIF-TX transfected cells appear to be retained intracellularly in this expression system.

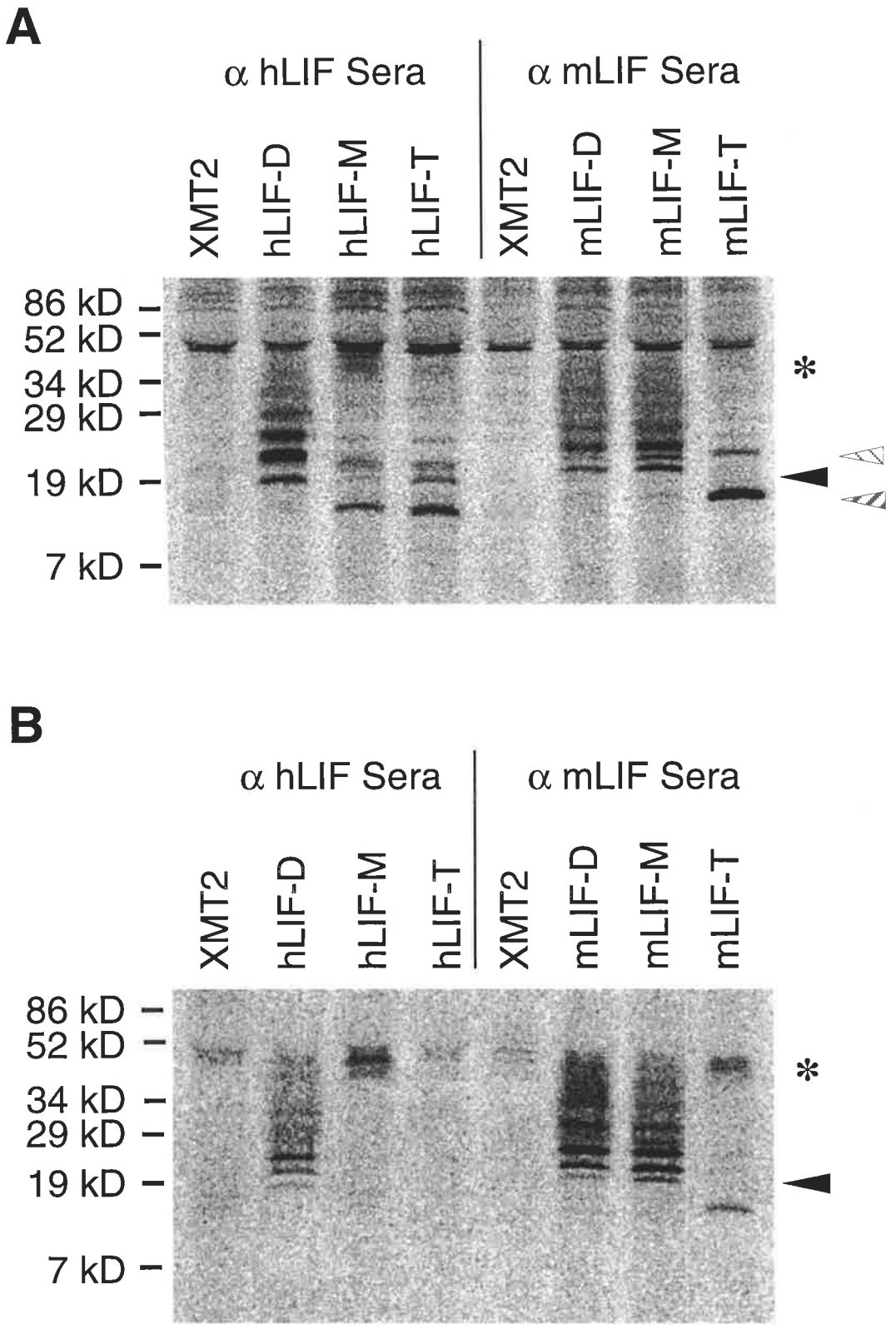
The proteins encoded by the alternate mLIF transcripts have been characterised (Metcalf, 1991; Rathjen et al., 1990a; Haines et al., 1999). Immunoprecipitation of cell lysates and conditioned media from Cos 1 cells overexpressing alternate mLIF transcripts allowed direct comparison of the proteins expressed from alternate hLIF and mLIF transcripts. Immunopreciptations from Cos 1 cells overexpressing alternate mLIF transcripts revealed that the mLIF-D and mLIF-M transcripts encoded secreted glycoproteins of 20-45 kD, analogous to the secreted hLIF-D proteins and that the mLIF-T transcript encoded predominantly intracellular

Figure 5.3 **Immunoprecipitation of LIF Proteins From the Cell Lysates and Conditioned Media of Cos 1 Cells Overexpressing Alternate hLIF and mLIF Transcripts**

Cos 1 cells were transfected with the indicated LIF expression vectors and subjected to immunoprecipitation 48 hours post-transfection using anti-mLIF-GST and anti-hLIF-GST antibodies as described in section 2.4.7. The 20+ kD secreted proteins encoded by hLIF-D, mLIF-D and mLIF-M are indicated by a solid arrowhead. An asterisk indicates the 45 kD secreted proteins encoded by hLIF-M. The 17 and 23 kD intracellular proteins encoded by the hLIF-M, hLIF-T and mLIF-T are indicated by thickly and thinly cross-hatched arrowheads respectively. Atypically high levels of mLIF-T proteins are detected in this immunoprecipitate of conditioned media, presumably as a result of cell lysis in culture. (Levels of extracellular mLIF-T bioactivity and protein, like hLIF-T, are generally very low, Figure 5.2.)

(A) Cell Lysates

(B) Conditioned Media


A
α hLIF Sera
α mLIF Sera
XMT2
hLIF-D
hLIF-M
hLIF-T
XMT2
mLIF-D
mLIF-M
mLIF-T
86 kD
52 kD
34 kD
29 kD
19 kD
7 kD
*
B
α hLIF Sera
α mLIF Sera
XMT2
hLIF-D
hLIF-M
hLIF-T
XMT2
mLIF-D
mLIF-M
mLIF-T
86 kD
52 kD
34 kD
29 kD
19 kD
7 kD
*

proteins of approximately 17 and 23 kD which appeared to be analogous to the intracellular proteins encoded by the hLIF-M and hLIF-T cDNAs (Figure 5.3).

5.2.4 N-Linked Glycosylation of Proteins Encoded by the Alternate hLIF Transcripts

Size heterogeneity among secreted hLIF-D proteins results from a series of post-translational modifications of a 20 kD polypeptide that includes addition of large amounts of N-linked carbohydrate (Gascan et al., 1989). To determine whether the secreted hLIF-M proteins produced in this system contained N-linked glycosides, metabolic labelling prior to immunoprecipitation was carried out in the presence of tunicamycin, which prevents the anchoring of the carbohydrate carrier dolichol phosphate in the endoplasmic reticulum, a prerequisite for initiation of N-linked glycosylation (Merlie et al., 1982). It thus inhibits N-linked glycosylation without interfering with protein secretion.

Immunoprecipitates of conditioned media from tunicamycin treated phLIF-DX contained apparently identical 20 and 22 kD proteins and an apparently identical set of proteins was seen in and phLIF-MX transfected cells (Figure 5.4). Cells expressing hLIF-M and hLIF-T produced 17 and 23 kD intracellular proteins that appeared to be unaffected by treatment. These data suggested that the secreted hLIF-D and hLIF-M proteins were based upon identical core polypeptides and differed only in their N-linked glycoside content. The lack of any affect of tunicamycin on the production or migration of the 17 and 23 kD intracellular proteins encoded by hLIF-M and hLIF-T indicated that they did not contain N-linked glycosides.

The mature hLIF-D protein is known to have an apparent molecular weight of 20 kD when completely de-glycosylated, and the size of its O-linked carbohydrate component is estimated to be 2-3 kD (Gascan et al., 1989). Therefore the 20 and 22 kD proteins immunoprecipitated from hLIF-D and hLIF-M overexpressing cells treated with tunicamycin were deduced to be the unmodified and O-glycosylated forms of the characterised mature hLIF core polypeptide respectively.

hLIF-D encoded proteins were approximately ten-fold more abundant than hLIF-M encoded proteins in these immunoprecipitates (Figure 5.4). These findings were in good accord with the ten-fold higher levels of LIF bioactivity present in the conditioned media of hLIF-D overexpressing cells when compared with hLIF-M overexpressing cells (Figure 5.4), indicating

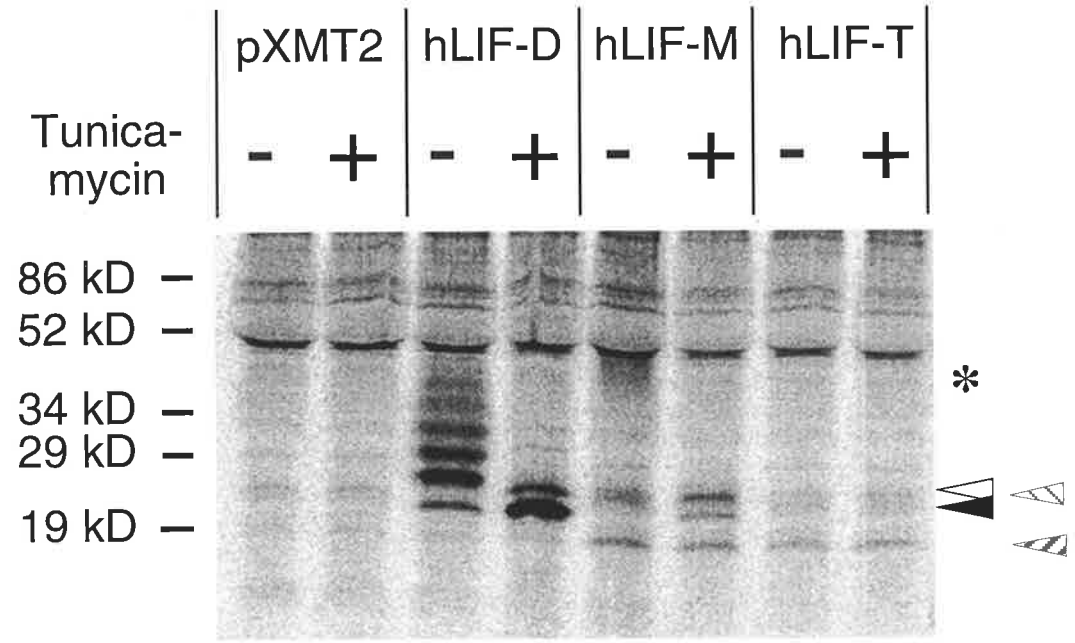
Figure 5.4 **Immunoprecipitation of hLIF Proteins from Cell Lysates and Conditioned Media of Cos 1 Cells Overexpressing hLIF Transcripts In the Presence and Absence of Tunicamycin**

Cos 1 cells were transfected with the indicated LIF expression vectors and subjected to immunoprecipitation 48 hours post-transfection using anti-hLIF-GST antibodies as described in section 2.4.7. ^{35}S metabolic labelling of cellular proteins was performed in the presence (+) or absence (-) of tunicamycin, an inhibitor of asparagine-linked glycosylation, as described in section 2.4.8. The 20 and 22 kD hLIF-D and hLIF-M secreted proteins produced by tunicamycin treated cells are indicated by a solid arrowhead and an open arrowhead respectively. An asterisk indicates the 45 kD secreted proteins produced by untreated hLIF-M transfected cells. The 17 and 23 kD intracellular proteins encoded by the hLIF-M and hLIF-T are indicated by thickly and thinly cross-hatched arrowheads respectively.

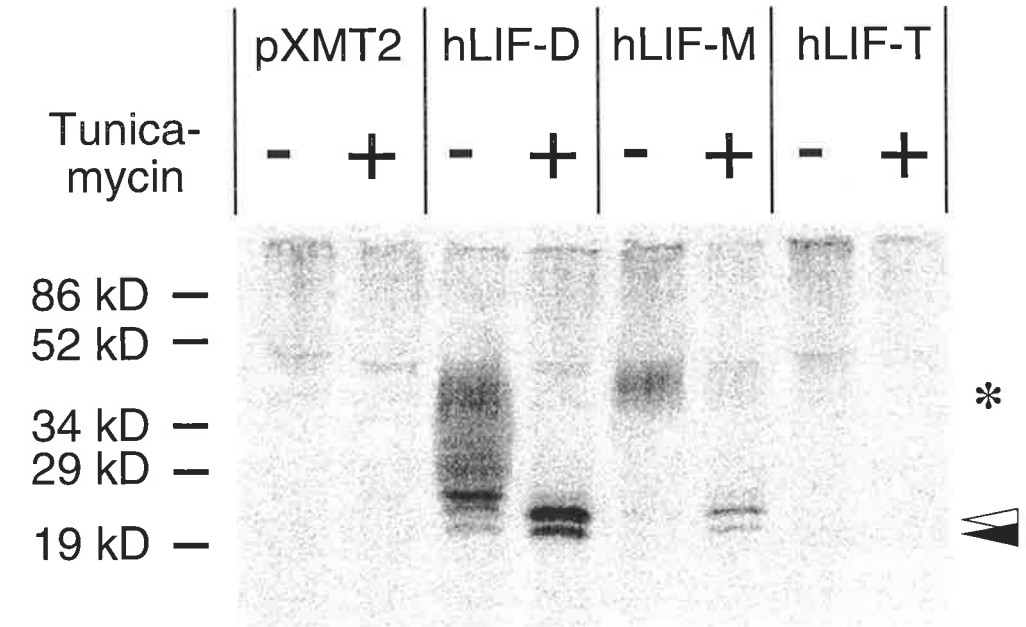
(A) Cell Lysates

(B) Conditioned Media

A


pXMT2
hLIF-D
hLIF-M
hLIF-T
Tunica-mycin
- + - + - + - +
86 kD
52 kD
34 kD
29 kD
19 kD
*

B


pXMT2
hLIF-D
hLIF-M
hLIF-T
Tunica-mycin
- + - + - + - +
86 kD
52 kD
34 kD
29 kD
19 kD
*

that the specific activities of the secreted LIF-D and LIF-M proteins in this assay were approximately equivalent.

5.2.5 The 40-45 kD hLIF-M and 20-45 kD hLIF-D Glycoproteins are Secreted Via a Golgi Apparatus-Mediated Mechanism

The production of secreted proteins from the hLIF-M transcript was unexpected, since the hLIF-M and hLIF-T transcripts had a single identical open reading frame initiated at an exon 2 AUG downstream of sequences encoding the signal peptide. It was therefore of interest to determine whether the secreted proteins encoded by hLIF-M trafficked the same signal-peptide dependent, Golgi apparatus-mediated secretory pathway as the hLIF-D encoded proteins. The drug monensin interferes with exocytotic vesicle traffic between the *trans* and *cis* Golgi networks, inhibiting the signal peptide-dependent secretion of proteins (Wilcox et al., 1982). Thus, immunoprecipitations of cell lysates and conditioned media from Cos 1 cells overexpressing alternate hLIF transcripts that had been metabolically labelled in the presence or absence of monensin were performed to determine whether secretion of the hLIF-M and hLIF-D proteins depended upon transit through the Golgi apparatus (Figure 5.5).

The presence of monensin during metabolic labelling did not appear to affect the level or bias of protein labelling as assessed by electrophoresis of labelled extracts (Figure 5.5 A and C) Immunoprecipitation of cell lysates and conditioned media from these cells revealed significant intracellular accumulation and reduced secretion of the hLIF-D and hLIF-M encoded glycoproteins in monensin treated cells. In the case of hLIF-D transfected cells, this effect was modest and was manifest as an approximately twofold increase in the abundance of these proteins immunoprecipitated from cell lysates with a cognate reduction in the levels of these proteins immunoprecipitated from conditioned media when comparing monensin treated cells with untreated cells (Figure 5.5 B and D). A similar, more dramatic, accumulation of cell-associated hLIF-M proteins occurred in the case of hLIF-M transfected cells (Figure 5.5 B and D). Approximately fivefold more LIF-M glycoprotein was precipitated from the cell lysates of monensin treated cells than untreated cells. The magnitude of these monensin-induced effects was similar to that reported by others (Rubartelli et al., 1990). This indicated that transit through the Golgi apparatus was a requirement for the secretion of both hLIF-M and hLIF-D encoded

Figure 5.5 **Immunoprecipitation of hLIF Proteins from hLIF-D, hLIF-M and hLIF-T Overexpressing Cells in the Presence or Absence of Monensin, an Inhibitor of Golgi Apparatus-Mediated Protein Secretion**

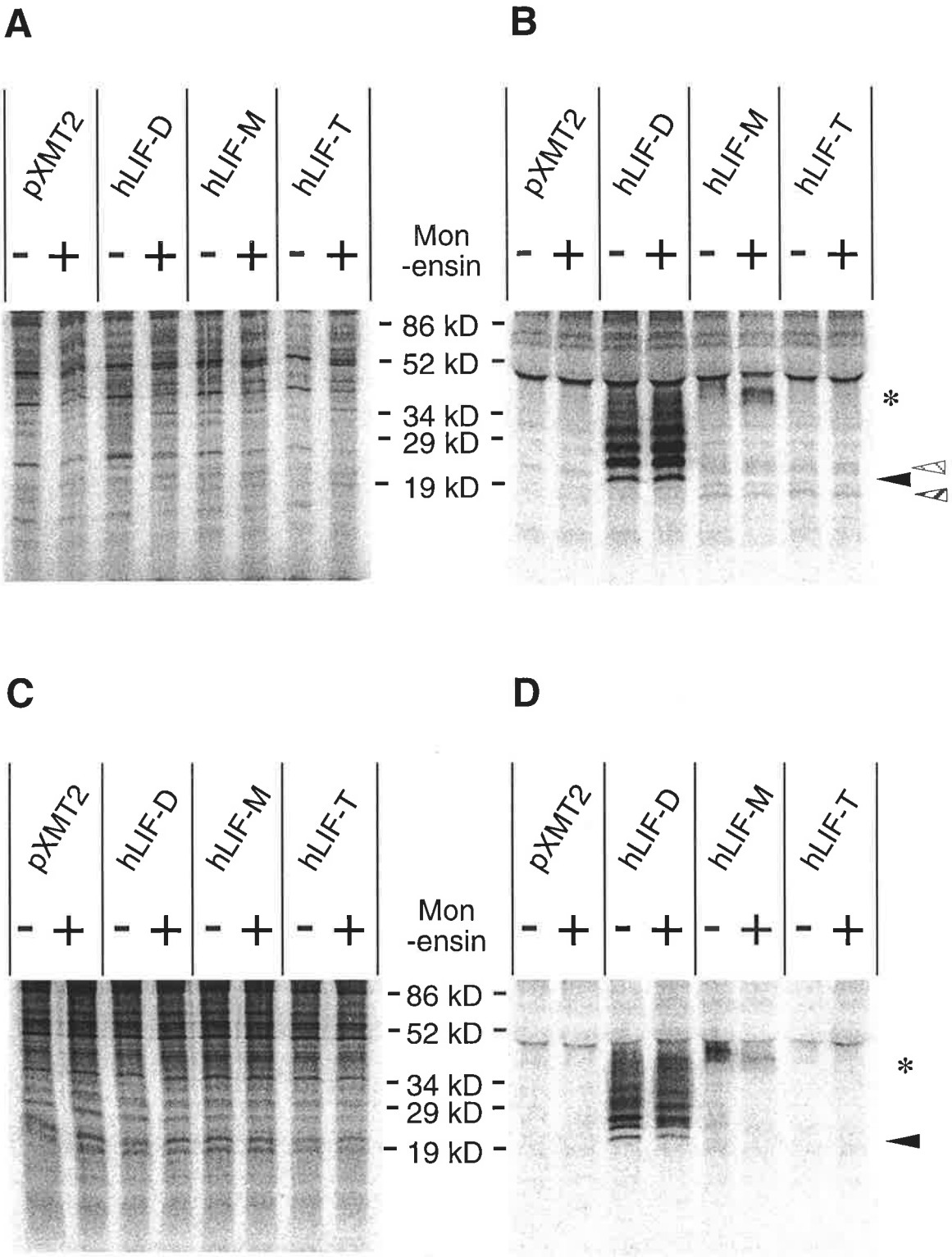
Cos 1 cells were transfected with the indicated LIF expression vectors and subjected to immunoprecipitation 48 hours post-transfection using anti-hLIF-GST antibodies as described in section 2.4.7. ^{35}S metabolic labelling of cellular proteins was performed in the presence (+) or absence (-) of monensin, an inhibitor of secretory vesicle trafficking in the trans-Golgi network, as described in section 2.4.8. The 20+ kD secreted proteins encoded by hLIF-D are indicated by a solid arrowhead. An asterisk indicates the 45 kD secreted proteins encoded by hLIF-M. The 17 and 23 kD intracellular proteins encoded by the hLIF-M and hLIF-T are indicated by thickly and thinly cross-hatched arrowheads respectively.

(A) Loading control for cell lysates. 1µl of labelled cell lysate from transfected Cos 1 cells was electrophoresed to indicate the relative abundance and labelling levels of cellular proteins present.

(B) Anti-hLIF-GST immunoprecipitates of transfected Cos 1 cell lysates.

(C) Loading control for conditioned media. 1µl of labelled conditioned media from transfected Cos 1 cells was electrophoresed to indicate the relative abundance and labelling levels of secreted proteins present.

(D) Anti-hLIF-GST immunoprecipitates of conditioned media from transfected Cos 1 cells.


A
B
pXMT2
hLIF-D
hLIF-M
hLIF-T
- +
Mon
-ensin
86 kD
52 kD
34 kD
29 kD
19 kD
*
C
D

glycoproteins and that both proteins were likely to follow a classical signal peptide-dependent secretory pathway. The presence of monensin during metabolic labelling did not appear to affect the 17 and 23 kD proteins encoded by hLIF-M and hLIF-T .

5.2.6 Translation of the Intracellular hLIF Proteins Initiates at the In-Frame AUG Encoded by Exon 2

Analysis of the hLIF-M and hLIF-T cDNA sequences predicted that the site of translational initiation in both transcripts was at the only in frame AUG found in a conserved position in the shared second exon. The primary translation product arising from translational initiation at this site had a predicted molecular weight of 17 kD. This was in good agreement with the immunoprecipitation of a 17 kD protein from phLIF-MX and phLIF-TX transfected cells, suggesting that this AUG was indeed a site of translational initiation for proteins produced from hLIF-M and hLIF-T transcripts. However, the possibility that the intracellular or other LIF proteins were produced by non-AUG mediated translation initiation (Hann et al., 1994) needed to be investigated.

To confirm the site of translational initiation for the intracellular hLIF proteins, hLIF-D and hLIF-M expression vectors, phLIF-DA^{-}X and phLIF-MA^{-}X, in which the exon 2 ATG had been mutated to GCG using mismatched primer PCR (Figure 5.6) were prepared (Figure 5.7). Immunoprecipitations revealed that levels of the secreted hLIF-D and hLIF-M proteins were unaffected by mutation of this AUG (Figure 5.8). However, production of the 17 and 23 kD intracellular hLIF-M proteins appeared to be completely abrogated by this mutation. This confirmed that this AUG was the site of translational initiation for the 17 and, possibly, 23 kD proteins encoded by hLIF-M and, by analogy, hLIF-T (Figure 5.8). It has also been demonstrated by mutational analysis that translation of the 17 kD protein encoded by mLIF-T is also very likely to initiate at an AUG in this position (Haines et al., 1999). The concurrent disappearance of the 23 kD protein when the exon 2 AUG is mutated suggested that this protein was either a post-translationally modified form of the 17 kD protein or a highly abundant endogenous protein that associated with the 17 kD protein.

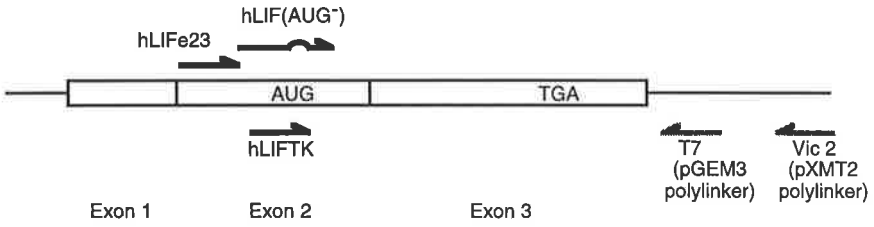
Figure 5.6 Positioning and Sequence of Primers used in the Creation of Specifically Modified and Mutated hLIF cDNAs

(A) Schematic showing positioning and orientation of PCR primers used in production of modified hLIF cDNAs for use in expression vectors with reference to phLIF-D (HpGEM1: Rathjen et al., 1990b).

(B) Sequence of primers shown in *A*. Subscripted numbers indicate the position of the first homologous nucleotide in the primer relative to the hLIF-D cDNA sequence reported by Moreau and co-workers (1988). Primer sequences mismatched to the unmodified hLIF cDNA (shown aligned with hLIF(AUG$^-$) and hLIFTK in bold) are underlined. Sequences preceding the subscripted nucleotide numbers are non-homologous and were added to provide convenient sites for restriction endonuclease digestion (*Eco* R1 for hLIFTK and *Kpn* 1 for hLIF e23). > Indicates that the primer is a 5' or "sense" primer. < Indicates that the primer is a 3' or "antisense" primer.

T7 and Vic 2 are 3' primers that hybridise to vector polylinker sequences.

A



B

hLIF e23 > $ATAGGTACCT_{65}GAGTTGTGCCCCTGCT_{80}$

hLIF(AUG⁻) > $_{130}CCTGTCAACGCCACCTGTGCCATACGCC$
$ACCCATGTCACAACAACCTCGCGAACCA_{185}$

hLIFTK > $GAAGATCT_{167}CAACAACATCATGAAC_{183}$

hLIF cDNA (unmodified) **$_{175}CTCATG_{180}$**

Figure 5.7 Construction of the phLIF-DA$^-$X and phLIF-MA$^-$X Expression Vectors

Schema illustrating the cloning strategy employed in construction of the mutated hLIF expression vectors: phLIF-DA$^-$X and phLIF-MA$^-$X. Production of these vectors is described in detail in section 2.3.22. The suffix "X" indicates the vector used is pXMT2. pXMT2 contains an SV40 origin of replication (open rectangle), an SV40 major late promoter (half arrowhead, thickened line), and the dihydrofolate reductase 3' untranslated region and polyadenylation signal (thickened line). Differential shading is used to indicate alternate first exons of hLIF and insertion of DNA fragments containing mutated sequences.

See Figure 5.6 for sequence and positioning of primers used.

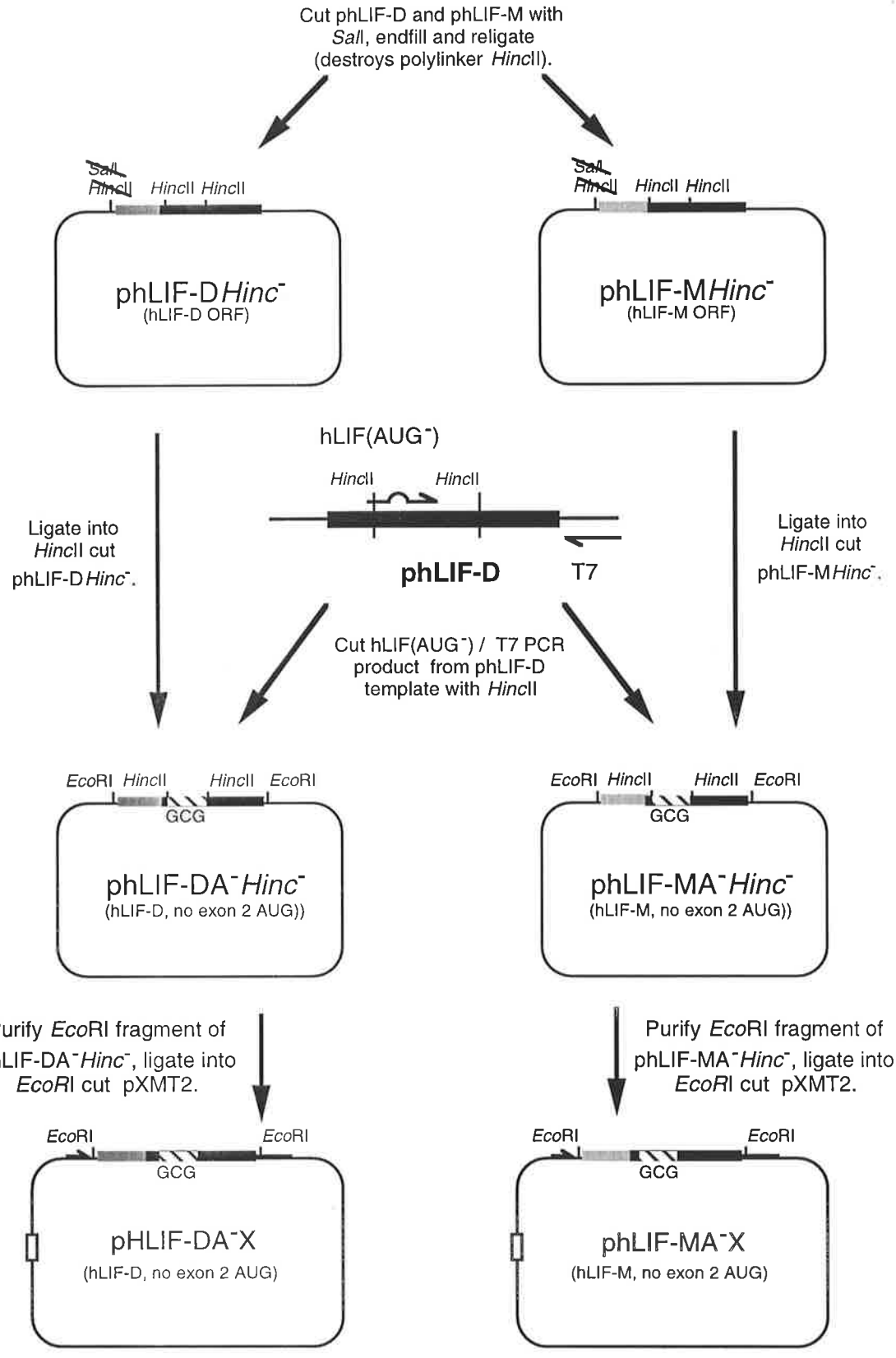
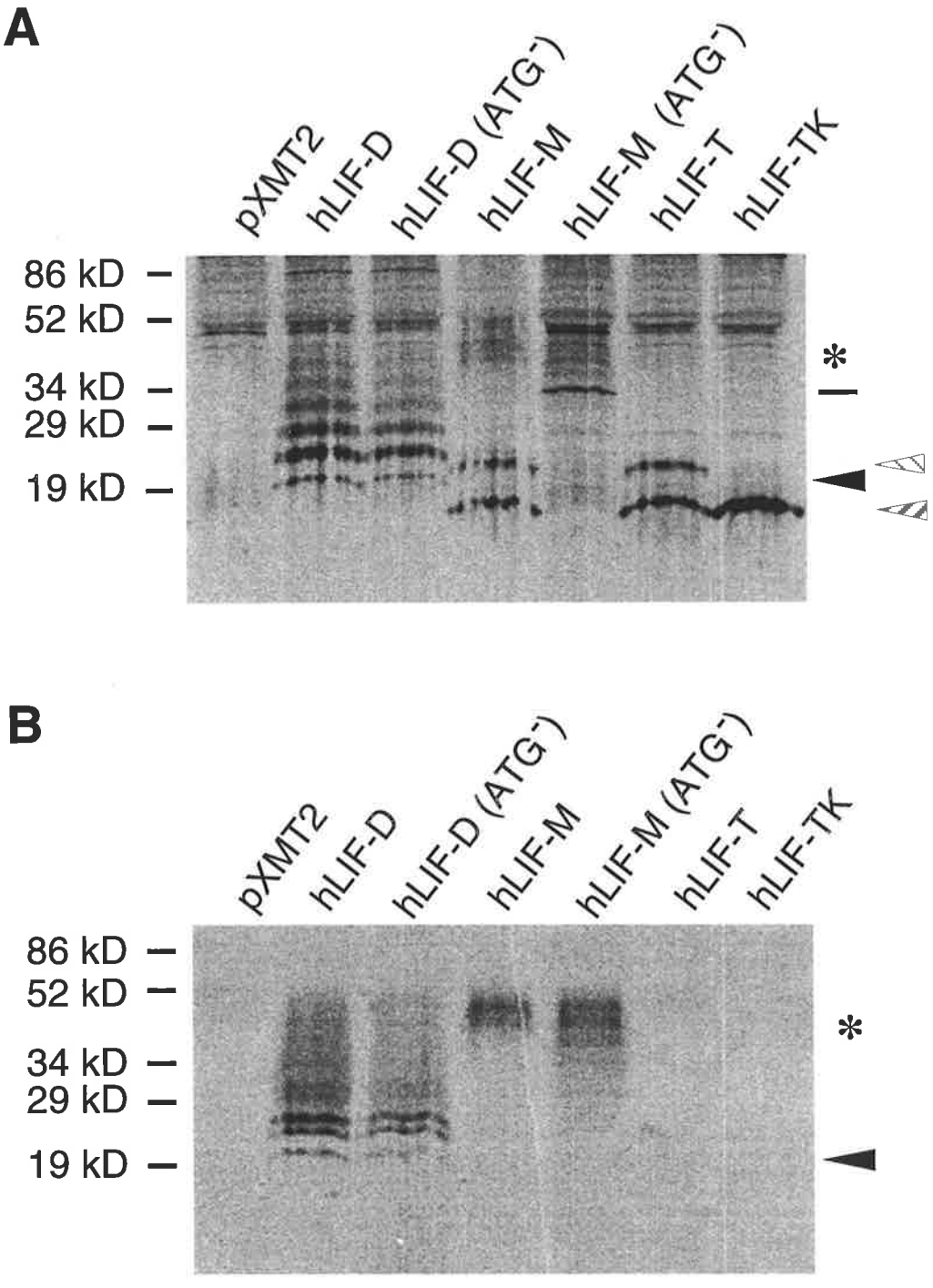

Cut phLIF-D and phLIF-M with
SalI, endfill and religate
(destroys polylinker HincII).
SalI
HincII
HincII HincII
phLIF-DHinc-
(hLIF-D ORF)
SalI
HincII
HincII HincII
phLIF-MHinc-
(hLIF-M ORF)
hLIF(AUG-)
HincII
HincII
phLIF-D
T7
Ligate into
HincII cut
phLIF-DHinc-.
Ligate into
HincII cut
phLIF-MHinc-.
Cut hLIF(AUG-) / T7 PCR
product from phLIF-D
template with HincII
EcoRI HincII
HincII EcoRI
GCG
phLIF-DA-Hinc-
(hLIF-D, no exon 2 AUG))
EcoRI HincII
HincII EcoRI
GCG
phLIF-MA-Hinc-
(hLIF-M, no exon 2 AUG))
Purify EcoRI fragment of
phLIF-DA-Hinc-, ligate into
EcoRI cut pXMT2.
Purify EcoRI fragment of
phLIF-MA-Hinc-, ligate into
EcoRI cut pXMT2.
EcoRI
EcoRI
GCG
pHLIF-DA-X
(hLIF-D, no exon 2 AUG)
EcoRI
EcoRI
GCG
phLIF-MA-X
(hLIF-M, no exon 2 AUG)

Figure 5.8 Immunoprecipitation of hLIF Proteins from Cos 1 Cells Transfected with Mutated hLIF Expression Vectors

Cos 1 cells were transfected with the expression vectors pXMT2, phLIF-DX, phLIF-D(ATG-), phLIF-M, phLIF-M(ATG$^-$), phLIF-TX, and phLIF-T_KX cell lysates and conditioned media were subjected to immunoprecipitation 48 hours post-transfection using anti-hLIF-GST antibodies as described in section 2.4.7. The 20+ kD secreted proteins encoded by hLIF-D are indicated by a solid arrowhead. An asterisk indicates the 45 kD secreted proteins encoded by hLIF-M and hLIF-M(ATG)$^-$. The 17 and 23 kD intracellular proteins encoded by the hLIF-M, hLIF-T , and hLIF-TK are indicated by thickly and thinly cross-hatched arrowheads respectively.

A horizontal bar indicates immunoprecipitation of an intracellular protein in hLIF-M(ATG)$^-$ cell lysates which results from translational initiation at an in-frame AUG codon present in pXMT2 (an in-frame TGA present immediately upstream of the hLIF-M cDNA in phLIF-MX precludes production of such proteins).


A
pXMT2
hLIF-D
hLIF-D (ATG⁻)
hLIF-M
hLIF-M (ATG⁻)
hLIF-T
hLIF-TK
86 kD
52 kD
34 kD
29 kD
19 kD
*
B
pXMT2
hLIF-D
hLIF-D (ATG⁻)
hLIF-M
hLIF-M (ATG⁻)
hLIF-T
hLIF-TK
86 kD
52 kD
34 kD
29 kD
19 kD
*

5.2.7 Translation of Secreted hLIF-M Proteins Appears to Initiate at Non-AUG Codons

Bioassays of conditioned media from phLIF-MA^{-}X transfected cells showed that levels of extracellular LIF activity were equivalent to those produced from phLIF-MX transfected cells (Figure 5.9). Since levels of the 40-45 kD secreted hLIF-M proteins visualised by immunoprecipitation also appeared to be unaffected by mutation of this AUG, it is these secreted hLIF-M proteins that must account for the high levels of LIF bioactivity in the media of phLIF-MX and phLIF-MA^{-}X transfected cells. This observation also demonstrates that translation of the secreted and intracellular hLIF-M proteins occurs independently.

Since the exon 2 AUG is the only in frame AUG in the hLIF-M open reading frame, the secreted hLIF-M proteins must initiate translation at a non-AUG codon(s). It is likely that this initiation codon(s) is located upstream of the sequence encoding the secretion signal peptide since the secreted hLIF-M encoded proteins were shown to traffic the classical signal peptide mediated pathway (section 5.2.5 and 5.3.2). The possibility that the secreted hLIF-M proteins arose from translational initiation within vector sequences can be excluded since the RACE-PCR cDNA insert used in preparing this construct contained an in frame TGA termination codon immediately upstream of the hLIF-M reading frame.

5.2.8 Sequences Contained in the hLIF-M First Exon are Necessary for Non AUG-Mediated Translational Initiation of the Secreted hLIF-M Protein

To further examine the role of the alternate first exons of the hLIF-M and hLIF-T transcripts in the production of secreted proteins from the hLIF-M transcript, an expression construct, phLIF-e23X, containing only exons 2 and 3 of the hLIF cDNA was prepared (Figures 5.6 and 5.10). This tested for the effect of the alternate hLIF-M and hLIF-T first exon sequences on production of the secreted hLIF-M proteins. Immunoprecipitation of cell lysates and conditioned media from phLIF-e23X transfected cells revealed that only the 17 and 23 kD intracellular hLIF proteins were translated from this construct (Figure 5.11). This was consistent with the low level of activity present in media conditioned by phLIF e23X transfected cells (Figure 5.9). This indicated that sequence information in exon 1 of the hLIF-M transcript was required to initiate and/or promote the translation of the secreted hLIF-M preprotein. These

Figure 5.9 **Assay of Media Conditioned by Cos 1 Cells Transfected with Mutated hLIF Expression Vectors for Maintenance of Murine ES Cells in an Undifferentiated State**

Cos 1 cells were transfected with parental vector (pXMT2) or parental vector containing the hLIF cDNAs: hLIF-D, hLIF-M, hLIF-D(ATG^-), hLIF-M(ATG^-), hLIF-e23, or hLIF-TK. Conditioned media from these cells was collected after 72 hours and assayed at dilutions between 1 in 10 and 1 in 20,000 for ability to maintain ES cell colonies in an undifferentiated state. ES cell colonies were scored as undifferentiated on the basis of their dome shaped morphology and staining for alkaline phosphatase activity (see Figure 5.2).

Assay results are presented in tabular format showing the actual dilutions performed.

(+) Undifferentiated ES cell colonies only,

(+/-) some differentiated ES cell colonies,

(-) differentiated ES cell colonies only.

	1/10	1/50	1/100	1/1 000	1/2 000	1/10 000	1/20 000
hLIF-D	+	+	+	+	+	+	+/-
hLIF-M	+	+	+	+	+/-	-	-
hLIF-D(ATG$^-$)	+	+	+	+	+	+	+/-
hLIF-M(ATG$^-$)	+	+	+	+	+/-	-	-
hLIF-e23	+	+/-	-	-	-	-	-
hLIF-TK	+	+/-	-	-	-	-	-
pXMT2	-	-	-	-	-	-	-

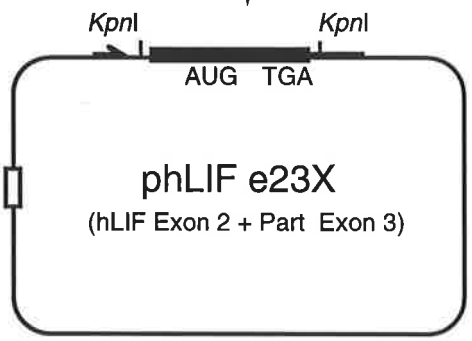
Figure 5.10 Construction of the phLIF e23X and phLIF-T_KX Expression Vectors

Schema illustrating the cloning strategy employed in construction of the hLIF expression vectors: phLIF e23X and phLIF-T_KX. Production of these vectors is described in detail in section 2.3.22. The suffix "X" indicates the vector used is pXMT2. pXMT2 contains an SV40 origin of replication (open rectangle), an SV40 major late promoter (half arrowhead, thickened line), and the dihydrofolate reductase 3' untranslated region and polyadenylation signal (thickened line).

See Figure 5.6 for sequence and positioning of primers used.

hLIFe23

Cut hLIFe23 / Vic 2 PCR product from phLIF-DX template with *Kpn*I, ligate into *Kpn*I cut pXMT2.



hLIFTK

Cut hLIFTK / Vic 2 PCR product from phLIF-DX template with *Kpn*I, ligate into *Eco*RI cut pXMT2.

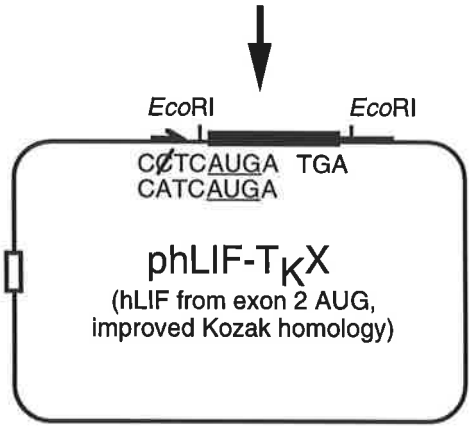
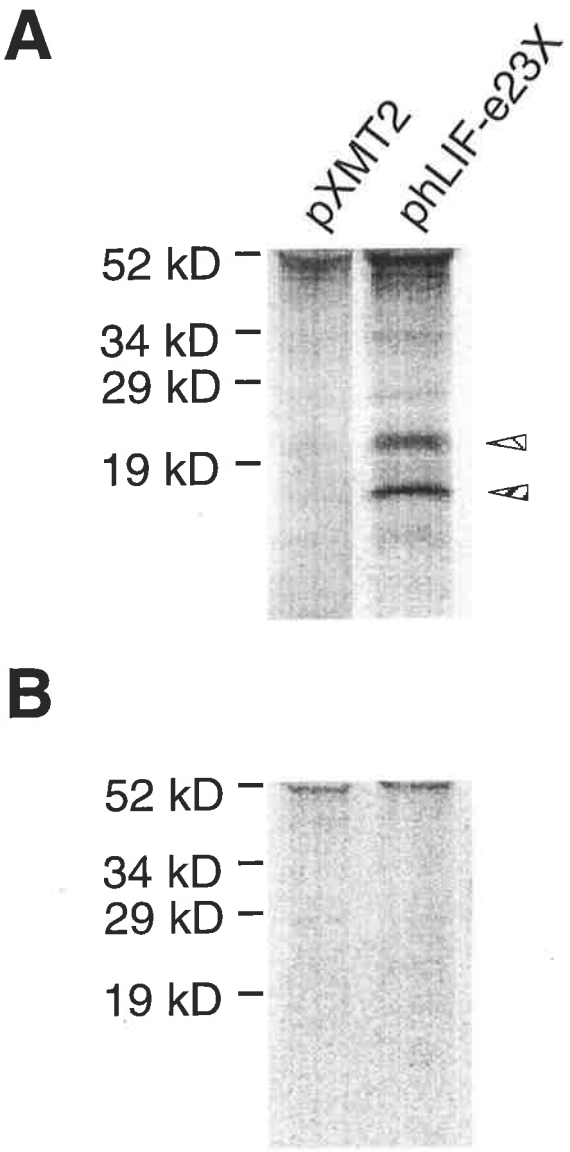


Figure 5.11 Immunoprecipitation of hLIF Proteins from Cos 1 Cells Transfected With the hLIF-e23 Expression Vector

Cos 1 cells were transfected with pXMT2 and phLIF e23X , which expresses an hLIF transcript containing only exons 2 and 3, and subjected to immunoprecipitation 48 hours post-transfection using anti-hLIF-GST antibodies as described in section 2.4.7. The 17 and 23 kD intracellular proteins expressed from the hLIF-e23 vector are indicated by thickly and thinly cross-hatched arrowheads respectively.

(A) Cell lysates.

(B) Conditioned media.


A
pXMT2
phLIF-e23X
52 kD
34 kD
29 kD
19 kD
B
52 kD
34 kD
29 kD
19 kD

findings also eliminate the alternative possibility that sequence information within exon 1 of the hLIF-T transcript acted to suppress translation of secreted hLIF proteins initiated within exon 2, where candidate non-AUG initiation codons are found (Figure 5.13).

5.2.9 Epitope Tagging of hLIF-D, hLIF-M, and hLIF-T Encoded Proteins

An expression construct, phLIF-DX.FLAG, in which sequences encoding a FLAG epitope tag had been engineered into the 3' end of the open reading frame of hLIF-D was kindly made available by co-workers. The constructs phLIF-MX.FLAG and phLIF-TX.FLAG express hLIF-M and hLIF-T encoded proteins containing carboxy-terminal FLAG epitope tags and were constructed by replacement of the 5' end of the hLIF-D cDNA in phLIF-DX.FLAG with the 5' end of the hLIF-M and hLIF-T cDNAs respectively (Figure 5.1). The ten amino acid FLAG epitope is recognised by commercially available monoclonal antibodies with proven capacities in immunoprecipitation analysis and western blotting (Guimaraes et al., 1995).

To determine whether addition of the carboxy-terminal FLAG epitope to hLIF proteins had any affect on their extracellularisation and/or activity, expression constructs for alternate hLIF transcripts encoding tagged and untagged proteins were transfected into Cos 1 cells. Media conditioned by these cells were collected 72 hours post-transfection and assayed for their ability to maintain murine ES cells in an undifferentiated state. No differences in the levels of extracellular LIF activity produced by cells overexpressing tagged and untagged constructs were observed (data not shown), indicating that addition of the carboxy-terminal epitope tag had little or no effect on the secretion and activity of extracellular hLIF proteins.

5.2.10 The 23 kD Intracellular hLIF Protein Appears to be a Post-Translationally Modified Form of the 17kD LIF Protein

Epitope tagged hLIF proteins could be detected by immunoprecipitation of conditioned media and cell lysates from Cos 1 cells overexpressing FLAG-tagged constructs using either the polyclonal GST-hLIF antisera (Figure 5.12) or the M2 anti-FLAG monoclonal antibody with identical results (data not shown). The hLIF proteins produced from these FLAG-tagged constructs appeared to be identical to those produced from the untagged constructs except that tagged proteins were approximately 1 kD larger than their untagged equivalents (Figure 5.12).

Figure 5.12 Immunoprecipitation of hLIF-T, hLIF-T.FLAG and mLIF-T Proteins from Overexpressing Cos 1 Cells

Cos 1 cells were transfected with the indicated LIF expression vectors and subjected to immunoprecipitation 48 hours post-transfection using anti-hLIF-GST antibodies as described in section 2.4.7. Thickly and thinly cross-hatched arrowheads indicate the 1kD "band shift" of the 17 and 23 kD proteins encoded by hLIF-T that is caused by addition of the FLAG epitope tag. The mLIF-T immunoprecipitate was electrophoresed in the lane adjacent to hLIF-T but is shown separated to allow the use of arrows.

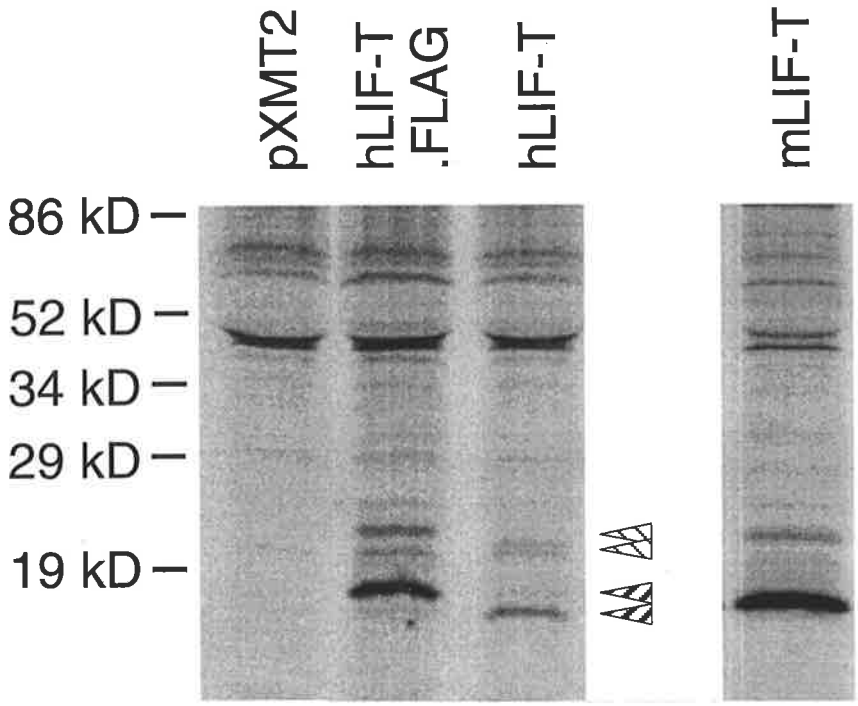

pXMT2
hLIF-T .FLAG
hLIF-T
mLIF-T
86 kD
52 kD
34 kD
29 kD
19 kD

Figure 5.13 Potential Non-AUG Translation Initiation Codons for Initiation of Secreted LIF-M Proteins in the LIF Genes of Eutherian Mammals

Alignment of cloned and predicted (section 3.3.1) nucleotide sequences from exon1 and the 5' end of exon 2 for the hLIF-M transcript of five species of eutherian mammal. Potential non-AUG translation initiation codons (Hann et al., 1994), some of which are conserved, are indicated in bold face type and underlined. The AUG translation initiation codon of mLIF-M is also indicated in the same way. In frame translation termination codons in the mLIF-M and pLIF-M first exons are boxed. The murine (m) and human (h) sequences are from Stahl and co-workers (1990). The porcine (p) and ovine (o) sequences are from Wilson and co-workers (1992). The bovine sequence is that of Kato and co-workers (1995).

mLIF-M (Exon1) C TAG TCC **CTG** GAA AGC TGT GAT TGG CGC GAG **ATG** AGA TGC A

hLIF-M (Exon 1) CT GGA AGC **GTG** TGG TCT GCG CTA G

pLIF-M (Exon 1) CT GGA AAG CTG TGA TCT **GTG** CTA G

bLIF-M (Exon1) AG CGA AAG **CTG** TGG TCT **GTG** CTA G

oLIF-M (Exon 1) TG CGC CCG **GTG** CCG CCT GAG CTA G

mLIF-M (Exon 2) GA ATT **GTG** CCC TTA **CTG** **CTG** **CTG** GTT **CTG** CAC TGG AAA CAC GGG GCA GGG AGC

hLIF-M (Exon 2) GA GTT **GTG** CCC --- **CTG** **CTG** TTG GTT **CTG** CAC TGG AAA CAT GGG GCG GGG AGC

pLIF-M (Exon 2) GA GTT **GTG** CCC --- **CTG** **CTG** **CTG** GTT **CTG** CAC TGG AAA CAC GGG GCA GGG AGC

bLIF-M (Exon 2) GA GTT **GTG** CCC --- TTG **GTG** **CTG** GTT CTC CAC TGG AAA CAC GGG GCC GGG AGC

oLIF-M (Exon 2) GA GTC **GTG** CCC --- **CTG** **CTG** **CTG** GTC TTG CAC TGG AAA CCC GGG GCG GGG AGC

For unknown reasons, significantly more tagged than untagged intracellular hLIF proteins were reproducibly immunoprecipitated from transfected Cos 1 cell lysates using the same antisera at the same dilution (Figure 5.12).

Addition of the 1kD C-terminal FLAG epitope tag increased the apparent molecular weight of both the 17 and 23 kD proteins (Figure 5.12). Furthermore, the 17 and 23 kD proteins could both be immunoprecipitated using the M2 anti-FLAG monoclonal antibody (data not shown). These observations demonstrated that the 23 kD protein contained the FLAG epitope and was not a co-precipitating protein. Taken together with the observed disappearance of both these proteins when the exon 2 AUG is mutated, these findings strongly supported the contention that the 23 kD protein was a post-translationally modified form of the 17 kD protein.

5.2.11 Production of the 23 kD Modified Form of the Intracellular hLIF-T Protein May be Assisted by Sequences Upstream of the In-Frame AUG in Exon 2

In an attempt to increase production and facilitate detection of intracellular hLIF proteins by immunofluorescence (discussed in section 6.2.1), a new construct, phLIF-T_KX, for the expression of intracellular LIF proteins was produced by mismatched primer PCR (Figures 5.6 and 5.10). This vector lacked LIF sequences upstream of the exon 2 AUG translation initiation codon and the sequence surrounding the AUG was engineered to more closely match the consensus sequence for mammalian translation initiation codons (Kozak, 1989) (Figure 5.10). When immunoprecipitations were performed on phLIF-T_KX transfected cells, increased levels of the 17 kD intracellular hLIF protein could be detected (Figure 5.8 and Figure 6.6). Surprisingly, however, the 23 kD intracellular LIF protein, was not clearly evident in these immunoprecipitates. It is thus possible that production of the 23 kD post-translationally modified protein could be at least partly dependent upon sequences in exon 2 of the LIF transcript upstream of the AUG translation initiation codon.

5.3 Discussion

5.3.1 Alternate hLIF Transcripts Encode Two Classes of Biologically Active, Differentially Compartmentalised Proteins.

A key finding of these studies was that the alternate hLIF transcripts encode two distinct sets of proteins that differ with respect to their compartmentalisation and glycosylation.

Immunoprecipitation studies and bioassays indicated that the hLIF-D transcript encoded secreted LIF proteins representing N-linked glycosylation variants of 20 kD and 22 kD core polypeptides that were deduced to be unmodified mature LIF and a variant of this protein containing O-linked glycosides.

The hLIF-T transcript encoded only intracellularly-retained LIF proteins of 17 and 23 kD which appeared to arise from translational initiation at the in-frame AUG in exon 2. An AUG is present in this region in all reported LIF gene sequences (Haines et al., 1999) including one from a marsupial mammal (Cui, 1998). The universal retention of an AUG in this position in the LIF genes of mammals for more than 200 million years of evolutionary history (O'Brien et al., 1990) may be indicative of a high degree of functional importance for the intracellular LIF proteins. Epitope tagging of the hLIF-T encoded proteins and mutagenesis studies confirmed that the 23 kD protein was a post-translationally modified form of the 17 kD LIF protein.

The hLIF-M transcript was shown to encode both intracellular and extracellular proteins. The intracellular hLIF-M proteins appeared identical to the intracellular hLIF-T proteins. This is likely to reflect their common translational origin at the in frame AUG in exon 2. Secreted proteins encoded by hLIF-M appeared to share an identical core polypeptide with the secreted proteins encoded by hLIF-D and differed only in their degree of N-linked glycosylation in this expression system. The compartmentalisation of these proteins was in good accord with the relatively high, intermediate and negligible levels of extracellular LIF activity produced from the hLIF-D, hLIF-M and hLIF-T transcripts respectively.

The LIF genes of mice and humans thus show conserved transcription, translation, and protein localisation since extracellular proteins are encoded by the human and murine LIF-D and LIF-M transcripts, and intracellular proteins are encoded by the LIF-T transcripts of both species.

LIF may now be grouped with at least two other cytokines which are produced in intracellularly and extracellularly localised forms by translation of independent transcripts. In the cases of the IL-1 receptor antagonist (Haskill et al., 1991; Jenkins et al., 1997) and IL-15 (Tagaya et al., 1997), the intracellular proteins are produced with leader peptides which are cleaved to yield a mature protein that, except for its lack of glycosylation, is identical to the secreted form. Only in the case of LIF do the mature secreted and intracellular polypeptides differ. Thus, three unrelated cytokines are now known to be expressed in intracellular and extracellular forms by independently regulated transcription of a single gene.

5.3.2 Production of Secreted Proteins from the hLIF-M Transcript

Since the secreted proteins encoded by hLIF-M and hLIF-D appear to contain an identical core polypeptide, their translation must initiate at an uncharacterised site upstream of the signal peptidase cleavage site (see Figure 8.1). There is no in-frame AUG in this position in the hLIF-M transcript, so translation of these secreted proteins must initiate at a non-AUG codon(s). The inclusion of an in-frame TGA in the RACE-PCR primer immediately upstream of the hLIF-M cDNA in the phLIF-MX construct excludes the possibility that the secreted hLIF-M proteins are produced as a consequence of translational initiation in upstream vector sequences. Secretion of the 40-45 kD hLIF-M encoded proteins could be inhibited by the drug monensin, implying that these proteins, like the secreted hLIF-D encoded proteins, trafficked a Golgi-mediated secretory pathway. This also implied that the site of translational initiation in the secreted hLIF-M protein was sufficiently upstream of the signal peptidase cleavage site to allow the production of a preprotein containing a functional signal peptide.

A number of CUG and GUG potential translation initiation codons (Hann et al., 1994) are present in the first exons and the 5' end of the second exons of the cloned and predicted LIF-M transcripts of five eutherian mammals (Figure 5.13) (Haines et al., 1999; Stahl et al., 1990; Willson et al., 1992). Atypical modes of translational initiation have been demonstrated for several important regulatory proteins including growth factors and cytokines (Hann, 1994; Hann et al., 1988; Hann et al., 1992; Bruenig et al., 1996; Vagner et al., 1995; Vagner et al., 1996). In many cases, the alternately initiated forms of these proteins are known to be functionally distinct (Hann et al., 1994; Bikfalvi et al., 1995; Blackwood et al., 1994; Arnaud et al., 1999) and can be

differentially localised (Bugler et al., 1991; Florkiewicz et al., 1991). In the case of FGF 2, four distinct proteins are produced by at least three independent translational mechanisms (Arnaud et al., 1999). Alternate translation of AUG-initiated and non-AUG-initiated proteins from a single transcript thus provides a mechanism for regulated expression of proteins that can differ both in their compartmentalisation and activity.

Secreted proteins encoded by the mLIF-M transcript can become localised to the extracellular matrix (Rathjen et al., 1990a). The hLIF-M transcript, like the mLIF-M transcript, encodes secreted proteins whose translation initiates upstream of the signal peptidase cleavage site. The lack of an in frame AUG in the putative LIF-M first exons of species other than the mouse has been interpreted as indicating a lack of biological relevance for the hLIF-M transcript (Willson et al., 1993; Gough et al., 1992). However, the translation of a secreted protein from the hLIF-M transcript by initiation at a non-AUG codon suggests that the presence of an in-frame AUG in the first exon of the mLIF-M transcript may be of little consequence since production of secreted LIF proteins appears to be dependent upon other information in the nucleotide sequence of the LIF-M first exon. The FGF 2 transcript, whose non-coding regions control several independent translational initiation processes, provides a precedent for this (Vagner et al., 1995; Arnaud et al., 1999).

5.3.3 Intracellular LIF Proteins

Forced secretion of the intracellular LIF protein by fusion to an N-terminal signal sequence dramatically increased the levels of extracellular LIF bioactivity (Haines et al., 1999), indicating that intracellular LIF proteins are capable of productive interaction with cell surface LIF receptors. However, the very low levels of extracellular LIF activity produced by hLIF-T and mLIF-T overexpressing cells (Haines et al., 1999), the negligible amounts of LIF proteins immunoprecipitated from hLIF-T and mLIF-T conditioned media, and the lack of an effect on extracellular hLIF-M activity when production of the 17 and 23 kD hLIF-M proteins was precluded by mutation, all indicate that the 17 and 23 kD proteins are not normally released from these cells. The very low levels of LIF activity detected in media conditioned by hLIF-T and mLIF-T overexpressing cells are thought to be released as a result of limited cell lysis. This is supported by the observation that mechanical lysis of mLIF-T-transfected cells dramatically

increased the levels of LIF activity seen in this assay (J. Rathjen, unpublished observations). A variety of extracellularly active proteins expressed without signal peptides, including IL-6 family cytokines, seem to be retained intracellularly yet can be released extracellularly by an uncharacterised mechanism(s) (Muesch et al., 1990; Mignatti et al., 1992; Lin et al., 1989; Pennica et al., 1995; Miyamoto et al., 1993). Thus, the intracellular hLIF proteins could be extracellularly active following release in response to environmental cues or by disruption of cell membrane integrity (McNeil et al., 1989). The possibility that an alternate mode of LIF action exists whereby intracellular signalling processes can occur independently of cell surface receptor signalling is considered in Chapters 6 and 8.

5.3.4 Expression of hLIF-M and hLIF-T Encoded Proteins and Germ Cell Tumourigenesis.

Deregulated LIF expression and autocrine LIF action are potentially significant in the establishment and/or maintenance of human germ cell tumours since LIF, in combination with other cytokines, will convert murine primordial germ cells into pluripotential stem cells (Matsui et al., 1992), maintains many proliferating stem and progenitor cell populations in vitro and *in vivo* (Escary et al., 1993; Smith et al., 1988; Williams et al., 1988; Matsui et al., 1991) and is involved in the self-renewal of murine embryonic stem cells (Dani et al., 1998). Murine EC cells also frequently acquire extra copies of chromosome 11, which carries the LIF gene (Kola et al., 1990).

The most abundant LIF transcript in at least four of the five human EC cell lines surveyed in Chapter 4 was hLIF-M. hLIF-T transcripts were also expressed at lower but consistent levels in these cell lines and, together, the hLIF-M and hLIF-T transcripts were more abundant than hLIF-D transcripts. It thus appears that intracellular LIF proteins are likely to be a major component of the LIF proteins expressed by human EC cells and may contribute to their tumourigenic phenotype. This contribution could involve autocrine and/or cell-autonomous signalling by the intracellular proteins and/or autocrine signalling by the secreted proteins. The ability of the 17 kD LIF protein to signal through cell surface receptors when its secretion or release is forced (Haines et al., 1999; J. Rathjen, unpublished results) and the expression of LIF receptor transcripts by all five EC cell lines surveyed suggest that autocrine and/or cell-autonomous LIF signalling may occur in EC stem cells.

The overlapping sets of hLIF proteins produced from the alternate hLIF transcripts comprise a continuum in terms of their potential modes of action: hLIF-D transcripts encode proteins that could act in a paracrine / autocrine fashion, while hLIF-M transcripts encode proteins with potentially paracrine / autocrine and cell-autonomous actions, and hLIF-T transcripts encode proteins with a possible cell-autonomous mode of action. Stringent transcriptional and post-transcriptional control of the production and action of hLIF proteins is indicated by these studies. Since highly regulated, combinatorial cytokine action is thought to be crucial to the establishment of cellular microenvironments such as "stem cell niches" (Smith et al., 1992), de-regulation of LIF production and/or action could be involved in the subversion of controls that normally maintain stem cell numbers, allowing growth of tumour cell populations.

Chapter 6 :

Novel Biological Activity of the Intracellular hLIF Protein

6.1 Introduction

Alternate transcripts produced from cytokine genes can encode proteins with distinct localisations and functions (Park et al., 1993; Pollock and Richardson, 1992; Nakagawa et al., 1996; Kimura et al., 1990; Parker et al., 1996). Immunoprecipitation analysis (section 5.2.3) demonstrated that the distinct sets of proteins encoded by the alternate hLIF transcripts differed in their localisation and thus potentially in their function. The truncated LIF proteins encoded by the hLIF-M and hLIF-T transcripts were distinct from the secreted LIF proteins encoded by the hLIF-D and hLIF-M transcripts in that they were found almost exclusively in whole cell lysates. These data did not, however, provide any information concerning the distribution of these proteins within the cell. As a first step toward elucidating the function of the proteins encoded by the novel LIF transcripts, an indirect immunofluorescence procedure was used to determine the subcellular compartmentalisation of these proteins within Cos 1 cells overexpressing them.

It was demonstrated using this approach that the sets of proteins encoded by the alternate hLIF transcripts had distinct subcellular localisations. Furthermore, it was found during these studies that Cos 1 and 293T cells overexpressing the intracellular hLIF proteins underwent an apoptotic cell death which was dependent upon caspase activity. The induction of a specific apoptotic process by the intracellular hLIF proteins represents an intriguing and novel biological activity, which is distinct from that of the secreted hLIF proteins. These findings were in good agreement with studies that revealed the apoptotic action of intracellular mLIF-T protein in Cos 1 and 293T cells (Haines, 1997, Haines et al., *Submitted*).

6.2 Results

6.2.1 Immunolocalisation of hLIF Proteins

Since endogenous levels of LIF expression are extremely low (Robertson et al., 1993), detection of cellular LIF protein was only achieved in cells overexpressing hLIF cDNAs (Chapter 5). Cos 1 cells transfected with hLIF expression vectors were stained with anti-hLIF-GST antibodies using an indirect immunofluorescence procedure (section 2.4.5). Stainings were performed two days after transfection. phLIF-DX transfected cells showed a cytoplasmic network

of staining with concentrations in the perinuclear region (Figure 6.1). This staining pattern suggested that hLIF-D encoded proteins were localised to the Golgi apparatus and endoplasmic reticulum, as would be expected for proteins whose secretion is inhibited by monensin (section 5.2.5). phLIF-MX transfected cells, which express secreted and intracellular proteins, exhibited two LIF staining patterns. Approximately half of the staining cells had a perinuclear, cytoplasmic network of LIF staining (upper cell in phLIF-MX panels, Figures 6.1). This staining pattern suggested the accumulation of predominantly secreted hLIF-M proteins in the Golgi apparatus and endoplasmic reticulum of these cells. The other half of the phLIF-MX transfected cells were rounded and exhibited uniform LIF staining throughout the cytoplasm and nucleus (lower cell in phLIF-MX panels, Figure 6.1). phLIF-TX transfected cells, which express only intracellular LIF proteins, showed a very low level of staining that was difficult to detect. Cells staining for hLIF-T expression resembled the rounded up, uniformly staining phLIF-MX transfected cells (Figure 6.1). Approximately eight fold fewer staining cells were seen in phLIF-MX and phLIF-TX transfections than in phLIF-DX transfections. In sum, these data suggested that the secreted hLIF proteins were exclusively cytoplasmic and localised to the Golgi apparatus and endoplasmic reticulum, as was suggested by inhibition of their secretion by monensin, while the intracellular hLIF proteins were distributed more uniformly throughout the cytoplasm and nucleus.

To identify the nuclei of staining cells and determine their nuclear morphology, all transfections were concurrently stained with the DNA-specific Hoechst 33258 dye (Figure 6.1; panels on right). The nuclei of phLIF-DX transfected cells were large, round, had a relatively uniform staining pattern, and appeared identical to the nuclei of untransfected cells. The nuclei of the phLIF-MX transfected cells exhibiting a cytoplasmic network of LIF staining also stained in a manner identical to the nuclei of untransfected cells (upper cell in phLIF-MX panels of Figure 6.1; upper cell in Figure 6.2 A and C). In contrast, the phLIF-MX and phLIF-TX transfected cells that were rounded up and stained uniformly for LIF protein generally had nuclei that appeared to be deteriorating and contained aggregated DNA (lower cell Figure 6.2 A and C; data not shown). Cells with this nuclear morphology were often raised out of the plane of focus for untransfected cells (Figure 6.2 C). Such nuclear morphologies have been associated with apoptotic cell death (Arends et al., 1990; Wylie et al., 1990; Savill et al., 1990). During apoptosis attachment to the substratum is gradually lost and the cells become raised and rounded, plasma and nuclear

Figure 6.1 Immunolocalisation of hLIF Proteins in Cos-1 Cells Transfected with Expression Vectors for hLIF-D, hLIF-M, and hLIF-T

Cos 1 cells were transfected with the expression vectors phLIF-DX (hLIF-D), phLIF-MX (hLIF-M) and phLIF-TX (hLIF-T). Cells were stained 48 hours post-transfection with anti-hLIF-GST antibodies and Hoechst 33258. Representative fields of view showing LIF and DNA staining are shown for each vector. Arrowheads indicate the nuclei of selected hLIF staining cells. Cos 1 cell transfection and hLIF immunolocalisation were performed as described in sections 2.4.2 and 2.4.5. Magnification 80x.

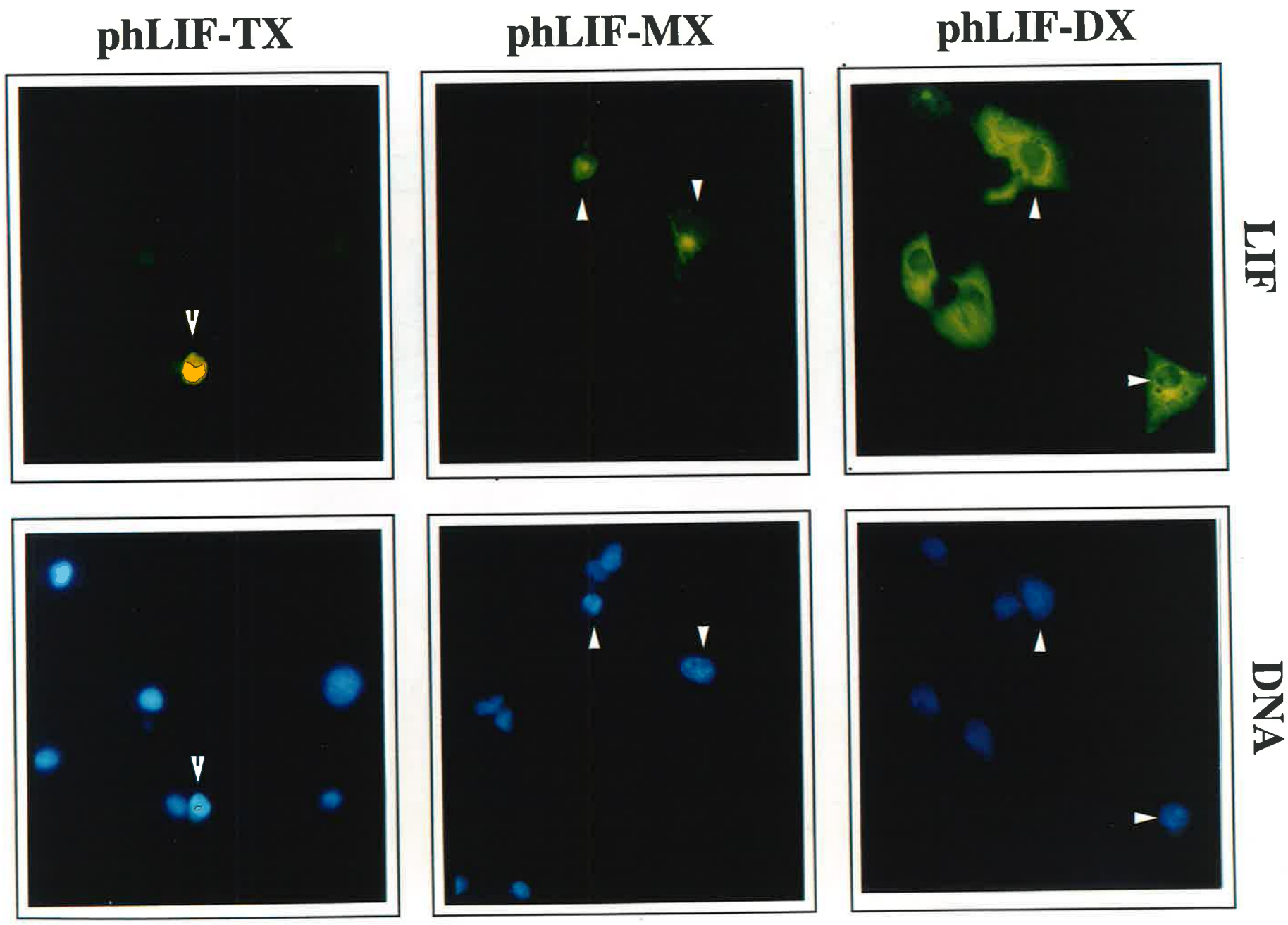
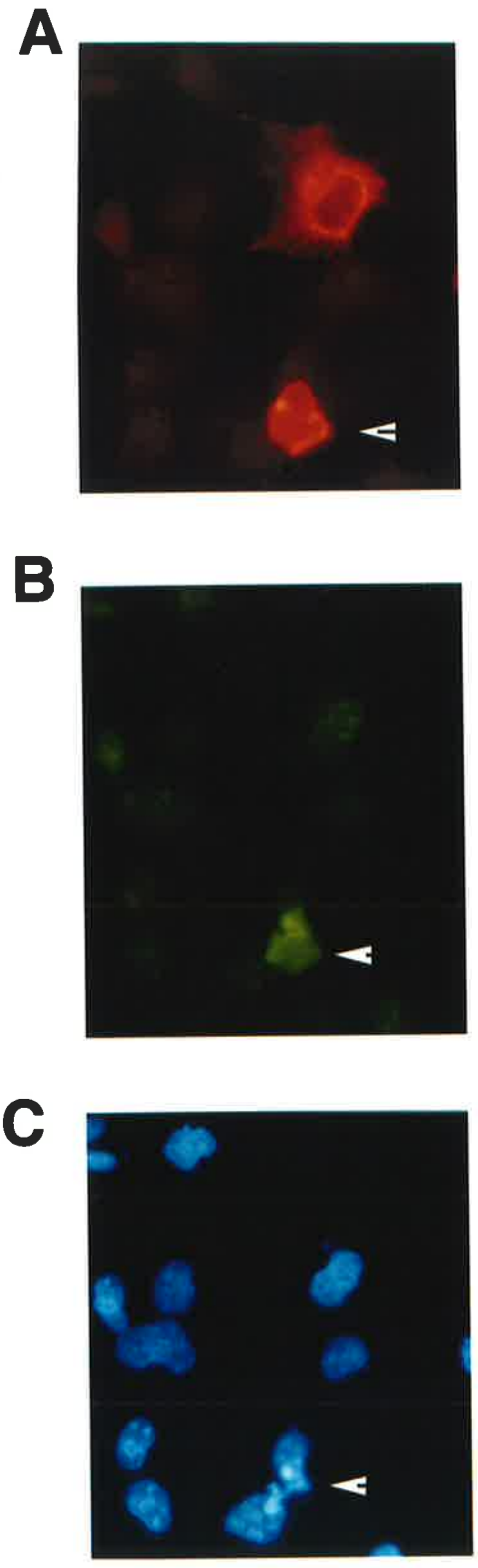

phLIF-TX
phLIF-MX
phLIF-DX
LIF
DNA

Figure 6.2 Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labelling (TUNEL) of phLIF-MX Transfected Cos 1 Cells

Cos 1 cells were transfected with phLIF-MX and stained for hLIF expression (A), oligo-nucleosomal DNA fragmentation (B) and DNA (C) using anti-hLIF antibodies, the TUNEL procedure, and Hoechst 33258 respectively. The same representative field of view is shown for each staining. Arrowheads indicate a cell staining for hLIF expression, nicked DNA ends and showing an apoptotic nuclear morphology (above plane of focus for other cells, between two healthy nuclei). Cos 1 cell transfections, hLIF immunolocalisation and TUNEL were performed as described in sections 2.4.2, 2.4.5 and 2.4.9 respectively. Magnification 80x.


A
B
C

membrane integrity is lost, and the nucleus condenses into chromatin aggregates and degenerates. The loss of phLIF-MX and phLIF-TX transfected cells to apoptotic cell death would provide an explanation for the eightfold reduction in numbers of LIF staining cells seen in these transfections when compared with phLIF-DX transfections. Furthermore, while these investigations were in progress, similar LIF staining patterns and nuclear morphologies suggestive of apoptosis were also observed in mLIF-T overexpressing Cos-1 cells (Haines, 1997). Use of the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) procedure (Gavrieli et al., 1992) confirmed that these mLIF-T overexpressing cells were undergoing apoptosis (Haines., 1997; Haines et al., *Submitted*).

6.2.2 Overexpression of Intracellular hLIF Proteins Induces Apoptosis in Cos 1 Cells

The TUNEL procedure was therefore used to determine whether cells overexpressing hLIF-M encoded proteins were undergoing apoptosis. phLIF-MX transfected cells were co-stained for LIF protein, oligo-nucleosomal DNA fragmentation, and DNA (Figure 6.2 A, B, and C). Cells with a cytoplasmic network of LIF staining showed no specific staining for oligo-nucleosomal DNA fragmentation and, as before, their nuclei appeared normal by Hoechst 33258 staining. However, rounded cells that stained uniformly for LIF showed specific staining for oligo-nucleosomal DNA fragmentation (arrow in Figure 6.2 B); exhibited nuclear morphologies consistent with apoptosis, including DNA aggregation (arrow in Figure 6.2 C); and resembled mLIF-T staining apoptotic cells. This finding provided the first indication that overexpression of intracellular hLIF proteins, like intracellular mLIF proteins, induced apoptosis in Cos 1 cells.

6.2.3 Apoptosis is Induced by Overexpression of Intracellular but not Extracellular hLIF Proteins and is Inhibited by CrmA but not Bcl2

The pathway of intracellular LIF induced apoptosis was investigated by co-transfection with replicating expression vectors for Bcl-2 (phBcl-2 RSV; Kumar et al., 1994) and the caspase inhibitor CrmA (pCXN2-CrmA; Niwa et al., 1991). The Bcl-2 and CrmA proteins inhibit distinct apoptotic pathways (Miura et al., 1995) and exert their effect at different levels in the signalling hierarchy regulating apoptosis (Hale et al., 1996; Jacobsen et al., 1997b). Cos 1 cells were co-transfected with hLIF expression vectors and either phBcl-2 RSV, pCXN2-CrmA, or pXMT2 at a

1:3 ratio and stained for hLIF proteins and DNA. In the case of cells overexpressing hLIF-D, the morphologies of the cells staining for LIF expression were as described above (section 6.2.1) and were not affected by the co-transfected plasmid (data not shown). Cells overexpressing hLIF-M, co-transfected with either pXMT2 or phBcl-2 RSV (Figure 6.3 A) showed the staining patterns and apoptotic morphologies noted above (sections 6.2.1 and 6.2.2), whereas cells co-transfected with pCXN2-CrmA (Figure 6.3 B) showed perinuclear, cytoplasmic LIF staining in the absence of nuclear staining for LIF. The nuclear morphology of cells co-transfected with phLIF-MX and pCXN2-CrmA appeared healthy and was identical to that of untransfected cells (Figure 6.3 B). In phLIF-MX and pCXN2-CrmA co-transfections the number of cells staining for LIF expression were also consistently increased by fivefold or more such that the numbers of LIF staining cells seen in these co-transfections were comparable to the numbers seen in phLIF-DX co-transfections. These effects were indicative of the rescue of apoptosing cells and antagonism of the apoptotic process by co-expression of the CrmA protein. Furthermore, since these effects were seen in co-transfections involving phLIF-MX vectors, which produce intracellular and secreted hLIF proteins, but not phLIF-DX vectors, which express secreted hLIF proteins, expression of intracellular hLIF proteins was found to correlate with the apoptotic effect.

Co-transfection with CrmA expression vectors thus confirmed that Cos 1 cells expressing intracellular LIF proteins were undergoing apoptosis. Moreover, it can be inferred that a specific apoptotic pathway was activated by expression of the intracellular LIF proteins since co-expression of CrmA, but not Bcl-2, was capable of antagonising the apoptotic process.

6.2.4 Quantifictation of Cos 1 Cell Apoptosis Induced by Overexpression of Intracellular LIF Proteins

To quantify the apoptotic effect of intracellular hLIF overexpression, a cell counting protocol was devised. To overcome the difficulties encountered in detection of hLIF-T encoded proteins by immunofluorescence (section 6.2.1), a β-galactosidase expression vector was co-transfected at a ratio of 5: 25: 70 with hLIF expression vectors and either pXMT2, phBcl-2 RSV or pCXN2-CrmA respectively. β-galactosidase expression was then used as a marker for hLIF expression. Counts of β-galactosidase positive cells were expressed as a ratio normalised to the counts obtained in the negative control (pXMT2) co-transfections termed the "apoptosis rescue

Figure 6.3 hLIF-Induced Apoptosis in Cos-1 Cells is Inhibited by Co-transfection with the Caspase Inhibitor CrmA but not Bcl-2

Cos 1 cells were co-transfected with phLIF-MX and either :

(A) pRSV-hBcl-2 (expression vector for the human Bcl-2 protein)

or

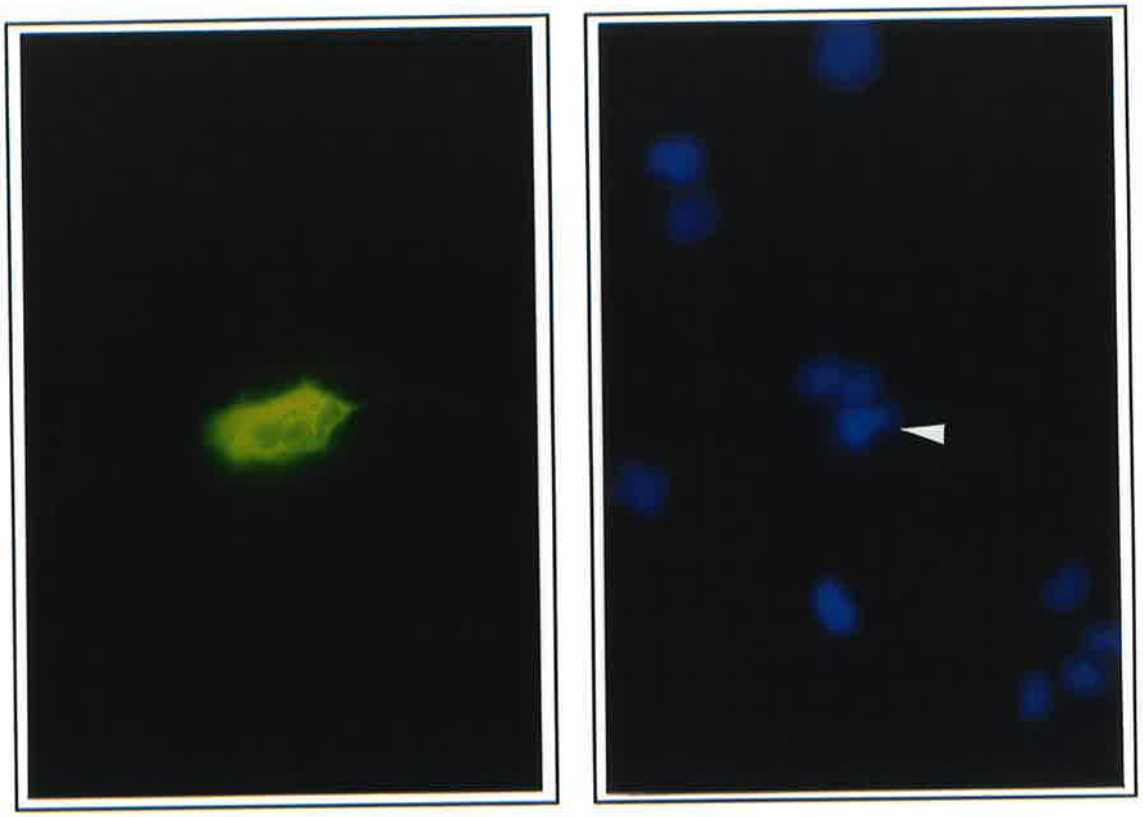
(B) pCXN2-CrmA (expression vector for the cowpox virus CrmA protein).

Representative fields of view showing anti-hLIF-GST (LIF) and Hoechst 33258 (DNA) staining are shown. The arrowhead in the DNA panel of *A* indicates the irregularly shaped nucleus of the LIF-staining cell. The ratio of co-transfected vectors was 1:3 (phLIF-MX : pRSV-hBcl-2 or pCXN2-CrmA). Transfection and immunolocalisation were performed as described in sections 2.4.2 and 2.4.5. Magnification 80x.

A

LIF

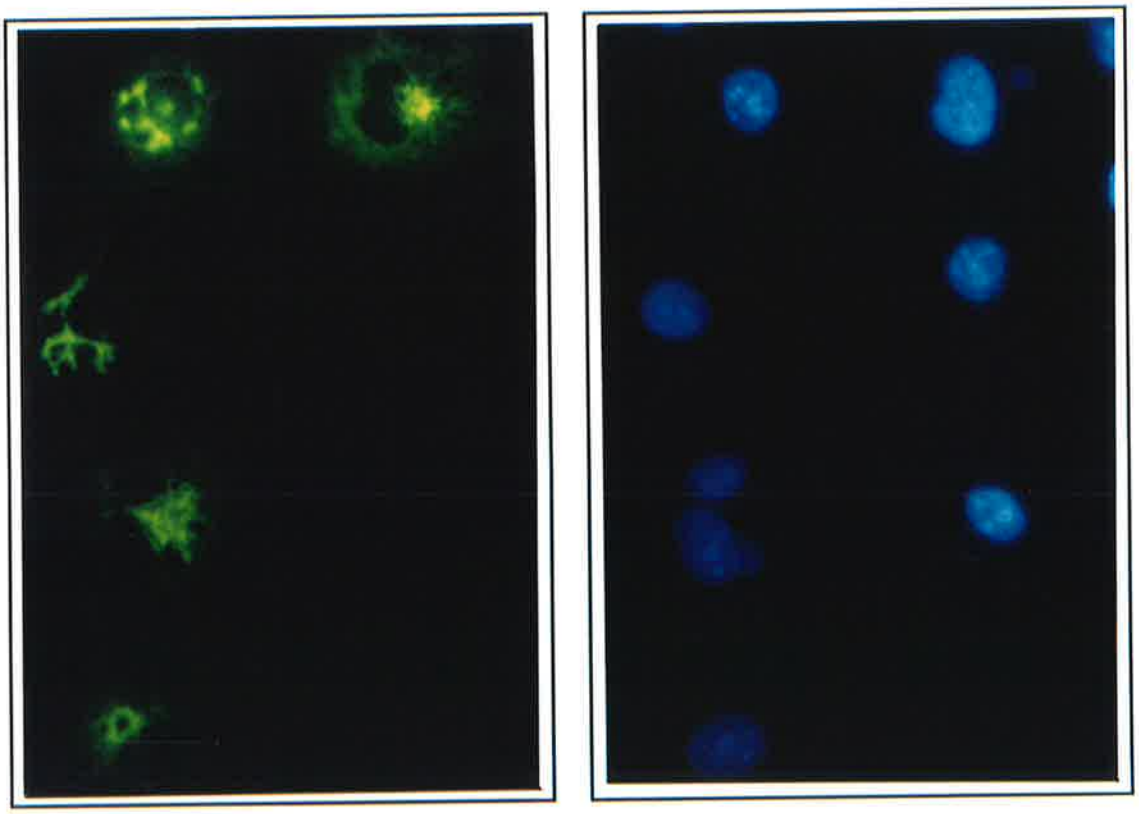
DNA



B

LIF

DNA



index". This was calculated for each hLIF expression vector by dividing the number of β-galactosidase positive cells seen in co-transfections with either phBcl-2 RSV or pCXN2-CrmA by the number of β-galactosidase positive cells seen in co-transfections with pXMT2. An apoptosis rescue index significantly greater than one for an hLIF expression construct therefore indicated that co-transfection with either phBcl-2 RSV or pCXN2-CrmA was rescuing cells from an apoptotic fate induced by expression of that particular construct.

The apoptosis rescue index of a range of hLIF expression vectors in co-transfections with pCXN2-CrmA and phBcl-2 RSV was determined in three replicate experiments. While the absolute apoptosis rescue indexes for each hLIF expression vector varied between experiments, the proportionality of apoptosis rescue indexes between vectors within each experiment remained highly consistent. Data from a representative experiment are shown in Figure 6.4. Co-transfection of vectors expressing the non-apoptotic proteins dihydrofolate reductase and alpha-1-4-galactosidase with pCXN2-CrmA yielded apoptosis rescue indexes of approximately 1.5 (Figure 6.4 A), indicating that CrmA expression rescued background apoptosis within the Cos 1 cell population. Co-transfections involving vectors from which intracellular hLIF proteins were expressed (phLIF-MX, phLIF-TX, and phLIF-T_KX; sections 5.2.3 and 5.2.11) and pCXN2-CrmA all had apoptosis rescue indexes of approximately 3.0 (Figure 6.4 A). CrmA expression was thus capable of rescuing Cos 1 cells expressing intracellular hLIF proteins from apoptotic cell death. This was further substantiated by the observation that co-transfections involving pCXN2-CrmA and phLIF-MA$^-$X, which did not produce intracellular hLIF proteins (section 5.2.6), had a mean apoptosis rescue index of only 1.2 (Figure 6.4 A). This confirmed that the production of intracellular hLIF proteins was necessary to induce the apoptotic effects of hLIF-M overexpression and that secreted hLIF proteins alone were unable to induce apoptosis. Co-transfections involving phLIF-DX and pCXN2-CrmA consistently yielded apoptosis rescue indexes of 2.0 or more whereas co-transfections involving phLIF-DA$^-$X and pCXN2-CrmA had apoptosis rescue indexes equivalent to those of constructs expressing negative control proteins. This suggested that low levels of intracellular hLIF proteins can be translated from the exon 2 AUG of the hLIF-D transcript and that these intracellular hLIF proteins were inducing a modest apoptotic effect in this system.

Figure 6.4 Apoptosis Rescue Indices of hLIF Expression Vectors in Transfected Cos 1 Cells

The β–galactosidase expression vector pCH110 (Pharmacia) was co-transfected at a ratio of 5: 25: 70 with hLIF expression vectors and pXMT2, phBcl-2 RSV or pCXN2-CrmA respectively. β-galactosidase expression was then used as a marker for hLIF expression. Counts of β-galactosidase positive cells were normalised to the counts obtained in the negative control (pXMT2) co-transfections by calculation of an "apoptosis rescue index". This was calculated for each hLIF expression vector by dividing the number of β-galactosidase positive cells seen in co-transfections with either phBcl-2 RSV or pCXN2-CrmA by the number of β-galactosidase positive cells seen in co-transfections with pXMT2. An apoptosis rescue index significantly greater than one for an hLIF expression construct therefore indicates that co-transfection with either phBcl-2 RSV or pCXN2-CrmA was rescuing cells from an apoptotic fate induced by expression of that construct.

Co-transfection assays are described in detail in section 2.4.10. Surviving transfected cells were stained for β-galactosidase activity as described in section 2.4.4. DHFR = dihydrofolate reductase (non-apoptosis inducing protein expressed from the pXMT2 negative control vector). αGal = a1-6-galctosidase expression vector (second pXMT2-based negative control vector).

(A) Apoptotic rescue indices of vectors when co-transfected with pCXN2-CrmA taken from a representative experiment of three performed.

(B) Apoptotic rescue indices of vectors when co-transfected with phBcl-2 RSV taken from a representative experiment of three performed.

A

Of Blue Cells Wiith Test cDNA + CrmA
Of Blue Cells Wiith Test cDNA + DHFR
0
1
2
3
4
5
DHFR
αGal
hLIF-D
hLIF-D(ATG⁻)
hLIF-M
hLIF-M(ATG⁻)
hLIF-T
hLIF-TK
Test cDNA

B

Of Blue Cells Wiith Test cDNA + Bcl 2
Of Blue Cells Wiith Test cDNA + DHFR
0
1
2
3
4
5
DHFR
αGal
hLIF-D
hLIF-D(ATG⁻)
hLIF-M
hLIF-M(ATG⁻)
hLIF-T
hLIF-TK
Test cDNA

Co-transfection of either control plasmids or hLIF expression constructs with phBcl-2 RSV all had apoptosis rescue indexes of approximately one (Figure 6.4 B). This confirmed that Bcl-2 expression was unable to antagonise either apoptosis induced by overexpression of intracellular hLIF proteins or the lower levels of background apoptosis occurring in Cos 1 cells.

6.2.5 Cos 1 Cells Accumulate Intracellular hLIF Proteins When Apoptosis is Inhibited by CrmA Co-transfection

To provide further confirmation that CrmA antagonised apoptosis induced by intracellular hLIF protein, accumulation of intracellular hLIF proteins from co-transfected cell populations was monitored by immunoprecipitation. Cos 1 cells were co-transfected with hLIF expression vectors and either pXMT2 or pCXN2-CrmA, and whole cell lysates were immunoprecipitated with anti-hLIF-GST three days post-transfection.

In the cases of co-transfections involving vectors that expressed predominantly secreted hLIF proteins (phLIF-DX, phLIF-DA$^-$X, and phLIF-MA$^-$X) and either pXMT2 or pCXN2-CrmA, similar levels of secreted hLIF glycoproteins were immunoprecipitated from the cell lysate (Figure 6.5). In the case of co-transfections involving hLIF expression vectors from which intracellular hLIF proteins were produced (phLIF-MX, phLIF-TX, and phLIF-T_KX) and pXMT2 or pCXN2-CrmA, levels of intracellular hLIF proteins accumulated to levels three- to five-fold higher in co-transfections with pCXN2-CrmA than in co-transfections involving pXMT2 (Figure 6.5). This accumulation closely paralleled the increase in numbers of staining cells seen in pCXN2-CrmA co-transfections by β-galactosidase marking and indicated that antagonism of apoptosis by CrmA allowed the accumulation of intracellular LIF proteins to levels that would otherwise induce cell death.

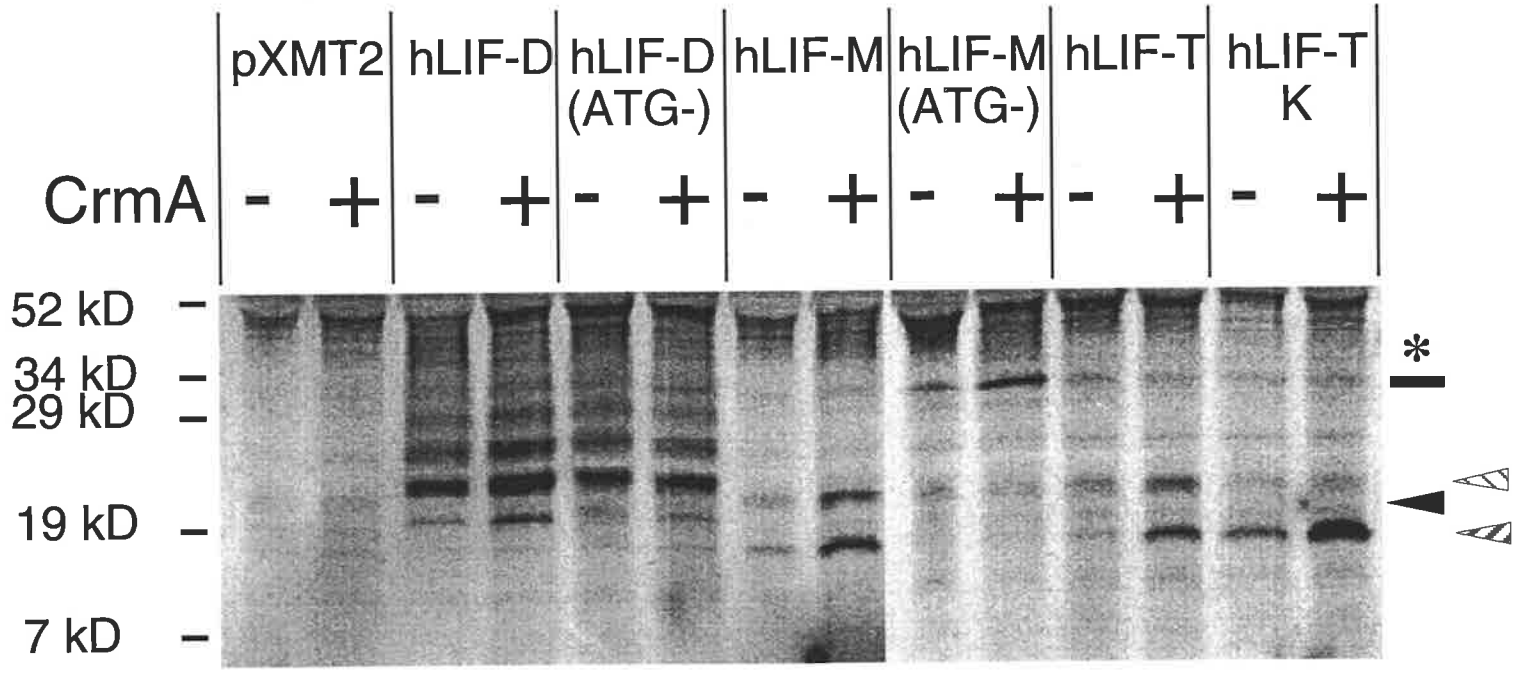
6.2.6 Human 293T Cells Overexpressing Intracellular but not Extracellular hLIF Proteins Undergo Apoptosis that is Antagonised by CrmA

In order to investigate the generality of these findings, human embryonic kidney 293T cells were transfected with expression vectors for intracellular hLIF proteins to determine whether this resulted in apoptosis that could be antagonised by CrmA. Since human 293T cells (Pear et al., 1993; Graham et al., 1977) and African green monkey Cos cells (Gluzman, 1981) are both

Figure 6.5 Accumulation of Intracellular hLIF Proteins in Cos 1 Cells Co-transfected with hLIF and CrmA Expression Vectors

Cos 1 cells were co-transfected with the indicated hLIF expression vectors and pCXN2-CrmA (+) (CrmA expression vector) or pXMT2 (-) (control). Immunoprecipitation of cell lysates was performed three days post-transfection as described in section 2.4.7. The ratio of co-transfected vectors was 1:3 (hLIF expression vector : pCXN2-CrmA or pXMT2).

The 20+ kD secreted proteins encoded by hLIF-D and hLIF-D(ATG$^-$) are indicated by a solid arrowhead. An asterisk indicates the 45 kD secreted proteins encoded by hLIF-M and hLIF-M(ATG)$^-$. A horizontal bar indicates immunoprecipitation of an intracellular protein in hLIF-M(ATG)$^-$ cells which results from translational initiation at an in-frame AUG codon present in pXMT2. The 17 and 23 kD intracellular proteins encoded by the hLIF-M, hLIF-T , and hLIF-TK are indicated by thickly and thinly cross-hatched arrowheads respectively.


pXMT2
hLIF-D
hLIF-D (ATG-)
hLIF-M
hLIF-M (ATG-)
hLIF-T
hLIF-T K
CrmA
- + - + - + - + - + - + - +
52 kD
34 kD
29 kD
19 kD
7 kD
*

competent for SV40 replication, the pXMT2-based expression vectors constructed during these investigations could also used for hLIF overexpression in 293T cells. Co-transfections of hLIF expression vectors with either pXMT2, phBcl-2 RSV, or pCXN2-CrmA were performed and stained for hLIF proteins and DNA two days post-transfection. In co-transfections involving phLIF-DX, the numbers, morphologies, and LIF staining patterns of cells staining for hLIF expression were very similar in cells co-transfected with either pXMT2 or pCXN2-CrmA (data not shown). However, when the phLIF-MX expression vector was co-transfected with pCXN2-CrmA (Figure 6.6 B), the number of cells staining for hLIF expression was up to eight-fold higher than in co-transfections with pXMT2 or phBcl-2 RSV (Figure 6.6 A). As was the case with Cos 1 cells (Figure 6.4 B), the majority of cells staining for LIF expression in the phLIF-MX, pCXN2-CrmA co-transfections showed cytoplasmic LIF staining and had nuclei that appeared healthy (Figure 6.6 B). In contrast, all cells staining for LIF expression in the phLIF-MX, pXMT2 co-transfections were rounded, stained uniformly for LIF protein and had abnormal nuclear morphologies that indicated that they were apoptotic. It was thus apparent that expression of intracellular hLIF proteins in the human 293T cell line also induced apoptosis that could be antagonised by CrmA expression.

6.3 Discussion

6.3.1 Alternate Subcellular Localisation of Secreted and Intracellular hLIF Proteins

Immunoprecipitation analysis (section 5.2.3) revealed that the alternate hLIF transcripts encoded two classes of proteins: secreted glycoproteins and truncated proteins that were retained within the cell. The immunofluorescence studies reported here further emphasise the distinct nature of these two classes of proteins. The cytoplasmic network of staining seen in hLIF-D and some hLIF-M overexpressing cells suggested that the secreted proteins produced from these transcripts were localised to the Golgi apparatus and endoplasmic reticulum, consistent with the finding that monensin, an inhibitor of the classical Golgi apparatus-mediated secretory pathway, is able to inhibit the secretion of hLIF-D and hLIF-M encoded proteins. It was, however, found that the hLIF-T expressing cells all resembled mLIF-T expressing cells (Haines, 1997; Haines et

al., *Submitted*) in that there was a uniform nuclear-cytoplasmic distribution of LIF protein. Two days after transfection, half the cells expressing hLIF-M exhibited this LIF staining pattern and their numbers increased with time post transfection. This uniform nuclear-cytoplasmic distribution of the hLIF-M and hLIF-T intracellular proteins was distinct from that of the hLIF-D secreted proteins which were localised within a cytoplasmic network and never detected in the nucleus. Thus, the human and murine intracellular LIF proteins appeared to have equivalent subcellular distributions.

Concurrently, it was established using laser scanning confocal microscopy that the intracellular mLIF-T proteins co-localised with nuclear DNA staining in transfected Cos cells (Haines et al., *Submitted*). Indeed, mutagenesis experiments had previously suggested that nuclear localisation of intracellular mLIF proteins may be required for their induction of apoptosis (Haines et al., submitted; discussed in Chapter 8). Moreover, co-expression of CrmA abolishes the nuclear component of LIF staining normally seen in cells expressing intracellular LIF proteins (Figures 6.3 and 6.6) and this correlates with inhibition of apoptosis. The nuclear-cytoplasmic distribution of the intracellular mLIF proteins thus seems to be functionally important. Nuclear localisation of intracellular LIF could occur by passive diffusion through the nuclear pore complex, which is permeable to molecules of up to 45 kD (Jans and Hassan, 1998), or by active import requiring nuclear localisation sequences. A conserved sequence (RKK in mouse and rat, KKK in other sequenced LIF genes) with homology to p53 and Max nuclear localisation sequences (Boulikas, 1993) is located at the C-terminus of the LIF protein, within a region required for receptor interaction and could serve as an intrinsic nuclear localisation sequence. Alternatively, intracellular LIF could be transported to the nucleus by interaction with another protein containing a nuclear localisation sequence.

6.3.2 Intracellularly Localised hLIF Proteins Exhibit a Novel Activity

Staining of phLIF-MX and phLIF-TX transfected cells using the TUNEL procedure demonstrated that, as with mLIF-T overexpressing cells (Haines et al., submitted), a high proportion of cells exhibited the type of DNA fragmentation associated with apoptosis. Furthermore, cells showing strong staining for oligo-nucleosomal DNA fragmentation also showed an apoptotic nuclear morphology when stained with the DNA-specific Hoechst 33258

Figure 6.6 **hLIF-Induced Apoptosis in Human Embryonic Kidney 293T Cells is Inhibited by Co-transfection with the Caspase Inhibitor CrmA but not Bcl-2**

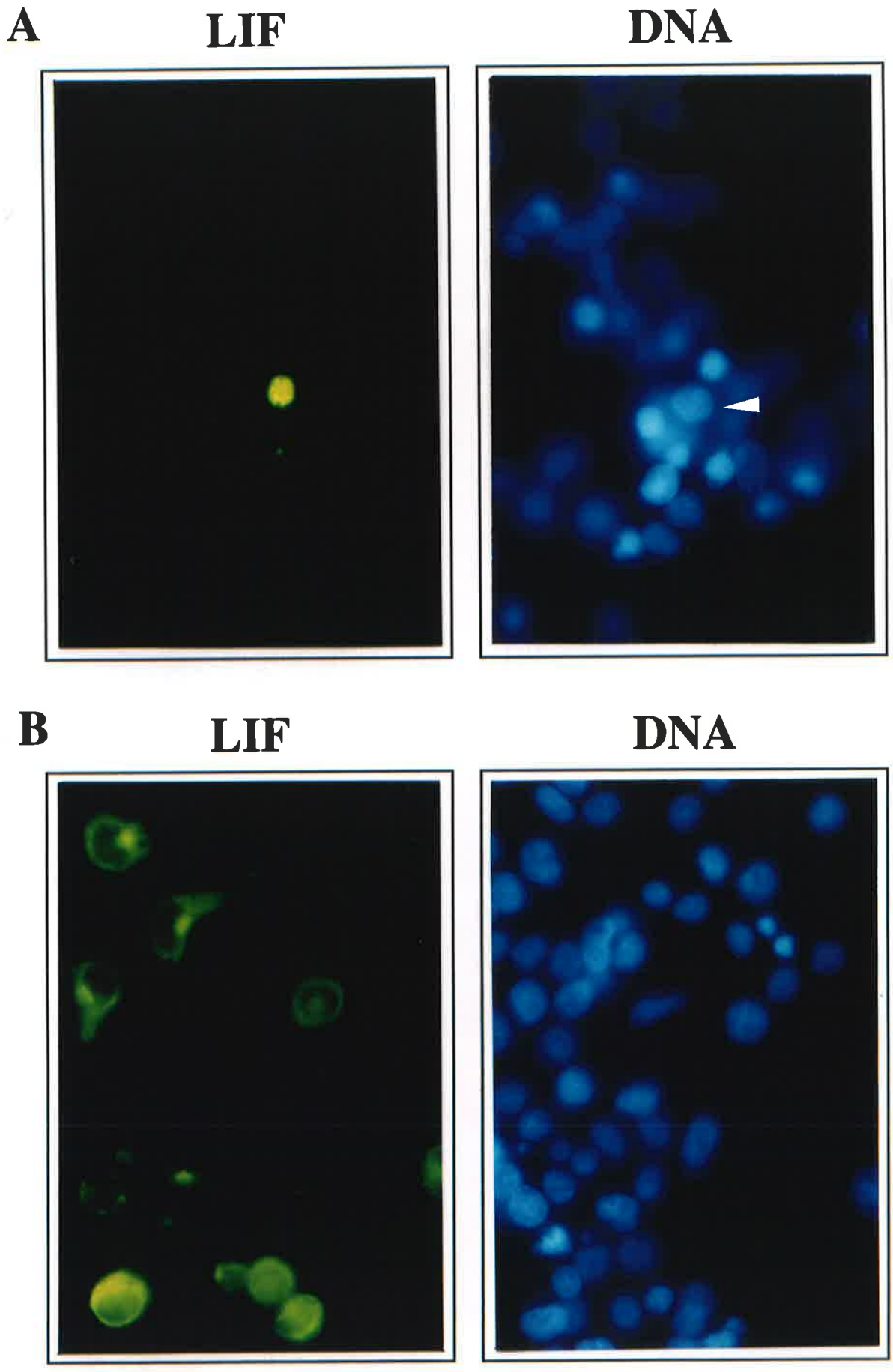
Human embryonic kidney 293T cells were co-transfected with phLIF-MX and either:

(A) pRSV-hBcl-2 (expression vector for the human Bcl-2 protein)

or

(B) pCXN2-CrmA (expression vector for the cowpox virus CrmA protein).

Representative fields of view showing anti-hLIF-GST (LIF) and Hoechst 33258 (DNA) staining are shown. An arrowhead indicates the nucleus of the LIF staining cell in *A*. The ratio of co-transfected vectors was 1:3 (phLIF-MX : pRSV-hBcl-2 or pCXN2-CrmA). $CaPO_4$-mediated transfection was performed as described in section 2.4.3 and immunolocalisation was performed as described in section 2.4.5. Magnification 80x.


A
LIF
DNA
B
LIF
DNA

dye. These cells contained either intensely staining, irregularly shaped aggregates of DNA or completely lacked DNA staining. LIF staining revealed that these cells were also shrunken and rounded. The number of cells staining in transfections involving vectors that expressed intracellular hLIF proteins were also much lower than in transfections involving vectors that expressed extracellular hLIF proteins. Taken together, these observations indicated that cells overexpressing intracellular LIF proteins were undergoing a classical apoptotic cell death process.

Co-transfection of hLIF expression vectors and expression vectors for the apoptosis inhibiting proteins Bcl-2 and CrmA confirmed that intracellular hLIF proteins were inducing a specific apoptosis when overexpressed in Cos 1 cells. In co-transfections involving hLIF expression vectors that induced apoptosis, the number and morphology of cells staining for LIF expression was unaffected when the co-transfected plasmid was a Bcl-2 expression vector or a negative control vector. However, when the co-transfected plasmid was a CrmA expression vector, a large increase in the number of staining cells was seen and these cells exhibited a morphology similar to that of hLIF-D overexpressing cells. Apoptosis induced by overexpression of intracellular hLIF proteins was thus antagonised by CrmA but not Bcl-2. CrmA is a serine protease inhibitor encoded by the cowpox virus genome and is capable of inhibiting several caspases required for the induction of specific apoptotic processes (Ray et al., 1992, Komiyama et al., 1995). The apoptotic pathway inhibited by CrmA is that downstream of the "death domain" receptors such as the tumour necrosis factor (Miura et al., 1995) and Fas (Longthorne et al., 1997) receptors. In contrast, Bcl-2 can inhibit apoptosis induced by a range of "stress" stimuli that CrmA cannot, including apoptosis induced by ionising radiation and dimethyl sulphoxide (Jacobsen et al., 1997b). These findings thus associated intracellular hLIF protein expression with apoptosis occurring via a specific pathway rather than as a response to toxicity and cellular stress. The accumulation of intracellular, but not secreted, hLIF proteins seen in immunoprecipitates from cells co-transfected with CrmA indicated that cells expressing CrmA could tolerate levels of intracellular hLIF proteins that would normally induce apoptosis. Co-transfection of CrmA also altered the subcellular distribution of intracellular hLIF proteins in Cos 1 and 293T cells such that LIF staining appeared to be exclusively cytoplasmic and was concentrated in the perinuclear region. This suggested that the antagonism of apoptosis induced

by intracellular hLIF proteins by CrmA may involve sequestration of intracellular LIF proteins and prevention of their entry to the nucleus.

Two days after transfection significant numbers of hLIF-M expressing cells exhibited a cytoplasmic network of LIF staining and healthy nuclear morphologies. This was unexpected given that these cells express high levels of apoptosis inducing intracellular hLIF proteins in addition to secreted hLIF proteins as assessed by immunoprecipitation (section 5.2.3). This staining pattern presumably reflects accumulation of secreted hLIF proteins in these cells and could be the result of translational bias toward production of secreted proteins or their stabilisation under some cellular circumstances. Regardless, the number of LIF-M staining cells exhibiting apoptotic morphologies and LIF staining patterns increased with time post-transfection, perhaps as a result of accumulation of intracellular LIF proteins (and apoptosis) in cells that previously showed the cytoplasmic staining pattern.

Co-transfections with CrmA also revealed that the numbers of cells staining for LIF expression were being reduced by induction of apoptosis in cells transfected with vectors expressing intracellular hLIF proteins. This reduction is likely to result from the loss of attachment of apoptotic cells either in culture or during the staining procedure and is an effect noted in several other studies of apoptotic cell systems (Chinnaiyan et al., 1995; Hsu et al., 1995; Kumar et al., 1994).

Transfection of human embryonic kidney 293T cells with expression vectors for intracellular hLIF proteins also induced an apoptotic cell death that was antagonised by CrmA expression and appeared to be similar to the apoptosis induced in Cos cells. This demonstrated that the apoptotic effect was not confined to Cos cells and also that it occurred in human cells - the species from which the expressed LIF cDNAs were originally isolated. A greater increase in the number of staining cells rescued in co-transfections with CrmA expression vectors was seen in 293T cells than in Cos 1 cells (eightfold as opposed to fivefold). Among other possibilities, this might indicate that human LIF proteins are more potent inducers of apoptosis in human cells than in cells from other species.

Cos 1 or 293T cells overexpressing only secreted hLIF proteins are not induced to apoptose and neither are Cos 1 cells expressing a truncated mLIF-T protein that is secreted because it has been engineered with a signal peptide and signal peptidase cleavage site (Haines,

1997; Haines et al., *Submitted*). However, Cos 1 cells expressing a non-secreted mLIF-D protein lacking a signal peptide are induced to levels of apoptosis similar to those seen in cells expressing the intracellular mLIF-T protein (Haines, 1997, Haines et al., *Submitted*). These observations indicate that the distinct activities of the intracellular and extracellular LIF proteins are a consequence of their different cellular localisations and not of differences between the proteins themselves.

6.3.3 The Significance of Intracellular LIF-Induced Apoptosis

The relevance of LIF-T induced apoptosis to *in vivo* LIF function depends on the levels at which the protein is expressed in individual cells. If the intracellular LIF protein is expressed at low levels throughout the tissues in which LIF-M and LIF-T transcripts are detected (Robertson et al., 1993; Haines et al., 1999; Voyle et al., 1999), then the low expression levels would suggest that apoptosis induced by overexpression may not be relevant. However, if expression is confined to a small subset of cells within a tissue, then the expression level in individual cells may be comparable to those experienced in *in vitro* overexpression experiments. Clarification of these issues will require that the cellular sites and levels of LIF-M and LIF-T transcript and, particularly, protein expression are determined. With this in mind, it is interesting to note that the spleen and thymus are significant sites of LIF transcript expression (Robertson et al., 1993) and also harbour large populations of lymphocytes undergoing apoptosis (Jacobsen et al., 1997a).

Apoptosis induced by overexpression of intracellular LIF proteins may occur by a direct or indirect mechanism. Intracellular LIF proteins could interact with a cytoplasmic apoptotic pathway causing its activation or they may interact directly with transcription factors that control an apoptotic response. Alternatively, the excess of intracellular LIF-T caused by overexpression may induce interaction with molecules that are not usually targets for LIF-T interaction. This may interrupt essential cellular pathways resulting in the cell becoming compromised and the indirect induction of apoptosis. The latter possibility seems less likely given that non-replicating mLIF-T expression vectors, which express at much lower levels, also induced apoptosis of transfected Cos 1 cells (Haines, 1997; Haines et al., *Submitted*). Regardless, intracellularly localised LIF proteins are capable of interaction with intracellular signalling pathways to produce a biological outcome in a manner that is distinct from the secreted LIF proteins.

6.3.4 Mode of Action of the Intracellular LIF Protein

Intracellular LIF appears to interact with and activate a cytoplasmic or nuclear signalling pathway that culminates in a cellular response. However, the outcome of this signalling need not necessarily always be apoptosis since the intensity and duration of the signal and the molecular constitution of the responding cell may influence the outcome of this signalling. The actions of intracellular LIF proteins, like extracellular LIF proteins, are likely to be pleiotropic. For example many intracellular regulatory molecules such as *myc* proteins have been implicated in a wide range of cellular events that are dependent on the identity of the expressing cell and its environment (Vastrik et al., 1994). In the cases of fibroblast growth factor 2 (Imamura et al., 1990; Wiedlocha et al., 1996, Arnaud et al., 1999), and Schwannoma-derived growth factor (Kimura et al., 1990), localisation of the cytokine to the nucleus is required to elicit a mitogenic response. In both these cases, activities mediated by receptor binding and by nuclear localisation of the ligand can be distinguished. The demonstration that IL-6 antisense oligonucleotides but not anti-IL-6 neutralising antibodies inhibit cellular proliferation has been interpreted as evidence for intracellular IL-6 activity, that is distinct from the activity of extracellular IL-6 (Barut et al., 1993; Lu & Kerbel et al., 1993; Roth et al., 1995; Kong et al., 1996). These observations are supported by the identification in peripheral blood mononuclear cells of a splice variant of the hIL-6 transcript encoding a predominantly intracellular protein with a disrupted signal sequence (Kestler et al., 1995). Furthermore, intracellular action of the hLIF protein has been suggested as an explanation for a transcriptional response in Hep3B cells that is resistant to neutralising antibodies (Baumann et al., 1993).

A putative protein-protein interaction domain has been identified in the intracellular mLIF protein and implicated in the induction of apoptosis by this protein (Haines, 1997; Haines et al., *Submitted*) (discussed in Chapter 8). This domain contains a conserved repeat of leucine residues that are well separated from regions of the LIF protein implicated in receptor binding. Mutation of the conserved leucines in this domain has shown that they are required for intracellular LIF-mediated apoptosis but not for extracellular LIF-mediated ES cell maintenance (Haines, 1997; Haines et al., *Submitted).* The identification of this domain and the discovery that it operates

independently of and is separable from the receptor binding domain has provided a first step in the elucidation of the mechanism underlying intracellular LIF action. Thus, signalling by intracellular hLIF proteins could independently augment and/or supplement signalling by extracellular hLIF proteins in a cell-autonomous fashion. Such signalling could play a role in the de-regulation of self-renewal processes seen during tumourigenesis in stem cell populations as well as in biologically important processes occurring in normal cells.

Chapter 7 :

Alternate Transcription of the LIF-Related Cytokine Oncostatin M

7.1 Introduction

Like LIF, oncostatin M (OSM) (Bruce et al., 1992) is a member of the structurally and functionally-related IL-6 cytokine family. Expression mapping and analysis of OSM bioactivity *in vitro* and *in vivo* suggest that this protein fulfils diverse biological roles. OSM is expressed in tissues of the haematopoietic system and in cells of haematopoietic origin (Malik et al., 1989; Yoshimura et al., 1996), and can act as a maintenance factor for a haematopoietic / endothelial cell precursor and for early definitive haematopoiesis (Mukouyama et al., 1998; Xu et al., 1998). Exogenous OSM can also stimulate an extra-thymic lymphopoietic pathway in adult mice (Clegg et al., 1996). Early embryonic gonads and Sertoli cells express OSM and OSM induces proliferation of post-migratory primordial germ cells *in vitro*, suggesting a function for this cytokine in germ cell development (Hara et al., 1998). OSM is also expressed in cartilage primordia and the nervous system during embryogenesis (Hara et al., 1998), and as an immediate early gene in response to cytokine stimulation of haematopoietic cell lines (Yoshimura et al., 1996). *In vitro*, OSM has been shown to induce proliferation of diverse tumour-derived cell lines (Horn et al., 1990), and maintain the pluripotency of murine embryonic stem cells (Hara et al., 1997; Rose et al., 1994).

Of the IL-6 family cytokines, OSM is most closely related to LIF in terms of its structure and function (Kitchen et al., 1998; Piquet-Pellorce et al., 1994; Robinson et al., 1994). The *Osm* and *Lif* loci are tightly linked on human chromosome 22 (Giovannini et al., 1993) and mouse chromosome 11 (Yoshimura et al., 1996), and the human loci are separated by only 18 kb (Jefferey et al., 1993). Evolutionary comparisons of the OSM and LIF cDNA sequences suggest that they arose through the recent duplication of an ancestral gene (Shields et al., 1995). There are also strong similarities in the exon-intron structure of the cloned human, murine and bovine OSM and LIF genes (Kato et al., 1995; Malik et al., 1995; Malik et al., 1989; Stahl et al., 1990; Yoshimura et al., 1996).

OSM and LIF have distinct but overlapping biological activities (Piquet-Pellorce et al., 1994) that at least partly reflect the distribution of different ligand-selective receptor subunits. While both LIF and human OSM can signal through a complex of the gp190 LIF receptor and gp130 (Gearing et al., 1992), human OSM can also activate a gp130/OSM receptor-β complex (Mosley, 1996). Mouse OSM does not interact with mouse gp190 but can signal through a

complex containing the murine gp130 and the c12 proteins (Ichihara et al., 1997; Lindberg et al., 1998). The murine c12 protein is 55% identical to human OSM receptor-β (Ichihara et al., 1997; Lindberg et al., 1998).

There is accumulating evidence for alternate transcription, compartmentalisation and activity of IL-6 family cytokines. Ciliary neurotrophic factor and cardiotrophin-1 are translated from transcripts that do not encode a secretory signal and appear to be localised within the cell (Lin et al., 1989; Pennica et al., 1995). A differentially spliced IL-6 transcript encodes a predominantly intracellular protein (Kestler et al., 1995) that could provide an explanation for the cell surface receptor-independent IL-6 signalling seen in a variety of cell systems (Barut et al., 1993; Lu & Kerbel, 1993; Roth et al., 1995; Kong et al., 1996). Analysis of LIF gene expression and organisation (Haines et al., 1999), presented in Chapters 3 and 4, identified alternate transcripts encoding intracellular proteins which were functionally distinct from secreted LIF proteins (Chapters 5 and 6; Haines et al., *Submitted*). Intracellular signalling by LIF proteins has also been suggested to underlie cell surface receptor-independent gene activation in hepatocarcinoma cells (Baumann et al., 1993). Finally, intracellular stores of preformed human OSM have been identified in polymorphonuclear neutrophils (Grenier et al., 1999). The molecular basis for the production of the intracellular OSM stores has not been determined.

The investigations of LIF gene transcription described in this thesis and elsewhere (Rathjen et al., 1990a; Haines et al., 1999), considered together with the close relationship between the LIF and OSM genes suggested that OSM transcripts might also undergo alternate regulation and processing. Since the human OSM cDNA clone was not readily obtainable, investigations of mouse OSM transcription were carried out using ribonuclease protection assay and RACE-PCR approaches similar to those employed in investigations of LIF expression. These studies revealed an abundant, alternately spliced transcript, mOSM 13, containing the first exon of mOSM spliced directly to the third exon. The mOSM 13 transcript showed regulated expression indicative of biological significance and was expressed at high levels in mouse bone marrow and spleen. Furthermore, structural considerations suggested that a potential translation product of the mOSM 13 transcript might be retained intracellularly and encode a naturally occurring antagonist of OSM signalling.

7.2 Results

7.2.1 mOSM Transcript Expression and Processing

Differential protection of riboprobes was used to investigate the expression, regulation and processing of the mouse OSM transcript. As previously characterised (Yoshimura et al., 1996), the murine OSM gene produces a single transcript consisting of three sequentially spliced exons (Figure 7.1 A). A riboprobe (Figure 7.1B) spanning the 5' end and all three exons of the mOSM transcript was generated from the plasmid pmOSM 123*ET* (section 2.3.20) and used to detect expression of mOSM mRNA in a variety of tissues and cell culture systems (Figure 7.2). The characterised mOSM transcript, containing exons 1, 2, and 3, was detected at highest levels in the bone marrow, spleen, and thymus samples. These tissues all harbour populations of haematopoietic and lymphopoietic cells and have been reported previously to express OSM transcripts (Yoshimura et al., 1996). Lower levels of this transcript were also detected in 16 day post-coitum embryos, D3 embryonic stem cell embryoid bodies eight days after seeding, both of which contain haematopoietic precursor populations. D3 embryonic stem cells and Nulli SCCI murine embryonal carcinoma cells also expressed mOSM transcripts. In contrast to the widespread distribution of LIF transcripts (Robertson et al., 1993), OSM transcripts could not be detected outside haematopoietic and lymphoid adult tissues (Figure 7.2).

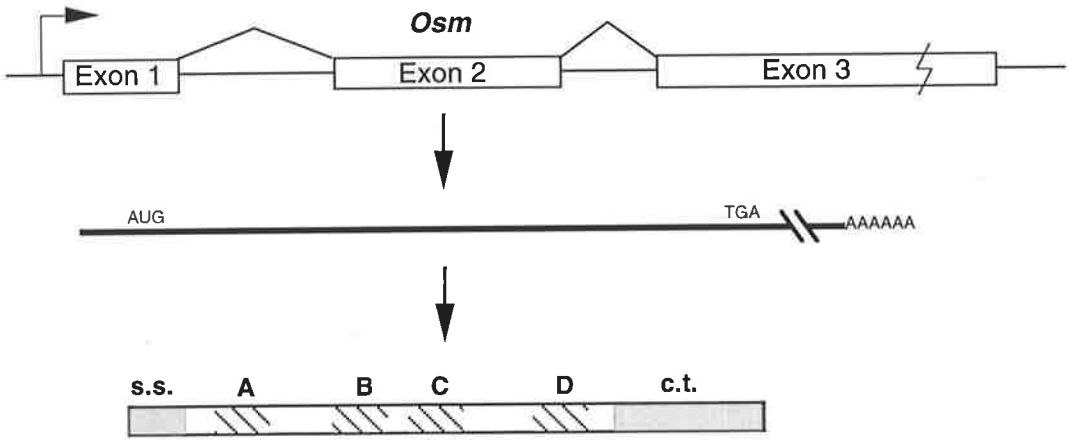
Most interestingly, additional smaller digestion products were visible in these assays (Figure 7.2) and corresponded in size to products predicted to result from protection of mOSM exons separately or in combination (Figure 7.1B). These digestion products were present at similar levels to the characterised mOSM transcript in tissues such as bone marrow and spleen where the levels of mOSM expression appear to be biologically relevant (Malik et al., 1989; Yoshimura et al., 1996). The variable abundance of these species in different samples (compare bone marrow and spleen; Figure 7.2) suggested that they arose from protection of novel alternate transcripts and were not artefactual. It thus appeared that the novel mOSM transcripts were regulated in a tissue-specific manner, suggesting that they were likely to be functionally distinct and biologically significant. The protection pattern observed could be explained by the existence of uncharacterised mOSM transcripts containing combinations of exons 1 and 2, exons 1 and 3, and exons 2 and 3.

Figure 7.1 The mOSM Gene and Digestion of an mOSM Specific Riboprobe.

(A) Schematic showing exonic organisation of the OSM gene, splicing of the OSM transcript, and salient features of the OSM protein. Initiation of translation at the exon 1 AUG of the mOSM transcript produces a secreted glycoprotein containing four αhelices (Yoshimura et al., 1996). s.s., secretion signal sequence; A, B, C, D, predicted α helical regions of mOSM (Kitchen et al., 1998); c.t., C-terminal region cleaved during maturation by specific proteolysis (Linsley et al., 1990).

(B) Origin and sizes of possible ribonuclease digestion products arising from the hybridisation of riboprobes generated from pmOSM 123*ET* to various combinations of the three characterised exons of the mOSM transcript (Yoshimura et al., 1996)

A


Osm
Exon 1
Exon 2
Exon 3
AUG
TGA
AAAAAA
s.s.
A
B
C
D
c.t.

B

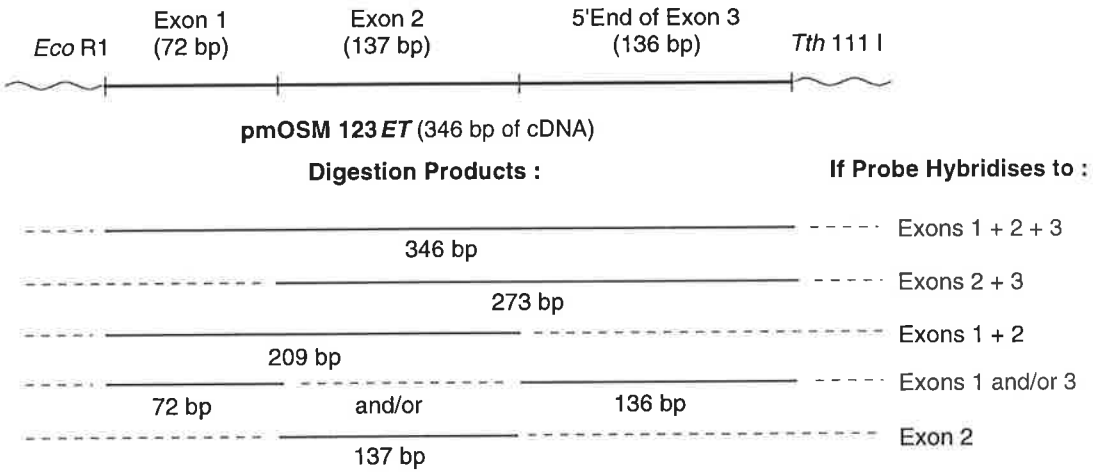
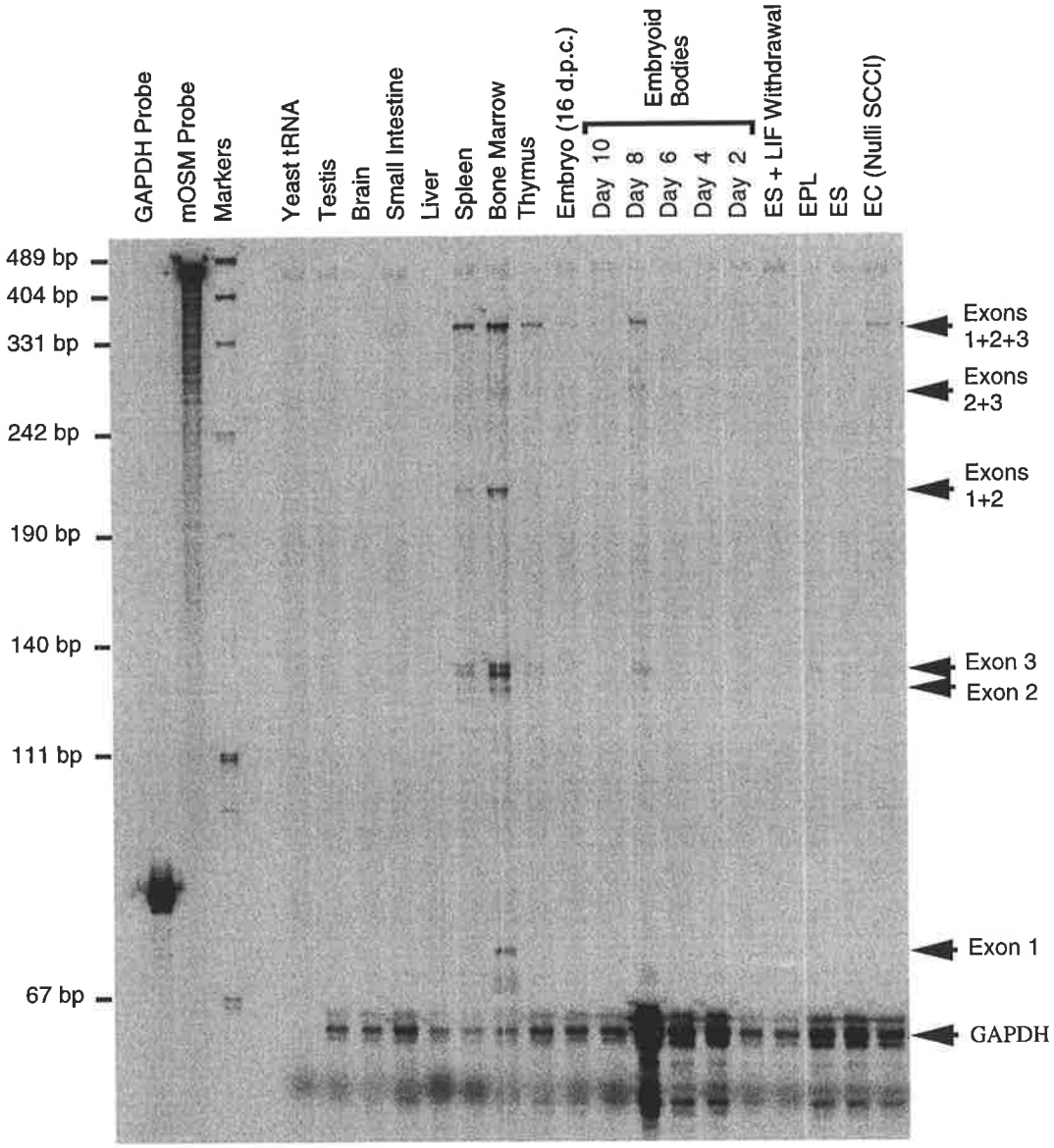

Exon 1
(72 bp)
Exon 2
(137 bp)
5'End of Exon 3
(136 bp)
Eco R1
Tth 111 I
pmOSM 123ET (346 bp of cDNA)
Digestion Products :
If Probe Hybridises to :
346 bp
Exons 1 + 2 + 3
273 bp
Exons 2 + 3
209 bp
Exons 1 + 2
72 bp
and/or
136 bp
Exons 1 and/or 3
137 bp
Exon 2

Figure 7.2 Expression of Alternate mOSM Transcripts *In Vivo* and *In Vitro*

Ribonuclease protection assay of mOSM transcript expression using the riboprobe generated from pmOSM 123*ET*. A murine GAPDH riboprobe was included to act as a control for RNA loading (section 2.3.20). Protected species are indicated whose sizes correspond to those expected from protection of the characterised mOSM (Exons 1+ 2 +3) and GAPDH (64 bp) transcripts. Protected species that correspond in size to those expected (Figure 7.1 B) when the probe hybridises to each of the three exons of the mOSM transcript alone or in combination are also indicated. The sizes of ^{32}P end-labelled pUC19 *Hpa* II DNA molecular weight markers are indicated at left.


GAPDH Probe
mOSM Probe
Markers
Yeast tRNA
Testis
Brain
Small Intestine
Liver
Spleen
Bone Marrow
Thymus
Embryo (16 d.p.c.)
Day 10
Day 8
Day 6
Day 4
Day 2
Embryoid Bodies
ES + LIF Withdrawal
EPL
ES
EC (Nulli SCCl)
489 bp
404 bp
331 bp
242 bp
190 bp
140 bp
111 bp
67 bp
Exons 1+2+3
Exons 2+3
Exons 1+2
Exon 3
Exon 2
Exon 1
GAPDH

7.2.2 Identification of a Novel Splice Variant of the mOSM Transcript

To screen for all possible alternately spliced transcripts containing one or a combination of the three characterised mOSM exons, 5' and 3' RACE-PCR strategies were devised utilising four mOSM-specific primers (Figure 7.3). Each of the four mOSM-specific primers was used in combination with the AP1 adaptor-specific primer (Clontech). While the characterised mOSM transcript was isolated from all reactions, a product representing a novel splice variant of the mOSM transcript in which mOSM exon 1 was spliced directly to exon 3 was isolated from exon 3-based 5' RACE -PCR reactions (Figure 7.4 A). The exon sequences and the exon 1 splice donor and exon 3 splice acceptor sites utilised in this variant transcript were as previously reported (Yoshimura et al., 1996). This transcript was termed the mOSM 13 transcript to distinguish it from the characterised mOSM transcript.

Splicing of exon 1 directly to exon 3 disrupts the OSM open reading frame by placing the previously reported reading frame (Yoshimura et al., 1996) of exon 3 out of phase by +1 nucleotides (Figure 7.4 A and B).

7.2.3 Validation of the mOSM 13 Transcript

The existence of the mOSM 13 transcript was confirmed by PCR and ribonuclease protection assay. RT-PCR amplification of mouse bone marrow cDNA using the MOGSP1/MOGSP4 primer combination (Figure 7.3) yielded products of approximately 890 and 760 bp (Figure 7.5C) consistent with the predicted sizes for mOSM and mOSM 13 cDNAs. The identity of these products was confirmed by cloning them directly into pGEM-T (Promega) and sequencing. The relative abundance of the products obtained indicated that the mOSM transcript was approximately four-fold more abundant than the mOSM 13 transcript in this sample.

Expression of the mOSM 13 transcript in mouse tissues and cell lines was investigated by ribonuclease protection (Figure 7.5B) using an antisense riboprobe transcribed from pmOSM13*ST*, a subclone of the mOSM 13 5'RACE cDNA, (section 2.3.20) (Figure 7.5A). The full length mOSM transcript was detected in bone marrow, thymus, spleen and embryonic stem cells as two fragments of 72 and 136 bp. Protection of a 208 bp fragment, corresponding in size

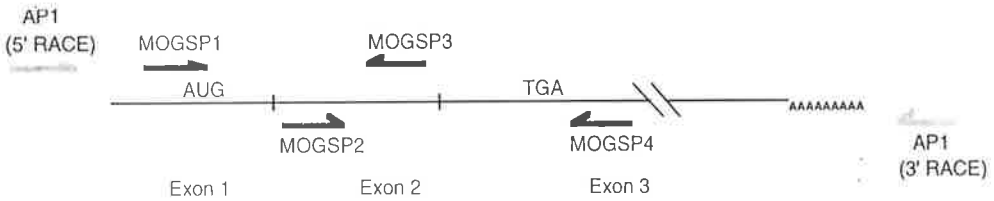
Figure 7.3 Positioning of Primers Used for RACE- and RT-PCR Cloning of mOSM cDNAs from Bone Marrow

(A) Schematic showing the previously characterised mOSM transcript with the position and orientation of primers used in 5' and 3' RACE-PCR and RT-PCR analysis of mOSM transcript production. This strategy enabled comprehensive screening for mOSM transcripts containing any one of the three characterised mOSM exons individually or in combination with characterised and/or uncharacterised exons. The AP1 primer (Clontech) hybridises to adaptor oligonucleotides ligated to the cDNA ends after second strand synthesis.

(B) Nucleotide and amino acid sequence of the previously characterised mOSM transcript (Yoshimura et al., 1996). Primer sequences (> indicates direction of priming) are shown in **bold** either above or below the mOSM cDNA sequence to indicate primers for sense or antisense strand synthesis respectively.

The amino acid sequence of the mOSM protein is indicated above the sense strand, the open reading frame is in UPPER CASE while the putative 5' and 3' untranslated regions is in lower case. Boundaries between exons are shown as thin vertical lines (|). The numbers at right denote the relative positions of the amino acids and nucleotides at the end of each line either in the protein or in the cDNA sequence respectively.

A



B

MOGSP1 > GCCAGA GTACCAGGA CCCAGTAT

```
                                                    M   Q   T   R    4
gtcacccc tgagaggca cgggccaga gtaccagga cccagtATG CAGACACGG          54

                                                MOGSP2 > ATCATG AGGATGGCA
  L   L   R   T   L   L   S   L   T   L   S   L   L   I   L   S   M   A   22
CTTCTAAGA ACACTGCTC AGTTTGACC CTCAGTCTC CTCATCCTG AGCATGGCA         108

CTGGCCAAT
  L   A   N   R   G   C   S   N   S   S   S   Q   L   L   S   Q   L   Q   40
CTGGCCAAT CGTGGCTGC TCCAACTCT TCCTCTCAG CTCCTCAGC CAGCTGCAG         162

  N   Q   A   N   L   T   G   N   T   E   S   L   L   E   P   Y   I   R   58
AATCAGGCG AACCTCACG GGGAACACA GAATCACTC TTGGAGCCC TATATCCGC         216
TTAGTCCGC TTGGAGTGC CCCTT < MOGSP3

  L   Q   N   L   N   T   P   D   L   R   A   A   C   T   Q   H   S   V   76
CTCCAAAAC CTGAACACA CCTGACCTG AGAGCTGCC TGCACCCAG CACTCTGTG         270

  A   F   P   S   E   D   T   L   R   Q   L   S   K   P   H   F   L   S   94
GCCTTCCCC AGTGAGGAC ACACTCCGG CAACTGAGC AAGCCTCAC TTCCTGAGC         324

  T   V   Y   T   T   L   D   R   V   L   Y   Q   L   D   A   L   R   Q   112
ACTGTGTAC ACCACACTG GACAGAGTC TTGTACCAA CTGGATGCT TTAAGACAG         378

  K   F   L   K   T   P   A   F   P   K   L   D   S   A   R   H   N   I   130
AAATTTCTG AAGACTCCG GCTTTTCCA AAGCTGGAC AGTGCCCGG CACAATATC         432

  L   G   I   R   N   N   V   F   C   M   A   R   L   L   N   H   S   L   148
CTCGGCATA AGGAACAAT GTTTTCTGC ATGGCCCGG CTGCTCAAC CACTCCCTG         486

  E   I   P   E   P   T   Q   T   D   S   G   A   S   R   S   T   T   T   166
GAGATACCT GAGCCCACA CAGACAGAC TCTGGGGCC TCACGGTCC ACTACAACA         540

  P   D   V   F   N   T   K   I   G   S   C   G   F   L   W   G   Y   H   184
CCAGATGTC TTTAATACC AAGATAGGC AGCTGTGGC TTTCTCTGG GGATACCAT         594

  R   F   M   G   S   V   G   R   V   F   R   E   W   D   D   G   S   T   202
CGCTTCATG GGCTCAGTG GGGAGGGTC TTCAGGGAA TGGGACGAT GGCTCCACA         648

  R   S   R   R   Q   S   P   L   R   A   R   R   K   G   T   R   R   I   220
CGCAGCCGG AGACAGAGC CCGCTCCGG GCCCGGCGC AAGGGAACC CGCAGAATC         702

  R   V   R   H   K   G   T   R   R   I   R   V   R   R   K   G   T   R   238
CGGGTCCGG CACAAGGGA ACCCGCAGA ATCCGGGTC CGGCGCAAG GGAACCCGC         756

  R   I   W   V   R   R   K   G   S   R   K   I   R   P   S   R   S   T   256
AGAATCTGG GTCCGGCGC AAGGGATCC CGCAAAATC AGACCTTCC AGGAGCACC         810

  Q   S   P   T   T   R   A   *                                       263
CAGAGCCCG ACGACCAGG GCCTAGgtt ccctggtag cctgaggac acactgaca         864

gacagcata gtctggtga tacaggatg tcgctctca gaggctttc aaagctgct         918
                T ATGTCCTAC TGCGAGAGT CTCCG < MOGSP4
```

Figure 7.4 Sequence and Potential Translation Products of the mOSM 13 cDNA.

(A) Three phase translation of the nucleotide sequence of the mOSM 13 cDNA in the region of the exon 1 - exon 3 splice junction (indicated by a vertical bar). Two potential open reading frames are indicated in **bold**. The ***italicised bold*** open reading frame continues for 125 amino acids and encodes the C-terminal region of the mature mOSM protein. Potential CUG translational initiation codons (Hann et al., 1994) in the OSM reading frame of exon 3 are underlined.

(B) Schematic of potential translation products of the mOSM 13 transcript. Initiation of translation at the exon 1 AUG of the mOSM transcript produces a secreted glycoprotein containing four αhelices (Yoshimura et al., 1996). Initiation of translation at the exon 1 AUG of the mOSM 13 transcript results in disruption of the OSM reading frame and premature termination of translation whereas initiation of translation at the first AUG in the OSM reading frame of exon 3 would produce a protein containing the C-terminal regions of mature mOSM. s.s., secretion signal sequence; A, B, C, D, predicted α helical regions of mOSM (Kitchen et al., 1998); c.t., C-terminal region cleaved during maturation by specific proteolysis (Linsley et al., 1990).

A

```
     G   Q   S   T   R   T   Q   Y   A   D   T   A   S   K   N   T   A   Q   S   A
       P   E   Y   Q   D   P   V   C   R   H   G   F   *   E   H   C   S   I   R   L
     A   R   V   P   G   P   S   M   Q   T   R   L   L   R   T   L   L   N   P   P
61   GCC AGA GTA CCA GGA CCC AGT ATG CAG ACA CGG CTT CTA AGA ACA CTG CTC AAT CCG CCT
                                                          Exon 1 | Exon 3

     S   K   T   *   T   H   L   T   *   E   L   P   A   P   S   T   L   W   P   S
       Q   N   L   N   T   P   D   L   R   A   A   C   T   Q   H   S   V   A   F   P
     P   K   P   E   H   T   *   P   E   S   C   L   H   P   A   L   C   G   L   P
121  CCA AAA CCT GAA CAC ACC TGA CCT GAG AGC TGC CTG CAC CCA GCA CTC TGT GGC CTT CCC

     P   V   R   T   H   S   G   N   *   A   S   L   T   S   *   A   L   C   T   P
       S   E   D   T   L   R   Q   L   S   K   P   H   F   L   S   T   V   Y   T   T
     Q   *   G   H   T   P   A   T   E   Q   A   S   L   P   E   H   C   V   H   H
181  CAG TGA GGA CAC ACT CCG GCA ACT GAG CAA GCC TCA CTT CCT GAG CAC TGT GTA CAC CAC

     H   W   T   E   S   C   T   N   W   M   L   *   D   R   N   F   *   R   L   R
       L   D   R   V   L   Y   Q   L   D   A   L   R   Q   K   F   L   K   T   P   A
     T   G   Q   S   L   V   P   T   G   C   F   K   T   E   I   S   E   D   S   G
241  ACT GGA CAG AGT CTT GTA CCA ACT GGA TGC TTT AAG ACA GAA ATT TCT GAA GAC TCC GGC

     L   F   Q   S   W   T   V   P   G   T   I   S   S   A   *   G   T   M   F   S
       F   P   K   L   D   S   A   R   H   N   I   L   G   I   R   N   N   V   F   C
     F   S   K   A   G   Q   C   P   A   Q   Y   P   R   H   K   E   Q   C   F   L
301  TTT TCC AAA GCT GGA CAG TGC CCG GCA CAA TAT CCT CGG CAT AAG GAA CAA TGT TTT CTG

     A   W   P   G   C   S   T   T   P   W   R   Y   L   S   P   H   R   Q   T   L
       M   A   R   L   L   N   H   S   L   E   I   P   E   P   T   Q   T   D   S   G
     H   G   P   A   A   Q   P   L   P   G   D   T   *   A   H   T   D   R   L   W
361  CAT GGC CCG GCT GCT CAA CCA CTC CCT GGA GAT ACC TGA GCC CAC ACA GAC AGA CTC TGG
```

B

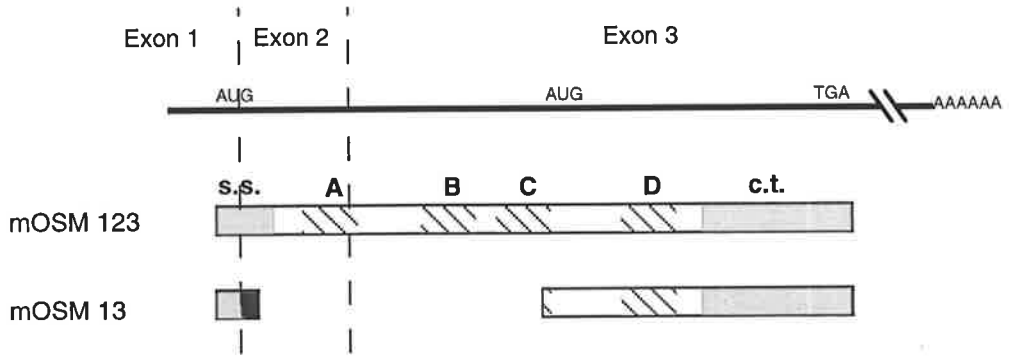
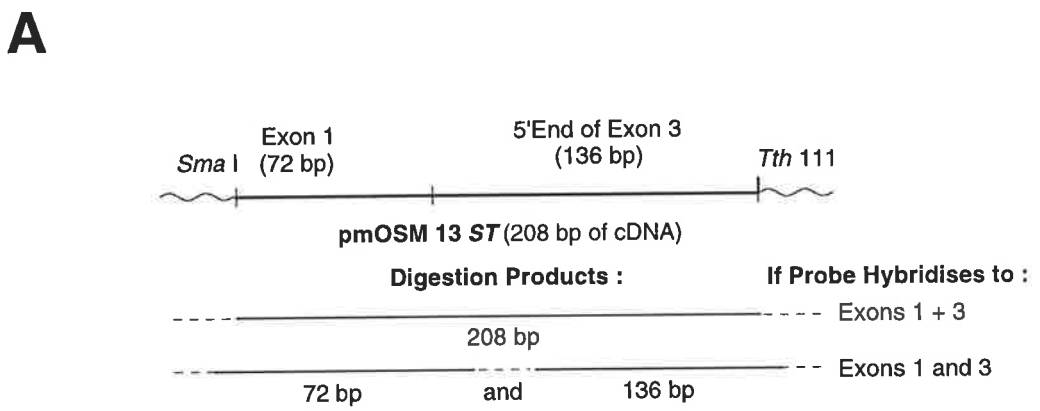


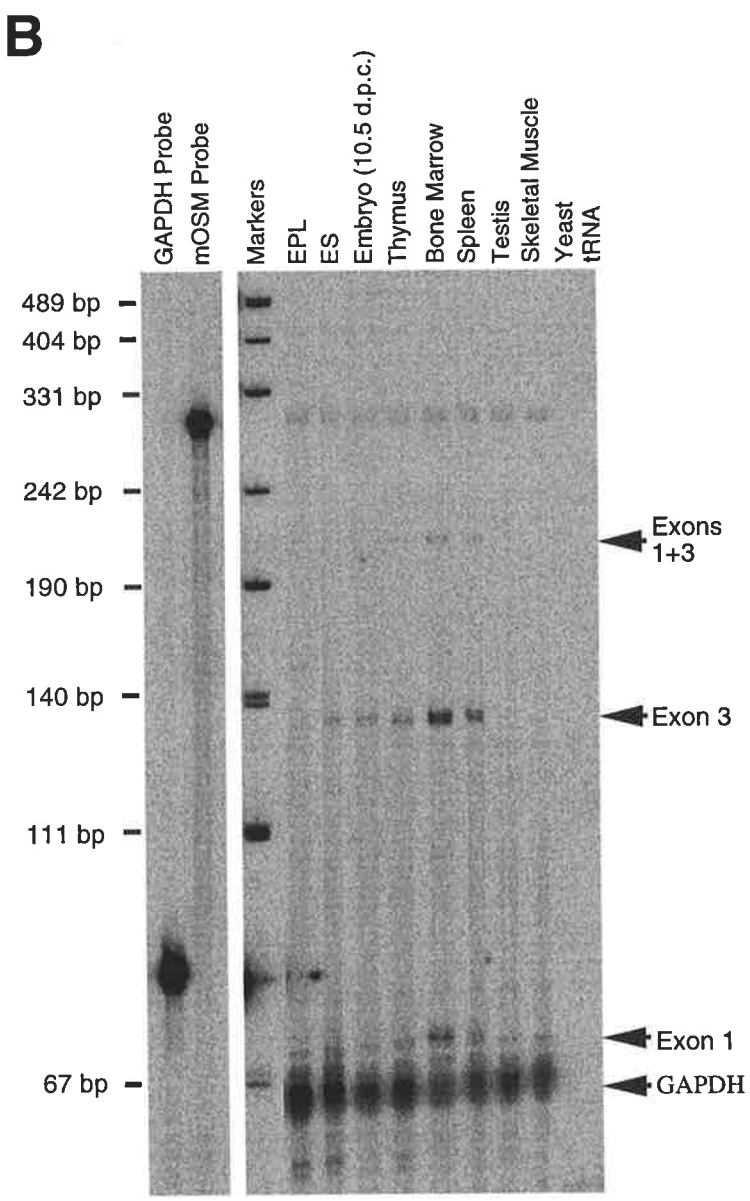
Figure 7.5 Validation and differential expression of the mOSM 13 transcript.

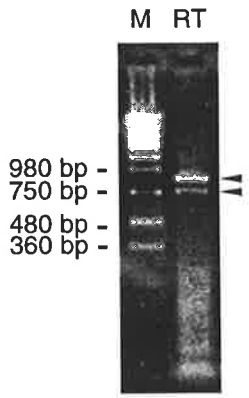
(A) Origin and sizes of possible digestion products arising from hybridisation of the riboprobe generated from pmOSM13*ST* to exons 1 and 3 of the mOSM transcript separately or in combination.

(B) Ribonuclease protection assay of mOSM transcript expression using the riboprobe generated from pmOSM13*ST* (section 2.3.20). A GAPDH riboprobe was included to act as a control for RNA loading. Protected species are indicated whose size corresponds to species predicted to arise from protection of the mOSM 13 (Exons 1 + 3) and mGAPDH transcripts. Protected species that correspond in size to those expected when the probe hybridises separately to exons 1 and 3 of the mOSM transcript are also indicated. The sizes of ^{32}P end-labelled pUC19 *Hpa* II DNA molecular weight markers are indicated at left.

(C) RT-PCR of mOSM transcripts using primers in exons 1 and 3. The primers MOGSP1 and MOGSP4 (Figure 7.2A) amplified two species (arrows) of approximately 890 and 760 bp from bone marrow cDNA. These correspond in size to products predicted for amplification of mOSM and mOSM 13 cDNAs respectively. M, phage SPP1 EcoRI DNA molecular weight markers; RT, RT-PCR of bone marrow cDNA.


A
Exon 1
(72 bp)
Sma I
5'End of Exon 3
(136 bp)
Tth 111
pmOSM 13 ST (208 bp of cDNA)
Digestion Products :
If Probe Hybridises to :
Exons 1 + 3
208 bp
Exons 1 and 3
72 bp
and
136 bp


B
GAPDH Probe
mOSM Probe
Markers
EPL
ES
Embryo (10.5 d.p.c.)
Thymus
Bone Marrow
Spleen
Testis
Skeletal Muscle
Yeast tRNA
489 bp
404 bp
331 bp
242 bp
190 bp
140 bp
111 bp
67 bp
Exons 1+3
Exon 3
Exon 1
GAPDH


C
M
RT
980 bp
750 bp
480 bp
360 bp

to the product predicted for the mOSM 13 transcript, was observed at highest levels in bone marrow and spleen and at lower levels in thymus. The presence of this band confirmed the existence of the mOSM 13 transcript *in vivo* and indicated that production of mOSM 13 transcripts did not result from de-regulation of transcription *in vitro*. Levels of the mOSM 13 transcript varied both between samples, and relative to the levels of mOSM transcription (compare ES cells, thymus, spleen and bone marrow; Figure 7.5B). As seen in the RT-PCR analysis, the mOSM 13 transcript was present in the bone marrow sample at levels about four-fold lower than the mOSM transcript. This suggested that the mOSM 13 and mOSM 123 transcripts were differentially and independently regulated in a tissue-specific manner and therefore likely to serve distinct, biologically significant functions.

7.3 Discussion

7.3.1 mOSM Transcript Expression and Regulation

Surveys of OSM transcript expression originally focused on adult haematopoietic tissues and cell lines (Malik et al., 1989; Yoshimura et al., 1996). We detected expression of the mOSM transcript in 10.5 day post-coitum embryos and ES cell embryoid bodies 8 and 10 days after seeding. This expression is likely to be associated with early definitive haematopoietic activities (Mukouyama et al., 1998; Xu et al., 1998). The mOSM transcript expression seen in 16.0 day post-coitum embryos is likely to reflect previously reported expression in the foetal gonads, thymus and cartilage primordia (Hara et al., 1998).

OSM expression was also detected in pluripotent cells. While signalling by IL-6 family cytokines is not absolutely required for pluripotent cell maintenance *in vivo* or *in vitro* (Dani et al., 1998; Stewart et al., 1992), the finding that D3 embryonic stem cells and NULLI SCCI embryonal carcinoma cells express the mOSM transcript suggests that mOSM, like LIF (Li et al., 1995), is a candidate autocrine maintenance factor for pluripotent cells in the early embryo. This is supported by the observation that OSM can replace LIF in the maintenance of murine embryonic stem cells *in vitro* (Hara et al., 1997; Rose et al., 1994). Unlike LIF transcripts (Rathjen et al., 1990b), mOSM transcripts were not upregulated during ES cell differentiation in

response to LIF withdrawal and mOSM is therefore unlikely to be involved in feedback renewal (Smith et al., 1992) of pluripotent cells.

mOSM expression could not be detected in a variety of tissues and cell systems including adult testis, brain, small intestine and spontaneously differentiated embryonic stem cells. In conjunction with the results of others it is therefore evident that the expression pattern of OSM is more restricted than that of LIF (Robertson et al., 1993), providing further evidence that these two cytokines serve distinct functions.

The isolation of the mOSM 13 transcript using multiple PCR-based approaches and its detection *in vivo* by ribonuclease protection confirm its existence and indicate that it is expressed as a relatively abundant, independently-regulated mOSM transcript, at least in haematopoietic tissues. In support of these findings, three mOSM transcripts, the smallest of which corresponds in size to that predicted for the mOSM 13 transcript, are detected in northern blot hybridisations performed by others (Yoshimura et al., 1996). The relative levels of mOSM 13 transcript expression varied in comparison to mOSM transcript expression, making it unlikely that the mOSM 13 transcript represents an irrelevant by-product of mOSM splicing.

7.3.2 Alternate Transcription is a Feature of IL-6 Family Cytokines

Variant transcripts in which exon 2 is omitted have been reported for other members of the IL-6 cytokine family. Human peripheral blood mononuclear cells express an IL-6 transcript in which exon 1 is spliced to exon 3 using unconventional splicing processes that preserve the open reading frame (Kestler et al., 1995). Alternate mouse and human LIF transcripts encoding differentially localised proteins have also been described (Haines et al., 1999; Voyle et al., 1999) and a human LIF transcript in which exons 1 and 3 are spliced together using the previously reported splice donor and acceptor sites has been identified in meningioma biopsies (Pergolizzi and Erster, 1994). As with the mOSM 13 transcript, this disrupts the open reading frame initiated in exon 1 and it is not clear what protein, if any, is encoded by the variant LIF transcript. The isolation, but not sequence of a cDNA representing an alternately spliced human cardiotrophin-1 transcript has been reported (Pennica et al., 1996) and variant ciliary neurotrophic factor transcripts are produced as a consequence of a naturally occurring mutation (Takahashi et al., 1994).

7.3.3 The mOSM 13 Transcript Potentially Encodes an Intracellular Protein Which Could Antagonise OSM Signalling

Initiation of translation at the AUG in exon 1 of the mOSM 13 transcript would result in loss of the OSM reading frame ten amino acid residues into the signal peptide and termination of translation nine residues later (Figure 7.4 A and B). However, initiation of translation at candidate AUG or CUG (Hann et al., 1994) codons in exon 3 could result in production of an N-terminally truncated mOSM protein lacking some or all of the A, B and C helices of the full-length OSM protein (Figure 7.4 A and B). Initiation of translation at downstream AUG and/or CUG codons is known to result in the production of alternate fibroblast growth factor 2 (Arnaud et al., 1999), IL-1 receptor antagonist (Malyak, et al., 1998) IL-15 (Tagaya et al., 1997) and LIF (Haines et al., 1999; Voyle et al., 1999) proteins. Any mOSM protein produced by translational initiation within exon 3 would also lack a functional N-terminal secretory signal sequence and could not be secreted from the cell by conventional mechanisms. This could result in the intracellular retention of OSM proteins as occurs with alternate IL-15 (Tagaya et al., 1997), IL-1 receptor antagonist (Malyak et al., 1998) and LIF (Haines et al., 1999; Voyle et al., 1999) proteins. Intriguingly, preformed intracellular stores of human OSM have been identified in polymorphonuclear neutrophils and production and release of biologically active intracellular OSM can be stimulated by granulocyte-macrophage colony stimulating factor and other agents (Grenier et al., 1999). Expression of human OSM 13 transcripts by these cells would provide a potential molecular explanation for the production of intracellular OSM proteins.

Among all known IL-6 family cytokines, N-terminal regions such as the A-B loop are implicated in specification of ligand-receptor sub unit interactions, and helices A and C appear to form site II, the "universal" binding site for gp130 (Heinrich et al., 1998; Grotzinger et al., 1997). Mutation of residues in the LIF A and C helices can create antagonists of LIF signalling, apparently by preventing recruitment of the gp130 receptor subunit into the signalling complex (Hudson et al., 1996). These findings are consistent with studies of the interactions between OSM and its receptor complex (Staunton et al., 1998; Radka et al., 1994; Kallestad et al., 1991). Thus, the N-terminally truncated OSM protein potentially encoded by the mOSM 13 transcript (Figure 7.4 B) might bind OSM receptor-β but not gp130, resulting in the prevention of receptor

complex assembly. The putative truncated OSM protein could then function as a naturally occurring competitive inhibitor of signalling by the full-length OSM protein. The predominantly intracellular protein encoded by the alternate IL-6 transcript in which exon 2 is skipped has also been suggested to function as competitive inhibitor of IL-6 signalling (Kestler et al., 1995).

Similar phenomena whereby alternate transcription and translation of cytokine RNAs results in the production of proteins exhibiting differential localisation and activity have been reported for a number of cytokines. Independently regulated translation of the FGF 2 transcript produces four differentially localised proteins (Arnaud et al., 1999). In the cases of the IL-1 receptor antagonist (Gabay et al., 1997; Muzio et al., 1995; Watson et al., 1995; Malyak, et al. 1998) and IL-15 (Tagaya et al., 1997) genes, alternate transcripts encode intracellular proteins that appear to be functionally distinct from the secreted proteins. Differential induction and modulation of the intracellular and extracellular forms of the IL-1 receptor antagonist transcripts and proteins have been demonstrated in neutrophils and monocytes (Malyak et al., 1998) and tissue specific differential regulation of alternate IL-15 transcripts is also known to occur (Tagaya et al., 1997). Similar phenomena have also been observed in the case of IL-6 family cytokines. The variant IL-6 transcript encoding a predominantly intracellular protein is also differentially regulated (Kestler et al., 1995) and the intracellular proteins produced from the independently regulated LIF-M and LIF-T transcripts of mice and humans (Haines et al., 1999; Voyle et al., 1999) are functionally distinct from the secreted LIF proteins (Haines et al., submitted). It will therefore be of interest to determine whether proteins potentially encoded by the mOSM 13 transcript can also have localisations and functions that are distinct from the mOSM encoded protein.

Chapter 8 :

Concluding Discussion

8.1 Introduction

The work presented in this thesis has demonstrated that alternate transcripts produced from the human LIF gene encode distinct but overlapping sets of proteins with novel biological activities and localisations (Figure 8.1). These findings were, in part, extended to the LIF and OSM genes of other mammals. The alternate transcriptional and translational processes characterised in this study make possible highly regulated production of intracellular and secreted LIF proteins from a single gene and constitute a mechanism for precise control of sites of LIF action. These findings add LIF to a small but growing list of cytokines produced in an intracellularly active form, and suggest new possibilities for the action of LIF *in vivo*, particularly in cell autonomous signalling processes that may operate during stem cell self-renewal and tumourigenesis.

8.2 Complex, Conserved Organisation of the LIF Gene

Three alternate LIF transcripts are produced from independently-regulated promoters in the LIF genes of humans, mice, pigs, and, evidently, all other eutherian mammals. These transcripts contain alternate first exons, located between the previously identified first and second exons (Stahl et al., 1990). The three alternate first exons are spliced to common second and third exons producing the LIF-D, LIF-M and LIF-T transcripts. Expression of alternate transcripts containing distinct first exons, transcribed from alternate promoters, has been observed for several cytokine and growth factor genes, including insulin-like growth factor-1 (Adamo et al., 1989) and acidic fibroblast growth factor (Philippe et al., 1992). Utilisation of shared and distinctive sequence elements conserved in the putative promoter regions of the hLIF-M and hLIF-T transcripts provides a mechanism for independently regulated expression of each LIF transcript.

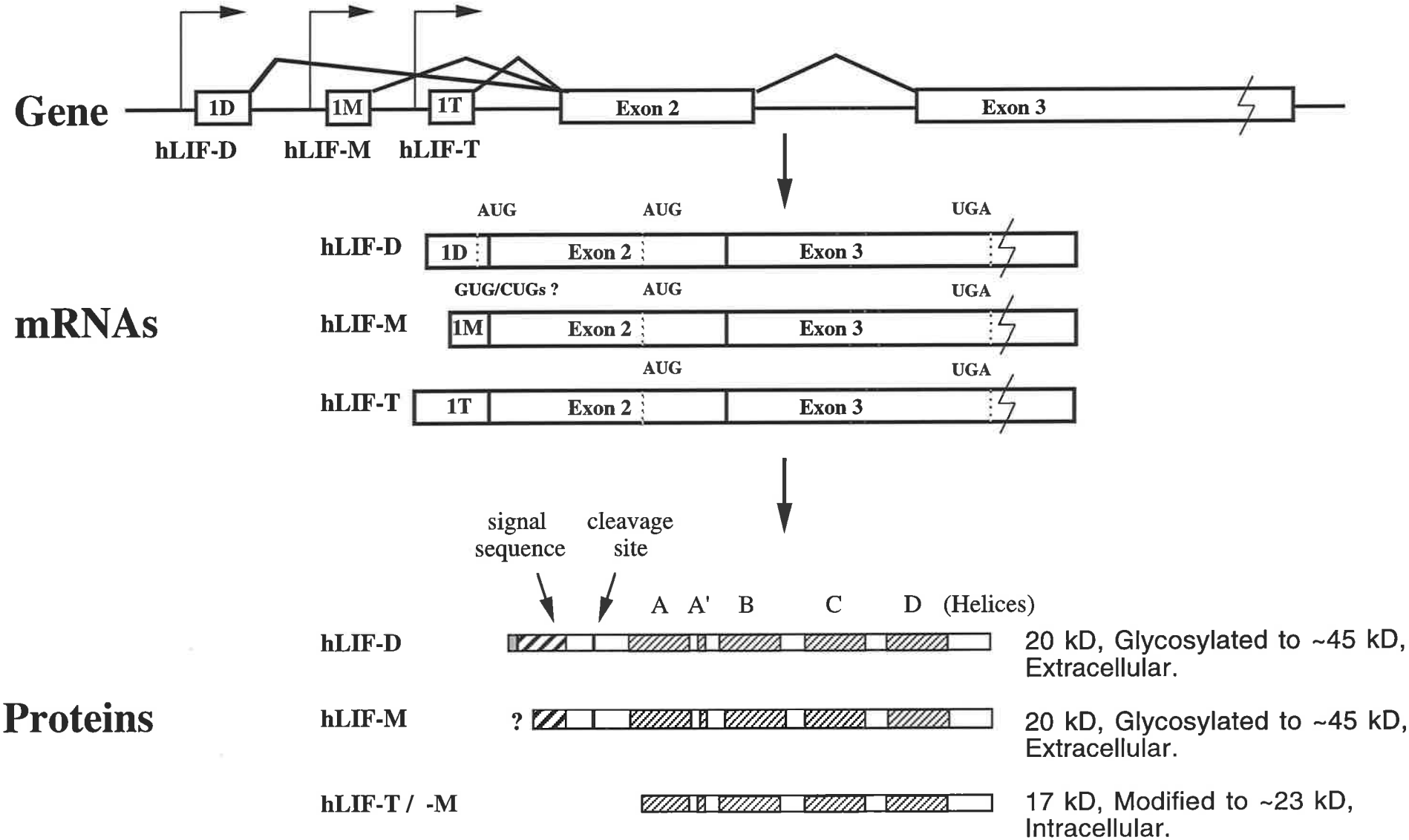
The cloned and predicted LIF-D transcripts of all characterised LIF genes (Gearing et al., 1987; Moreau et al., 1988; Yamamori et al., 1989; Willson et al., 1992) contain in-frame ATG translational initiation sites in their first exons. Murine LIF-M (Rathjen et al., 1990a) is unique among the ten cloned and predicted LIF-M and LIF-T transcripts in that it also contains an in-frame ATG translational initiation codon. Other LIF-M and LIF-T transcripts lack such a codon and often contain in-frame translational termination codons. The protein produced from the

Figure 8.1 Organisation and Expression of the Human LIF Gene

Three transcripts, hLIF-D, hLIF-M, and hLIF-T, with distinct first exons are produced from the human LIF gene, apparently as a result of alternate promoter usage. The three alternate first exons can be spliced to shared second and third exons. This arrangement of the LIF gene appears to occur in all eutherian mammals.

The exonic constitution of the three hLIF transcripts is shown with the longer third exon shown shortened relative to the other exons. The positions of AUG translational initiation codons and UGA translational termination codons that form the boundaries of the hLIF-D, hLIF-M and hLIF-T open reading frames are indicated. The translation initiation site of the secreted hLIF-M protein (indicated as "GUG/CUGs ?") is presently unknown (see Figure 5.12). The mature, secreted hLIF-D and hLIF-M proteins are based upon identical 20 kD mature, core polypeptides and appear to differ only in terms of their N-linked glycoside content. The N-terminally truncated, intracellularly retained proteins produced from the hLIF-T and hLIF-M transcripts appear to be based upon an identical 17 kD polypeptide produced as a result of translational initiation at a conserved in-frame AUG in the second exon.

The thickly cross-hatched region of the hLIF-D and hLIF-M proteins contain the core hydrophobic region of its secretion signal sequence which is cleaved at the indicated site to produce the mature protein. The lightly cross-hatched regions contain sequences that form α helices in the crystallised mLIF-D protein (Robinson et al., 1994).


Gene
1D
1M
1T
Exon 2
Exon 3
hLIF-D
hLIF-M
hLIF-T
mRNAs
AUG
AUG
UGA
hLIF-D
1D
Exon 2
Exon 3
GUG/CUGs ?
AUG
UGA
hLIF-M
1M
Exon 2
Exon 3
AUG
UGA
hLIF-T
1T
Exon 2
Exon 3
signal sequence
cleavage site
A A' B C D (Helices)
Proteins
hLIF-D
20 kD, Glycosylated to ~45 kD, Extracellular.
hLIF-M
?
20 kD, Glycosylated to ~45 kD, Extracellular.
hLIF-T / -M
17 kD, Modified to ~23 kD, Intracellular.

mLIF-D transcripts is a secreted, freely-diffusible molecule cleaved by a signal peptidase to produce a mature, glycosylated protein with a 20 kD core polypeptide (Gearing et al., 1987). The protein translated from the mouse LIF-M transcript is localised to the extracellular matrix following overexpression *in vitro* (Rathjen et al., 1990a). As with the mLIF-D transcript, the hLIF-D transcript encodes secreted glycoproteins based upon a 20 kD mature polypeptide (section 5.2.3; Gascan et al., 1989). The human LIF-M transcript, like mouse LIF-M, encodes secreted, glycosylated proteins based on a 20 kD core polypeptide apparently from a non-AUG initiation codon located upstream of sequences encoding the signal peptidase cleavage site. Although the site of translation initiation for the secreted LIF-M proteins has not been determined, potential non-AUG initiation codons exist in the LIF-M first exons and at 5' end of the second exon in all five eutherian mammals for which sequence is available (Figure 5.12). Translation of secreted proteins from the hLIF-M transcript required sequence information, but not necessarily initiation codons, present in the first exon. It was also demonstrated that the human LIF-M and the mouse and human LIF-T transcripts encode proteins that initiate from the first in-frame ATG in exon 2, producing an N-terminally truncated 17 kD intracellular form of the LIF protein that can be post-translationally modified to produce a 23 kD protein. Thus at least three distinct translational processes appear to be involved in the production of the alternate LIF proteins.

The existence and relevance of the LIF-M transcript in species other than the mouse has been questioned by other workers (Stahl et al., 1990; Willson et al., 1992). These results thus provide a resolution to this debate and raise the possibility that secreted, possibly extracellular matrix-associated, proteins similar to those encoded by mLIF-M and hLIF-M may be produced in other mammalian species.

The utilisation of alternate transcripts to produce intracellularly and extracellularly localised cytokines also occurs in the cases of the IL-15 (Tagaya et al., 1997) and the IL-1 receptor antagonist (Gabay et al., 1997) genes. In these cases alternate promoter usage directs differential splicing processes but these intracellular proteins contain vestigial signal sequences and, unlike the intracellular LIF proteins, are not truncated relative to their secreted protein counterparts. Unfortunately, there are presently no data concerning the biological functions of the

intracellular IL-15 and IL-1 receptor antagonist proteins. It will be of interest to determine whether common themes exist in the action, and not just production, of intracellular cytokines.

8.3 Alternate Transcriptional and Translational Processes Allow Precise Regulation of LIF Activity

The complex organisation of the human and mouse LIF genes results in the production of three alternate transcripts that yield proteins with distinct cellular localisations. Alternate compartmentalisation of many cytokines and growth factors has been demonstrated (Chintala et al., 1994; Park et al., 1993; Pollock and Richardson, 1992; Nakagawa et al., 1996; Derynck et al., 1984; Kimura et al., 1990; Parker et al., 1996). Restriction of the factor to an extracellular matrix-associated, membrane-associated or a diffusible fate provides mechanism for regulation of the site of action. The existence of differentially localised LIF proteins provides possible mechanisms for controlling the target cells of LIF action. The diffusible LIF protein would act in a paracrine / autocrine fashion, as demonstrated by LIF expression in endometrial glands during blastocyst implantation (Stewart et al., 1992; Cullinan et al., 1996). Cellular sites of action of LIF-D could be controlled by regulation of LIF receptor or gp130 expression (Nicholls et al., 1996; Saito et al., 1992) or by expression of LIF binding proteins (Mereau et al., 1993; Layton et al., 1992). Localisation of the secreted mLIF-M, and possibly other LIF-M, proteins to the extracellular matrix could permit juxtacrine LIF signalling, possibly involving the matrix localised LIF binding protein (Mereau et al., 1993). The intracellular LIF proteins, identified in these studies, which are produced from the hLIF-M and hLIF-T transcripts, could provide a mechanism for cell-autonomous, autocrine LIF action. Additionally, intracellular retention of LIF proteins may underlie a mechanism for regulated paracrine LIF action where release of the cytokine from within the cell would only occur after application of an extracellular stimulus, or following cell lysis. Any LIF protein released, by any mechanism, into the extracellular environment could also act on the releasing cell population in an autocrine fashion. The complex arrangement of the mammalian LIF gene thus provides the basis of a precise regime controlling the location of LIF protein production and function.

8.4 Potential *In Vivo* Roles for the Intracellular LIF Protein

The identification of alternate LIF transcripts able to encode distinct but overlapping sets of intracellular and extracellular proteins suggests the existence of hitherto unappreciated complexity in the function of the mammalian LIF gene *in vivo*.

In vitro and gain of function experiments have identified many potential functions for the secreted forms of LIF (Hilton et al., 1992). Many of the effects observed in these experiments are not observed *in vivo* in mice lacking a functional LIF gene (Stewart et al., 1992). Moreover, some effects of LIF gene disruption in mice, including their reduction in size and maternal infertility (Stewart et al., 1992), were not predicted by existing *in vitro* and gain of function studies. Additional more subtle defects in LIF deficient mice have subsequently been reported (Escary et al., 1993; Chesnokova et al., 1998; Kurek et al., 1997; Rao et al., 1993). However, the action of the LIF gene *in vivo* remains poorly understood at the molecular and cellular level.

The complexity of the phenotype of LIF knockout mice may partly be a consequence pleiotropy and redundancy within the IL-6 cytokine family (Piquet-Pellorce et al., 1994). Indeed, the role of LIF in supporting motor neuron function seems to be masked by ciliary neurotrophic factor in LIF knockout mice (Sendtner et al., 1996). The defects present in LIF knockout mice are thus very diverse and likely to be quite numerous. When considering this it is worth noting that production of all three alternate mLIF transcripts is abolished in the LIF knockout mice produced to date. Furthermore, some aspects of the LIF knockout phenotype, such as the reduction in numbers of haematopoietic stem and progenitor cells (Escary et al., 1993), do not appear to be recapitulated in the LIF receptor knockout phenotype (Ware et al., 1995; Li et al., 1995). This suggests that these aspects of the LIF knockout phenotype might result from receptor-independent actions of the LIF protein. It is also possible that loss of some intracellular functions of the LIF gene can be compensated for by the intracellular actions of other IL-6 family cytokines. Regardless, it seems likely that at least part of the complex phenotype of LIF knockout mice results from the absence of intracellular LIF proteins. It will thus be important in future studies to attribute different aspects of the LIF knockout phenotype to specific products of the LIF gene.

The intracellular LIF molecule could act as a store of cytokine able to be released into the extracellular environment and act via cell surface receptors. In this regard it is interesting to note that active mLIF-T proteins can be released by mechanical cell lysis (J. Rathjen, unpublished observations). This would provide a possible mechanism for LIF release in response to injury or stress. A role for LIF in the response to nerve (Rao et al., 1993; Banner and Patterson, 1994; Curtis et al., 1994) and muscle (Kurek et al., 1996; Kurek et al., 1997) damage has been suggested by studies of LIF knockout mice. Intracellular LIF could also be secreted in response to environmental cues via an unconventional mechanism (Muesch et al., 1990) not operative in the Cos cell overexpression system. For example, the LIF-related cytokine ciliary neurotrophic factor lacks a secretion signal sequence and is not secreted from Cos cells (Lin et al., 1989), yet it exerts at least some of its effects by signalling through cell surface receptors (De Chiara et al., 1995). Either mechanism of release could create high extracellular LIF concentrations unable to be produced by *de novo* synthesis. This could be important in situations of intense cytokine signalling, such as during the inflammatory responses accompanying disease states such as arthritis (Alexander et al., 1994).

An alternative or additional mode of action for the intracellular LIF protein could be cell-autonomous and intracellular, involving a signalling pathway distinct from that of the secreted LIF protein.

8.5 A Distinct Mechanism for Intracellular LIF Protein Action

The induction of apoptosis by intracellular LIF protein is intriguing in that it appears to involve cellular signalling pathways distinct from those associated with cell surface receptor-mediated LIF activity. The molecular basis of mLIF-T induced apoptosis in Cos cells was closely examined by Bryan Haines (Haines, 1997; Haines et al., *Submitted*). Searches for potential intracellular protein-protein interaction motifs in LIF identified a conserved leucine rich region, well separated from regions implicated in receptor subunit interactions (Robinson et al., 1994; Hudson et al. 1996). This motif contained a heptad repeat of five conserved leucine and isoleucine residues and was present in all species from which LIF cDNAs have been cloned,

including the marsupial *Sminthopsis crassicaudata* (Cui et al., 1998) (Figure 8.2A). Other conserved, non-heptad, leucines residues were also noted in this region. Conservation of this distinct motif throughout more than 200 million years of evolution (O'Brien & Graves, 1990) argued that it was functionally important. When represented on an alpha helical wheel diagram (Figure 8.2 B), it was found that this leucine-rich region had the potential to form an amphipathic, helical surface similar to that formed in leucine zipper domains. Leucine zippers are common in regulatory proteins and mediate protein-protein interactions among many nuclear transcription factors (Kerppola and Curran, 1991). The positioning of the heptad and non-heptad conserved leucines and isoleucines within this motif was also consistent with the formation of a leucine-rich repeat protein-protein interaction domain (Kobe & Diesenhofer, 1994) (Figure 8.2C).

To determine whether this motif was involved in the intracellular activity of the LIF protein, conserved and non-conserved leucine and isoleucine residues within this motif were mutated (Figure 8.3 A) to alanine and the apoptotic activity of the engineered proteins assayed using overexpression in Cos cells. Substitution of heptad and some non-heptad conserved leucines and isoleucines in this motif for alanine resulted in loss or dramatic reductions in the apoptotic activity of the intracellular LIF proteins (Figure 8.3B). The same mutations of the secreted LIF proteins did not affect their ability to be secreted and maintain embryonic stem cells by signalling through cell surface receptors (Figure 8.3B). Thus, these mutations disrupted the intracellular activity of the LIF protein without affecting the extracellular activity or folding of the LIF protein. Intriguingly, immunolocalisation of the mutant intracellular LIF proteins revealed that inhibition of apoptosis correlated with loss of nuclear localisation of the LIF protein. Nuclear staining for LIF protein is also lost when apoptosis is antagonised by CrmA co-expression. These observations raised the possibility that the apoptotic action of LIF required nuclear localisation.

The leucine rich region spans parts of the B and C helices and the B-C loop of the structure determined for the extracellular LIF protein (Robinson et al., 1994). Formation of either a leucine zipper or a leucine-rich repeat would therefore require that the intracellular form of the LIF protein underwent a different folding process. Translation of the alternate LIF transcripts encoding intracellular proteins by cytoplasmic ribosomes, rather than the endoplasmic reticulum-bound ribosomes that produce secreted proteins, could result in the folding of the intracellular

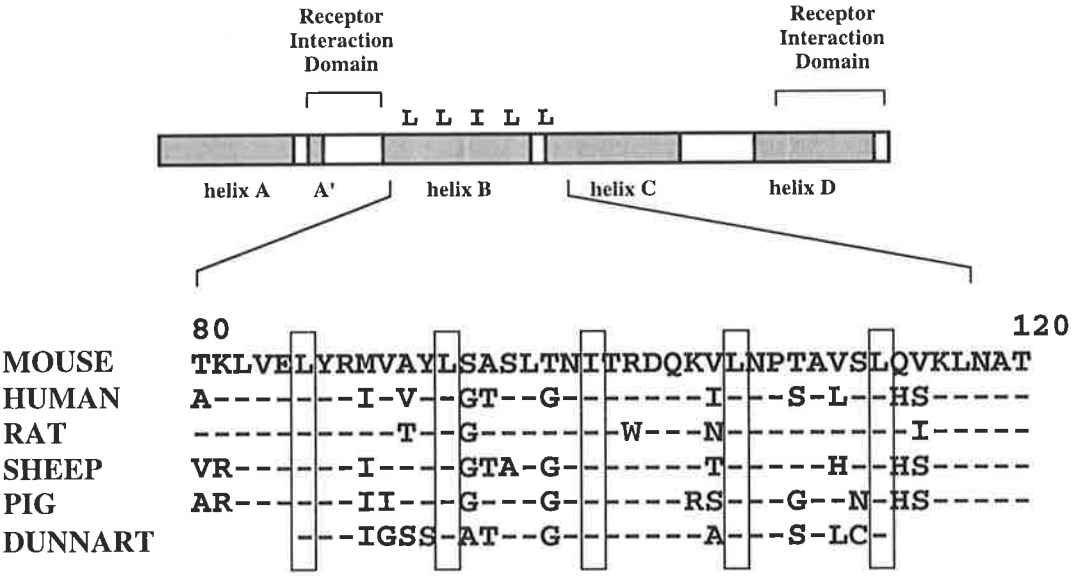
Figure 8.2 Putative Protein-Protein Interaction Domains in the Mammalian LIF Protein

(A) Schematic of the LIF protein showing the position of the conserved leucine zipper-like structure relative to the receptor interaction domains in five eutherian mammals and one marsupial. Other LIF amino acid sequences deduced from genomic and cDNA clones are: human (Moreau et al., 1988); ovine and porcine (Willson et al., 1992); bovine (Kato et al., 1995); and dunnart (Cui, 1998). Dashes indicate residues conserved with the mouse LIF gene. Boxes indicate conserved leucine residues. Helices in the LIF secondary structure (Robinson et al., 1994) are shaded. Numbers indicate corresponding residues in the mouse LIF-D sequence described by Gearing and co-workers (1987).

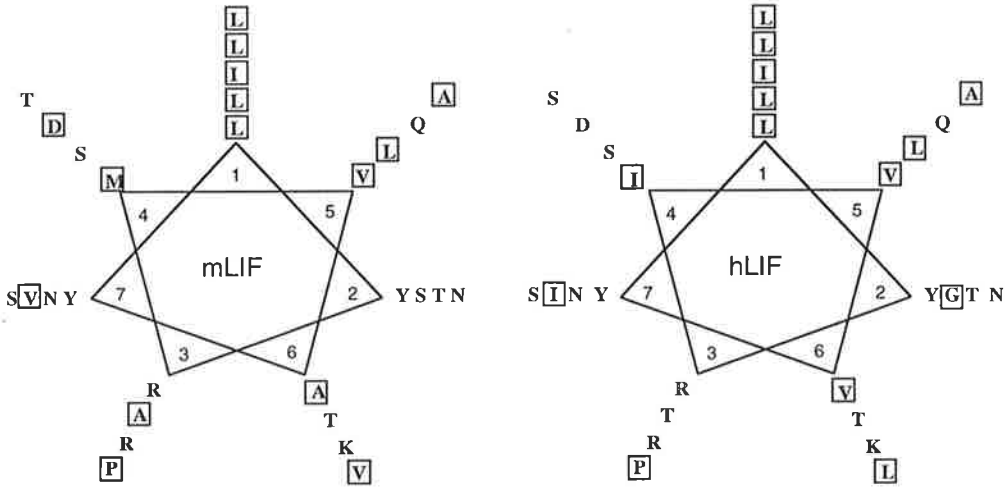
(B) Amphipathicity of α helices in the region of potential leucine zipper motifs present in the human and murine LIF and OSM proteins represented as wheel diagrams. Hydrophobic residues are boxed. Repeated leucines are positioned at residue 1.

(C) Alignment of the mouse LIF leucine-rich motif with the consensus sequence for a leucine-rich repeat (Kobe & Diesenhofer, 1994). Asterisks indicate residues identified by mutation as required for intracellular LIF activity. The valine residue that is non-essential for intracellular LIF activity is underlined. Numbers indicate corresponding residues in the mouse LIF-D sequence described by Gearing and co-workers (1987).

A


Receptor Interaction Domain
Receptor Interaction Domain
L L I L L
helix A
A'
helix B
helix C
helix D
80
120
MOUSE TKLVELYRMVAYLSASLTNITRDQKVLNPTAVSLQVKLNAT
HUMAN A---------I-V--GT--G-------I---S-L--HS-----
RAT ----------T--G--------W---N---------I-----
SHEEP VR--------I----GTA-G-------T-----H--HS-----
PIG AR---------II---G---G-------RS---G--N-HS-----
DUNNART ---IGSS-AT--G-------A---S-LC-

B


mLIF
hLIF

C

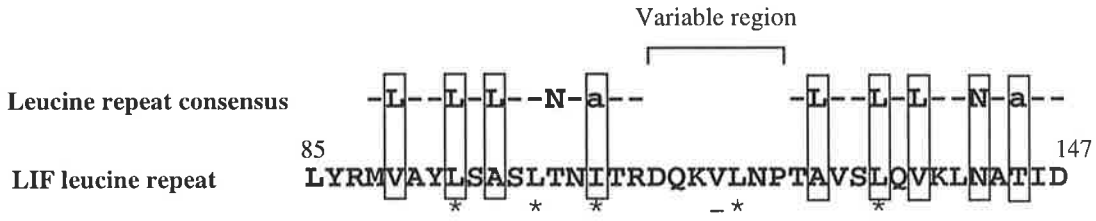
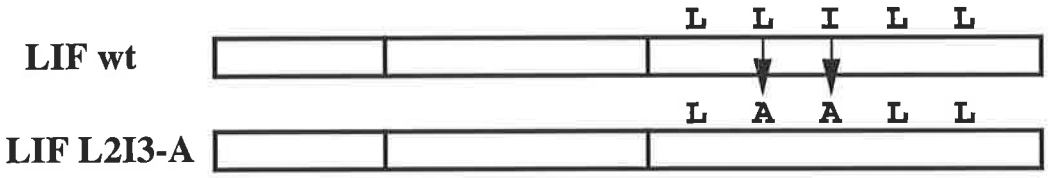

Variable region
Leucine repeat consensus
-L--L-L--N-a--
-L--L-L--N-a--
85
LIF leucine repeat
LYRMVAYLSASLTNITRDQKVLNPTAVSLQVKLNATID
147

Figure 8.3 Mutating the Leucine Repeat Motif of the Mouse LIF Protein Abrogates Intracellular but not Extracellular LIF Activity

(A) Mutation of the wild type mLIF-D and mLIF-T (LIFwt) proteins to give the LIF L2I3 forms of these proteins was performed by primer-directed PCR mutagenesis. This converted the second leucine and the third isoleucine in the heptad repeat of the mLIF-D and mLIF-T proteins to alanines.

(B) LIFwt and LIF L2I3 proteins (mLIF-D and mLIF-T forms of both) were assayed for their intracellular (Cos 1 cell immunostaining) and extracellular (ES cell maintenance assay) activities. The conclusions reached are indicated inside the appropriate quadrants.

A



B

	Intracellular Activity (mLIF-T Form)	Extracellular Activity (hLIF-D Form)
LIF wt	Nuclear/cytoplasmic APOPTOTIC	ACTIVE
LIF L2I3-A	Cytoplasmic NON-APOPTOTIC	ACTIVE

LIF protein under different conditions. For example, the intracellular environment is reducing such that disulphide bonds do not form, while the cytosolic and endoplasmic reticulum compartments contain alternate chaperonins capable of influencing the structure adopted by proteins (Frydman & Hohfeld, 1997). Intriguingly, the C-terminal end of the B helix and the B-C loop have been shown by amide exchange to comprise the most plastic region of the LIF molecule which is otherwise extremely rigid (Yao et al., 1999). Since this region is contained within the leucine repeat required for intracellular LIF activity, its plasticity could promote unzipping of the helical bundle and facilitate protein-protein interactions and the adoption of alternate structures.

LIF action through interaction with proteins involved in intracellular signalling pathways would represent a distinct mode of action from that of other intracellular cytokines. In cases where the mode of action of intracellular cytokines has been characterised, it seems that the cytokine undergoes cell surface receptor-mediated internalisation and traffics to the nucleus as a complex with its receptor (Jans & Hassan et al., 1998; Curtis et al., 1990; Lobie et al., 1994). However, there is some evidence suggesting the existence of a receptor-independent mode of intracellular cytokine trafficking and signalling. FGF 2, for example, has been shown to alter transcription in cell-free systems (Nakanishi et al., 1992; Bouche et al., 1989) and to interact with DNA and intracellular proteins other than its receptor (Amalric et al., 1994; Kolpakova et al., 1998). With regard to the apoptotic intracellular action of LIF, a variety of nuclear transcription factors containing leucine zippers have been implicated in apoptosis, including c-*Myc* (Janicke et al., 1996), ces-2 (Metzein et al., 1996), par-4 (Johnstone et al., 1996), SREBP-1 and SREBP-2 (Wang et al., 1996), CHOP (Matsumoto et al., 1996) and the E2A-HLF oncogene (Inaba et al., 1996). Such factors are therefore candidate interaction partners for the intracellular LIF protein.

These studies thus establish LIF as a unique new member of a growing class of growth factors and cytokines that are found within the cell and appear to have an intracellular action (Jans & Hassan, 1998; Prochiantz and Theodore, 1995). The finding that LIF acts in the intracellular and extracellular environments via different mechanisms and regions of the protein also contributes to an increasing body of evidence suggesting that single proteins can serve multiple functions that confound existing classification systems (Jeffery, 1999).

Since LIF is a highly pleiotropic cytokine, it is possible that apoptosis represents just one of a range of possible outcomes of intracellular LIF signalling. The outcome of intracellular LIF signalling is likely be dependent on the molecular constitution of the responding cell, signals received from the extracellular environment, and the level or stability of intracellular LIF proteins present. Intracellular regulatory molecules such as *myc* proteins have been implicated in a wide range of cellular events and the result of their activity is heavily influenced by many such contextual factors (Vastrik et al., 1994).

8.6 Potential Roles for Intracellular LIF Proteins in Stem Cell Maintenance and Tumourigenesis

There is much evidence implicating the IL-6 family cytokines, including LIF, in the processes of stem cell self-renewal and tumourigenesis. The finding that alternate LIF transcripts encoding intracellularly-retained proteins predominate in some tumour cell lines, particularly embryonal carcinoma cell lines, is therefore intriguing because of the potential for these LIF proteins to form cell autonomous or autocrine signalling loops. Such signalling processes could occur regardless of whether these intracellularly retained proteins are released from the cell. Intracellular or cell-surface receptor mediated LIF signalling could be involved in the early stages of tumourigenesis, playing a role in the removal of constraints upon the self-renewal of tumour cells. During the evolution of the resultant tumour cell population, positive selection pressure for the retention of these signalling processes would generally remain since cells retaining them would be less dependent on the external environment for continued proliferation. Under tumourigenic conditions, a selective advantage may be conferred upon cells expressing high levels of transcripts encoding intracellular LIF proteins and acquisition or retention of high levels of LIF-M and/or LIF-T transcript expression could thus be an important feature of many tumour cell populations. Such cell autonomous or autocrine LIF signalling might also occur in normal cell populations where they could play a role in the regulation of stem cell self renewal or other processes. However, the characteristic LIF transcription profile of human embryonal carcinoma cell lines in which transcripts encoding intracellular proteins predominate suggests that embryonal carcinomas provide a suitable starting point for investigation of intracellular LIF action during tumourigenesis.

The potential involvement of cell-autonomous LIF signalling loops in tumourigenesis is supported by the large body of evidence suggesting that IL-6 can act as an intracellular mitogen in a variety of tumourigenic and non-tumourigenic contexts. The evidence for intracellular IL-6 signalling is based on the ability of antisense oligonucleotides, which can enter the cytoplasm and prevent translation of IL-6 transcripts, but not of anti- IL-6 neutralising antibodies, which can sequester extracellular IL-6, to inhibit the growth of particular cell populations. Leukaemic hairy cells (Barut et al., 1993), malignant melanomas (Lu & Kerbel, 1993), ovarian carcinomas (Watson et al., 1993) and a choriocarcinoma germ cell tumour cell line (Kong et al., 1996) have all been shown to be growth-inhibited by such antisense oligonucleotides but not neutralising antibodies. Furthermore, intracellular IL-6 mitogenic signalling also seems, on the basis of the same evidence, to operate in non-transformed human cells including fibroblasts, vascular smooth muscle cells, and mesangial cells (Roth et al., 1995). A neutralising antibody-resistant, receptor-dependent transcriptional response to LIF has also been reported in hepatocarcinoma cells (Baumann et al., 1993). Thus there are precedents for intracellular signalling and action of LIF and other IL-6 family cytokines and it seems that this manner of signalling can influence the proliferation of tumour cells.

8.7 Alternate Transcript and Protein Production by Other IL-6 Family Cytokines

Accumulating evidence suggests that other IL-6 cytokine genes are alternately transcribed and that these alternate transcripts may encode intracellular proteins. The finding that the murine OSM gene, which is closely linked and highly homologous to the LIF gene, undergoes alternate transcriptional regulation adds significantly to this evidence. There are parallels between the production of the mOSM 13 transcript, in which exon 2 is absent, and the production of LIF (Pergolizzi et al., 1994) and IL-6 (Kestler et al., 1995) transcripts which also lack exon 2. The IL-6 transcript lacking exon 2 has been shown to encode a predominantly intracellular protein and it seems possible that alternate translational processes might produce intracellular proteins from the equivalent OSM and LIF transcripts. Other IL-6 family cytokines might thus share features of

the alternate transcriptional and translational processes elaborated in these studies of the LIF and OSM genes reported here.

The discovery of intracellular stores of OSM proteins in human neutrophils that can undergo regulated release (Grenier et al., 1999) is of great interest since such stores could be produced by translation of OSM 13 transcripts. It is also possible that there are functional analogies between the intracellularly-retained OSM and LIF proteins. Just as the intracellularly-retained LIF proteins may undergo regulated release, intracellular OSM proteins may have an alternate intracellular signalling role similar to that postulated for the intracellular LIF protein. In this regard, it is worthy of note that the murine, human, and bovine OSM proteins all contain an amphipathic leucine-rich motif similar to, and located in an equivalent position to, that required for intracellular signalling by the LIF protein (Figure 8.4). It will therefore be of great interest to determine the nature of any proteins encoded by the OSM 13 transcript and to determine their mode(s) of action.

8.8 Future work

The production of distinct, differentially localised proteins from each of the three alternate transcripts of the LIF gene provides a unique and powerful tool for analysing intracellular and extracellular cytokine activity. The ability to express the intracellular and extracellular proteins independently *in vitro* and *in vivo*, together with the ability to modify the encoded proteins by mutation, provides an excellent model system for the study of intracellular cytokine action.

8.8.1 Demonstration of an Endogenous Intracellular LIF Protein

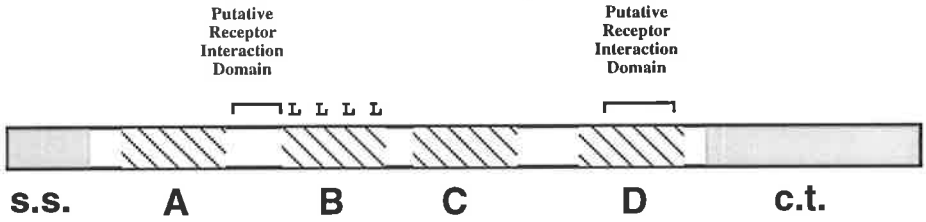
The nature of proteins produced from the LIF-M and LIF-T transcripts has been determined using *in vitro* overexpression systems, however, the production of proteins from these transcripts *in vivo* remains to be confirmed. It would also be of interest to determine whether the proteins produced from the alternate LIF transcripts differ among expressing cell types in terms of their localisation and/or post-translational modification. It is possible for example that only some cell types are competent for production and/or extracellular matrix deposition of secreted hLIF-M encoded proteins or that post-translational modification of the intracellular LIF protein

Figure 8.4 Putative Protein-Protein Interaction Domains in the Murine, Human, and Bovine OSM Proteins

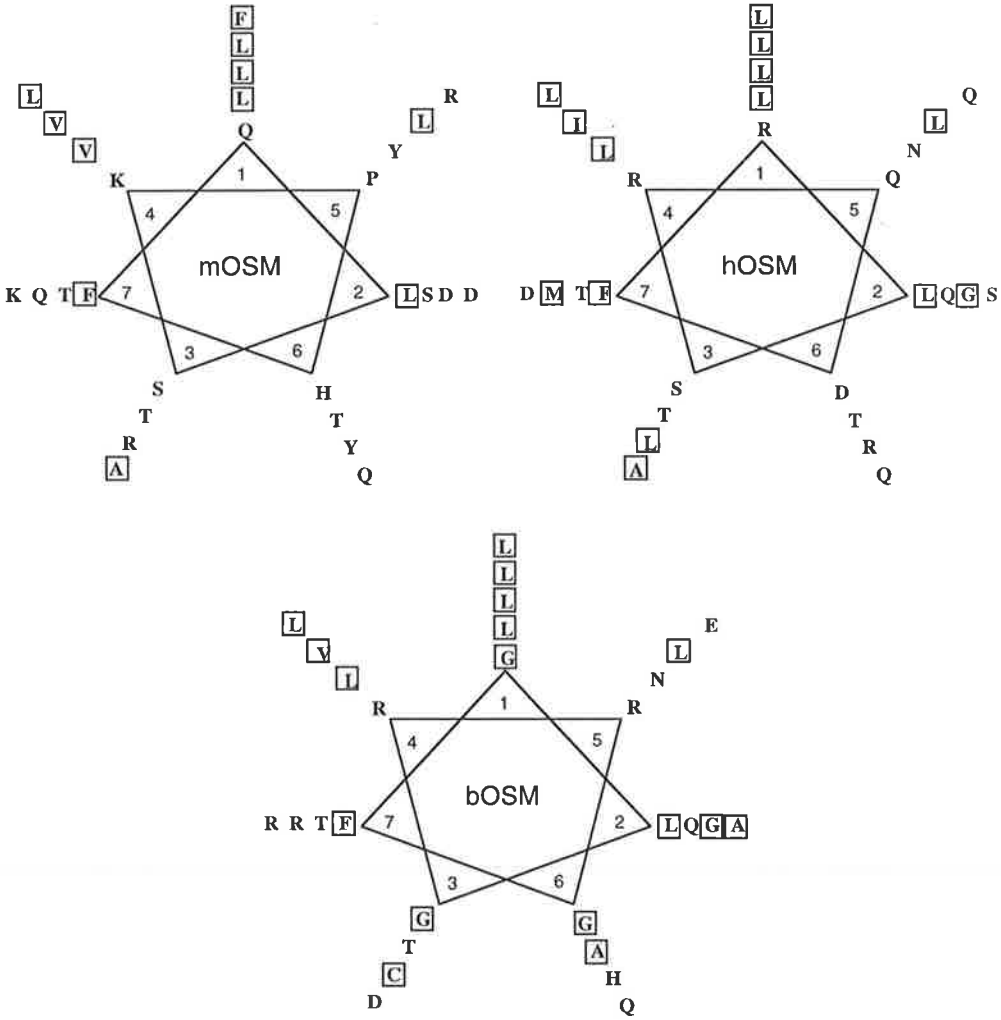
(A) Schematic of the OSM protein showing the position of a conserved leucine heptad repeat relative to the predicted receptor interaction domains (Kallestad et al., 1991; Staunton et al., 1998). Predicted helices, A, B, C, D, in the OSM secondary structure (Kitchen et al., 1998) are cross-hatched. s.s., secretion signal sequence; c.t., C-terminal region cleaved during maturation by specific proteolysis (Linsley et al., 1990)

(B) Amphipathicity of α helices in the region of potential leucine zipper motifs present in the human and bovine OSM proteins represented as wheel diagrams. Hydrophobic residues are boxed. Repeated leucines are positioned at residue 1. mOSM, hOSM, and bOSM sequences are those of Yoshimura and co-workers (1996), Malik and co-workers (1989), and Malik and co-workers (1995) respectively.

A


Putative
Receptor
Interaction
Domain
L L L L
Putative
Receptor
Interaction
Domain
s.s.
A
B
C
D
c.t.

B


mOSM
hOSM
bOSM

affects its interaction with other proteins. Sensitive immunoprecipitation analysis or affinity column techniques could be used to confirm the existence of the intracellular LIF protein *in vivo* either in cultured cell lines or in tissue.

8.8.2 Extracellular Localisation of the Secreted hLIF-M Protein

It is not known whether the secreted proteins translated from the hLIF-M transcript can, like the secreted mLIF-M proteins (Rathjen et al., 1990a), become localised to the extracellular matrix. This possibility could be tested by assay (Rathjen et al., 1990a) and immunoprecipitation (Taipale et al., 1994) of extracellular matrix preparations from appropriately transfected cells. With improved methodologies, it might also be possible to extend such studies to non-transfected cells. Since the secreted proteins produced from the hLIF-M transcript appear to have a distinct translational origin from the secreted proteins produced from the mLIF-M transcript, it will be important to establish, using the approaches mentioned above, whether there is any functional equivalence of these proteins.

8.8.3 Analysis of LIF gene expression

Determination of the *in vivo* function of the different LIF transcripts will be difficult until their sites of expression are elucidated at the cellular level. The sensitivity of *in situ* hybridisation techniques has been sufficient to detect expression of LIF transcripts in some contexts (Nichols et al., 1996; Conquet & Bruket, 1990; Cullinan et al., 1996) but not for the detection of the generally low levels of LIF expression in other tissues. Furthermore, *in situ* hybridisation is not likely to be capable of distinguishing between the alternate LIF transcripts. A gene-targeting-based approach to studying LIF transcript expression *in vivo* has therefore been devised (Whyatt et al., 1997). This approach introduces a β-galactosidase reporter gene into each of the alternate first exons and common second exon of the LIF gene so that those cells expressing β-galactosidase activity can be stained -indicating their expression of the targeted LIF transcript(s). This yields information on the exact sites of LIF transcript production. Since β-galactosidase translation generally initiates at the same sites as the LIF proteins targeted in these constructs, this approach also indicates probable sites of LIF protein production and action. Elucidation of the

precise cellular sites where alternate LIF transcripts and proteins are expressed should allow a more informed approach to functional studies of LIF action *in vivo* and *in vitro*.

8.8.4 Analysis of Intracellular LIF Protein Function

Diverse *in vivo* effects of LIF have been identified by inactivation of the LIF gene in mice. It would thus be of interest to generate mice unable to produce specific LIF transcripts and compare their phenotype to that of mice lacking a functional LIF gene. Since the β-galactosidase marker gene inserted in the alternate first exons of the LIF gene contains a polyadenylation signal at its 3' end (Whyatt et al., 1997), mice homozygous for such tagged LIF alleles could be produced that would entirely lack specific alternate LIF transcripts and their translation products. This may provide information on the role of individual LIF transcripts on developmental decisions *in vivo*. To further study LIF function in vivo, subsequent rounds of tag-exchange gene targeting (Whyatt et al., 1997) could be performed to introduce more subtle mutations at the LIF locus. For example, intracellular and extracellular protein activities could be separately inactivated by introducing leucine repeat mutations to abolish intracellular activity or receptor binding mutations to abolish extracellular activity. This might allow the identification of biological processes with specific requirements for differential localisation and activity of the LIF protein.

Analysis of LIF deficient ES cells has also provided some information about LIF function and stem cell differentiation and renewal during embryogenesis (Dani et al., 1998; Sanderson, 1997; and J. Rathjen, unpublished observations). Similar studies using ES cells lacking expression of specific LIF transcripts could yield more information on the roles of particular LIF gene products in these processes. Differential gene expression analysis of cells either overexpressing or lacking the LIF-T transcript could be employed to analyse possible downstream genes affected by the intracellular LIF protein. Expression vectors with inducible promoters (Whyatt et al., 1993) would be used in this analysis in order to obtain directly comparable cell populations and avoid possibly deleterious effects of constitutive intracellular LIF overexpression. This approach could be particularly powerful if LIF knockout cell lines, such as ES cells, were used. Changes in gene expression induced by expression of intracellular LIF on a LIF knockout background could be examined using differential display PCR analysis or oligonucleotide micro-arrays. This would allow the identification of genes activated or repressed

by expression of the intracellular LIF protein and perhaps indicate which cellular signalling pathways interact with the intracellular LIF protein.

8.8.5 Action of LIF During Tumourigenesis

Since the initial studies of LIF transcript expression were performed using a small range of transformed cell lines, it will be important to try to extend these findings by surveying LIF transcript expression using more cell lines and tumour biopsy material. In the cases of germ cell tumours, it would be of interest to determine the LIF transcription profile of carcinoma *in situ* and seminoma biopsies. If the expression of LIF-M and LIF-T transcripts is significant in germ cell tumourigenesis, it would be expected that these early stage tumours would have similar LIF transcription profiles to the GCT cell lines. This information would ideally be related to the expression of LIF transcripts during normal germ cell development. Comparing the abilities of antisense oligonucleotides directed against each of the exon 1 splice variants of the hLIF transcript and anti-hLIF neutralising antibodies to affect the growth of GCT or other cell lines might also be a practical means to establish the existence of intracellular LIF signalling loops in these tumour cell populations.

8.8.6 Characterisation of Proteins Encoded by the mOSM 13 Transcript

It will be important to determine the nature of the proteins, if any, that are encoded by the mOSM 13 transcript. No antibodies specific to the characterised mouse OSM protein have yet been reported. An initial approach to determining the protein products of the mOSM 13 transcript would therefore be to insert sequence encoding an epitope tag at the 3' end of the open reading frame in the mOSM 13 cDNA (ahead of the cleaved C-terminal peptide). Expression vectors containing this cDNA could be transfected into Cos cells and labelled proteins expressed from the cDNA immunoprecipitated using epitope-specific monoclonal antibodies. Studies similar to those performed in chapter 5 could be performed to further characterise any mOSM 13 encoded proteins.

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Addendum

p. 19 *(Insert sentence before second to last sentence of first paragraph.)*

In the case of polypeptide extracellular signalling molecules, interaction with cell surface receptor proteins may occur while the polypeptide is bound to the membrane of the signalling cell, bound to surrounding extracellular matrix, or in a freely diffusible form.

p.30 *(Insert sentence after second sentence of third paragraph.)*

The cleavage of these membrane-associated preproteins to form diffusible signalling molecules appears to occur in a regulated fashion yet these preproteins are capable signalling while still in their membrane-bound form.

p.35 *(Insert sentence after second to last sentence of s1.3.1.)*

This use of the term "asymetric division" is however somewhat misleading since it does not necessarily imply that differentiated and undifferentiated daughter cells are produced during the same round of stem cell division.

p.38 *(Insert phrase at end of second sentence of final paragraph of s1.3.4)*

...and the methylation statuts of other imprinted genes in EG cells varies with respect to the developmental stage at which the EG cells were derived and is different from that of ES cells (Tada, T., Tada, M., Hilton, K., Barton, S.C., Sado, T. and Surani, M.A., 1998, *Dev. Genes Evol.*, 207:551-61).

p.58 *(Insert sentences at end of first paragraph of s1.6.2)*

However, an alternative model of adult-onset GCTs suggests that primordial germ cells or even earlier pluripotential cells that have undergone defective or incomplete development persist in later life and accumulate genetic changes that result in their neoplastic transformation (Raepert-De Meyts, E., Jorgenson, N., Brondum-Nielson, K., Muller, J. and Skakkebeck, N.E., 1998. *APMIS* 106:198-204). In fact, the variation in the epidemiological, pathological and histological manifestion of GCTs suggests that they could have multiple cellular origins.

p.165 *(Insert sentences after first sentence of first paragraph.)*

The possibility that the apoptotic effect of the intracellular LIF proteins may occur only in the context of their overexpression must also be considered. Nevertheless, the apoptotic effect does indicate that the intracellular LIF proteins are cappable of interaction with important intracellular signalling pathways.

p.169 *(Insert paragraph as first paragraph of s8.8.4.)*

An important next step in the characterisation the alternate LIF proteins will be the study of their sub-cellular distribution using confocal immunofluorescence microscopy. This technique has been employed with the mLIF proteins and allows colocalisation with markers for particular intracelular compartments and cellular components to be studied. For example, mLIF-D staining was found to colocalise with Alexa 594-conjugated concanavilin A, a lectin which binds specificly to glycoproteins of the endoplasmic reticulum (Haines, B., Voyle, R.B., & Rathjen, P.D, 2000, *Mol. Biol. Cell* 11:1369-1383). Employment of this technique to determine the localisation of the hLIF proteins at the sub-cellular level would provide important clues as to their possible function.

Table 1.2 *(Add to legend at bottom.)*

SS= Signal Sequence, NLS= Nuclear Localisation Sequence

Figure 4.1 *(Insert at end of legend.)*

Note that the use of the polylinker *Eco* R1 and *Hin* dIII restriction sites to produce linearised transcription templates ensures that the size of undigested and protected probe fragments can be distinguished by differences in their length (36 and 42bp diffences for phLIF-$MS1_{RACE}$ and phLIF-$TS1_{RT}$ respectively).

Figure 5.12 *(Insert at end of legend.)*

Proteins were immunoprecipitated from Cos 1 cell lysates.No LIF proteins were detected in immunoprecipitates of media conditioned by these cells.