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Complex Conserved Organization of the Mammalian Leukemia Inhibitory Factor Gene: Regulated Expression of Intracellular and Extracellular Cytokines

Bryan P. Haines, Roger B. Voyle, Tricia A. Pelton, Regan Forrest, and Peter D. Rathjen

Leukemia inhibitory factor (LIF), a secreted glycoprotein belonging to the IL-6 family of pleiotropic cytokines, is extensively involved in modulating hematopoiesis and immunity. We have undertaken a detailed analysis of LIF genomic organization and gene transcription and investigated the proteins expressed from alternate transcripts. Previously unidentified LIF transcripts, containing alternate first exons spliced onto common second and third exons, were cloned from murine embryonic stem cells, human embryonal carcinoma cells, and primary porcine fibroblasts. Based on sequence homology and position within the genomic sequence, this confirmed the existence of the LIF-M transcript in species other than the mouse and identified a new class of transcript, designated LIF-T. Thus, a complex genomic organization of the LIF gene, conserved among eutherian mammals, results in the expression of three LIF transcripts (LIF-D, LIF-M, and LIF-T) differentially expressed from alternate promoters. The first exon of the LIF-T transcript contained no in-frame AUG, causing translation to initiate downstream of the secretory signal sequence at the first AUG in exon two, producing a truncated LIF protein that was localized within the cell. Enforced secretion of this protein demonstrated that it could act as a LIF receptor agonist. Regulated expression of biologically active intracellular and extracellular LIF cytokine could thus provide alternate mechanisms for the modulation of hematopoiesis and immune system function. The Journal of Immunology, 1999, 162: 4637–4646.
to these factors (23). Many growth factors and cytokines have been shown to be localized within the cell, often in the nucleus. In some cases, there is direct evidence that this localization is required for particular biological activities (24–26).

In this regard, the cellular distribution of IL-6 family cytokines is intriguing. Although LIF, oncostatin-M, IL-11, and IL-6 can all be found in the extracellular space, ciliary neurotrophic factor and cardioporphin-1 are expressed without signal sequences and have no known mechanism for secretion from the cell, although both are able to act via specific cell-surface receptors (27, 28). An alternately spliced human (h) IL-6 transcript, identified in PBMC, appears to encode an intracellular IL-6 protein that lacks a functional signal sequence (29). Furthermore, the resistance of cytokine activity to neutralizing Abs has been interpreted as evidence for the possible existence of intracellular LIF protein in human hepatoma cells (30), for intracellular autocrine action of IL-6 in the proliferation of leukemic hairy cells and choriocarcinoma (25, 31), and in melanoma progression (32). Thus, although IL-6 family cytokines can signal through cell surface receptor complexes containing the gp130 receptor subunit, there is evidence for alternative intracellular localization and action of some of these proteins.

In this work, we describe a detailed investigation of LIF gene organization and transcription. We report a complex arrangement of the LIF gene that is conserved among eutherian mammals that provides a mechanism for the alternate localization of cytokines within or outside the cell. Three alternative first exons can be spliced to common second and third exons, yielding three transcripts whose transcription is regulated independently. LIF transcripts that contain an ATG in exon 1 code secreted LIF proteins, while those that lack an ATG in exon 1 initiate translation downstream of the signal sequence within exon 2, producing a truncated, biologically active LIF protein that is localized within the cell. Conservation of this genomic arrangement across species and the differential expression of the individual LIF transcripts in vitro and in vivo implies an important biological role for the intracellular LIF protein.

**Materials and Methods**

**PCR-cloning of novel LIF transcripts**

A mLIF-T cDNA was cloned by rapid amplification of cDNA ends PCR (RACE-PCR) on chick embryonic stem (ES) cell cDNA as described by Rathjen et al. (20). The PCR product was digested with XhoI/SmaI, purified from agarose, and cloned into SalI/SmaI-digested pT7T3 19U (Pharmacia, Piscataway, NJ) to give the plasmid pmLIF-T-TSI. LIF clones were sequenced by double-stranded dyeoxy chain termination sequencing using a T7 sequencing kit (Pharmacia). A LIF-T cDNA was cloned by constructing the 3′ SmaI/EcoRI fragment of the mLIF cDNA from pDR1 (20) into Smal/EcoRI-digested pmLIF-TSI to produce pmLIF-T.

cDNAs for porcine (p) transcripts plIF-T and plIF-M, hLIF-T, and mLIF-T were amplified by RT-PCR. A total of 500 ng oligo d(T) primer was used in a 20-μL reaction containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1 mM DTT), dNTPs (1.5 mM each), RNasin (40 U), oligo(dT) primer (500 ng), and 5 μL avian myeloblastosis virus RT (Molecular Genetic Resources, Tampa, FL). cDNA was diluted 10-fold in water, and 5 μL was used in a 20-μL PCR reaction containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin), 0.5 mM dNTPs, 20 pmol of each 5′ and 3′ primers, and 0.5 U Taq polymerase (Bresatec, Adelaide, Australia) reactions were cycled at 94°C for 5 s, 55°C for 5 s, and 72°C for 60 s for 45 cycles using a FTS-1 capillary thermal cycler (Corbett Research, Mortlake, Australia). PCR products were analyzed by Southern blot using an oligolabeled LIF cDNA probe (nucleotides 11–657; Ref. 33) from pDR1.

plIF-T was amplified from porcine primary fibroblast RNA using the 5′ primer 5′-AAAGAATTCATGGAAGCCAGGAAGCGGTGTAAG3′ (pLIF-TRT2) and the 3′ primer 5′-AAGAATTCACCTGGCCAGCAUGCGACCTGGA3′ (pLIF-3UT), followed by amplification using the 5′ primer plIFLTRT2 and a nested 3′ primer 5′-GGCGCCAGGCGGACACTGACAT

T294-3′. A plIF-T DNA was cloned by digestion of PCR products with EcoRI and PvuII and ligation into EcoRI-digested plBluescript II KS+ (Stratagene, La Jolla, CA) to give plIF-TPII. plIF-M was amplified using the 5′ primer 5′-TAGAATTCCTGGAAGCCAGGAAGCGGTGTAAG3′ and the 3′ primer plIF-3UT and was cloned by digestion of PCR products with EcoRI and ligation into EcoRI-digested plBluescript II KS+ to give plIF-M. Nucleotides are specified as in Willson et al. (22) with numbers positioned 3′ of engineered restriction sites. hLIF-T was amplified using the 5′ primer 5′-GAAGAACCTGGCTACCTCGACCTC3′-3′ and 3′ primer ATAGGATCCGGCGTTGACCCTTTG3′. An hLIF-T cDNA was cloned by digesting PCR products with EcoRI and Smal and cloning into Smal/EcoRI-cut plBluescript II KS yielding the plasmid plIF-TTI. Nucleotides are specified as in Stahl et al. (34).

mLIF cDNAs were amplified using the primers 3′-CTTTGCCTTCGGGGTTGAUGTT-5′ (538G) and 5′-GGCGTTGACCCTTTG3′-3′ (583G). mLIF-specific PCR was conducted using the LIF-T 5′ primer 5′-GACATTCCCTGGCTACCTC3′-3′ (2360) and 583G. Nucleotides are specified from Stahl et al. (34).

**Nucleic acid manipulations**

DNA manipulations and cloning were conducted using standard techniques (38).

Expression vectors were based on the vector pXMT2 (20). Deletion mutants mLIF-I and mLIF-269 were isolated from mLIF RACE-PCR cloning reactions. Complete open reading frames were constructed in pT7T3 19U by cloning the 3′ SmaI/EcoRI fragment of mouse LIF from pDR1 (20) into Smal/EcoRI-digested mLIF-91 and mLIF-269 to produce the plasmids pmLIF-91 and pmLIF-269. Expression vectors for mLIF-T, mLIF-91, and mLIF-269 were generated by digesting pmLIF-T, pmLIF-91, and pmLIF-269 with PstI and EcoRI and cloning the LIF cDNA into PstI/EcoRI-digested pXMT2, producing the expression vectors pmLIF-TX, pmLIF-91X, and pmLIF-269X, respectively. The mLIF-D expression vector, pDR10, has been described previously (20). The LIF EXTRA cDNA was constructed by excising an EcoRI LIF-D fragment from pDR1 and cloning it into EcoRI-cut pUC18/BanII, a pUC18 vector modified by digestion, end-filling, and destruction of the BanII restriction site. PCR was conducted, the resulting plasmid pLIF-D BanII was digested ATAGAAGCCCTTGAAGCCAGATCGAAC3′ and a T3 primer (Stratagene, La Jolla, CA). The PCR product, which contains mLIF sequence downstream of residue 151 (33), was digested with BanII/HindIII and fused with the LIF-D signal sequence/proteolytic cleavage site by cloning into BanII/HindIII-digested pmLIF-D BanII to produce pmLIF-D EXTRA. An expression vector for LIF-T EXTRA (pmLIF-T_EXTRA) was produced by cloning the EcoRI LIF fragment from pmLIF-T_EXTRA into EcoRI-cut pXMT2.

The vectors pmLIF-DHI and pmLIF-THI were used for antisense probe preparation. pmLIF-DHI was constructed by digesting pDR2 (20) with EcoRI and HindIII, end-filling, and cloning the 5′ fragment of the mLIF-D cDNA (position 1 to position 168; Ref. 33) into Smal-digested pT7T3 19U. pmLIF-THI was constructed by digesting pmLIF-D with PstI and HindIII, end-filling, and cloning the 5′ fragment of the mLIF-D cDNA (position 1 to position 225, equivalent to position 168 of the mLIF-D cDNA; Ref. 33) into Smal-digested pT7T3 19U.

RNA from cultured cells was isolated by the method of Edwards et al. (39). Tissue RNA isolated from day 16.5 postcoytoembryo and adult CBA strain mice was isolated according to the method of Chomczynski and Sacchi (36). RNAs from Ehrlich-Lettre ascites carcinoma cells, STO and C3H 10T/12 embryonic fibroblasts, and PYS 2 and CP1 ES cells were described by Rathjen et al. (20). Preparation of RNA from GCT 27/C4 embryonal carcinoma cells, 293T adenovirus (SV40-transformed kidney fibroblasts, and HeLa epithelial carcinoma cells is to be described elsewhere (71).

**Antisense riboprobes and RNAse protection analysis**

mLIF-D-specific antisense RNA probes were generated from HindIII-linearized pDR2 (20) and EcoRI-linearized pmLIF-DH. An mLIF-T-specific probe was generated from mLIF-THI. Radioactive mLIF riboprobes were transcribed with T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany) using the method of Rathjen et al. (20), except that riboprobe transcription reactions contained 250 μCi of [-32P]UTP (Bresatec). The rat glyceraldehyde-3-phosphate dehydrogenase (GAP) riboprobe was prepared as described previously (37) using 20 μCi of [-32P]UTP and 0.1 mM unlabeled UTP. mLIF-M and mLIF-D riboprobes containing sequence from exon 1 to the unique Smal site in exon 3 were transcribed from plIF-M1S and plIF-TS1 (71). mLIF-specific riboprobes were prepared in essentially the same manner as mLIF-specific
riboprobes except that pHIF-MS1 was linearized with EcoRI and transcribed using T7 RNA polymerase, and pHIF-TS1 was linearized with BamHI and transcribed using T3 RNA polymerase (Boehringer Mannheim). A riboprobe for hGAP was prepared by linearization of pGAPM (Dr. G. Goodall, Adelaide, Australia) with Ddel followed by transcription with T7 RNA polymerase as for the rat GAP riboprobe. RNase protections were conducted on 10–20 μg of total RNA as described previously (20), except that all digestions were conducted at 37°C for 1 h and RNase digestion products were visualized by phosphorimagier analysis (ImageQuant software package, Molecular Dynamics, Sunnyvale, CA).

**Cos 1 cell transfection**

Cos 1 cells were cultured in DMEM containing 10% FBS. For transfection, Cos 1 cells were grown to near confluence, harvested by trypsinization, washed twice with electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 6 mM glucose), and resuspended in electroporation buffer at 1 × 10⁶ cells/ml. A total of 5 × 10⁶ cells were added to an electroporation cuvette (Bio-Rad, Hercules, CA) containing 50 μl FBS, 50 μl salmon sperm DNA (10 mg/ml), and 10 μg experimental plasmid DNA and were incubated at 4°C for 10 min. Cells were electroporated at 270 volts (capacitance, 250 μF) using a Bio-Rad Gene Pulser electroporator, incubated at room temperature for 10 min, and grown in 10-cm plates containing 10 ml of DMEM/10% FBS.

**Protein analysis**

For analysis by Western blot, transfected Cos 1 cells were cultured for 72 h, harvested, and protein extracts produced by cell lysis using a single-detergent lysis buffer and separated by SDS-PAGE on 15% polyacrylamide gels. Proteins were transferred to nitrocellulose using a semidyrid Western transfer apparatus (Pharmacia) according to the manufacturer’s instructions. Western blots were blocked with buffer 1 (100 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1% Tween 20) containing 2% BSA overnight, washed with buffer 1, and incubated with a 1/250–1/500 dilution of anti-LIF polyclonal Ab (a kind gift of Dr. A. G. Smith, Centre for Genome Research, Edinburgh, U.K.) in buffer 1 overnight. After incubation, filters were washed three times with buffer 1 and incubated with alkaline phosphatase-conjugated anti-rabbit secondary Ab (1/10,000 dilution in buffer 1; Sigma, St. Louis, MO) for 3 h. Filters were subsequently washed once with buffer 1 and twice with buffer 2 (100 mM Tris-HCl, pH 9.5, 100 mM HCl, 5 mM MgCl₂) and developed by the addition of 300 μg/ml nitroblue tetrazolium and 200 μg/ml 5-bromo-4-chloro-3-indoly phosphate (BCIP) in buffer 2.

For analysis by immunoprecipitation, transfected Cos 1 cells were cultured for 48 h, washed once in PBS and once in methionine/cysteine-deficient DMEM, then starved in methionine/cysteine-deficient DMEM supplemented with 2 mg/l-glutamine for 30 min and labeled in 2 ml of labeling mix (2 ml methionine/cysteine-deficient DMEM/complete DMEM (14:1), 2 mM l-glutamine, 50 μCi Tran-35S Label (ICN, Costa Mesa, CA)) for 5 h. Following labeling, immunoprecipitation of samples was conducted at 4°C. Labeled cells were washed once with PBS before the addition of 1 ml NP40 lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF) and incubation for 30 min with agitation. Cells were harvested and centrifuged at 15,000 rpm for 10 min to pellet cell debris. The cell lysate was transferred to a fresh tube and precleared by incubation with 100 μl of 10% protein A Sepharose 4-100 (Pharmacia) slurry for 30 min with gentle agitation followed by centrifugation. A 1/50 dilution of caprylic acid-purified anti-mLIF polyclonal Ab was added to the supernatant in a fresh tube and incubated for 2 h with agitation. Then, 100 μl of 10% protein A Sepharose slurry, previously blocked by incubation with 2% nonfat milk powder for 2 h, was then added and incubated a further 2 h with agitation. The Sepharose beads were pelleted by centrifugation and washed three times with lysis buffer (3 × 1 ml). Immunoprecipitates were electrophoresed under reducing conditions on 18.75% denaturing gels by standard techniques (38) and dried onto blotting paper before phosphorimagier analysis (ImageQuant software package, Molecular Dynamics, Sunnyvale, CA).

**LIF genomic sequence analysis**

Analysis of LIF genomic sequences for putative transcription factor binding sites was conducted using MadInspector to search the Transfac transcription factor database (39).

**Results**

**Identification and cloning of a novel mLIF transcript: mLIF-T**

LIF expression in cultured mouse cell lines was investigated using the RNase protection system described by Rathjen et al. (20) (Fig. 1A). In addition to bands at 369 and 349 bp, representing the mLIF-D transcript, and 345 bp, corresponding to transcripts that diverge from mLIF-D at the exon 1/exon 2 boundary (mLIF-M/T). Antisense probes derived from pDR2 protect bands of 369 bp, corresponding to the mLIF-D transcript, and 345 bp, corresponding to transcripts that diverge from mLIF-D at the exon 1/exon 2 boundary (mLIF-M/T). Antisense probes derived from pmLIF-DH1 yield protection products of 158 bp and 134 bp, respectively. B, RNase protection analysis of 10 μg of RNA from Ehrlich ascites (EA), STO, 10T1/2, PYS 2, and ES cells using an antisense mLIF riboprobe transcribed from pDR2. C, RNase protection of 20 μg of MBL-5 ES cell RNA using an antisense mLIF riboprobe transcribed from pmLIF-DH1. Size differences at the exon 1/2 boundary are due to incidental homology at the 3′ ends of the alternate mLIF-D and mLIF-M first exons.

**LIF biological activity assay**

Transfected Cos 1 cells plated into 10-cm plates containing DMEM/10% FBS and cultured for a 24-h recovery period were washed once with incomplete ES cell medium and incubated for 48 h in 10 ml of incomplete ES cell conditioned medium. Conditioned medium was removed and passed through 2-μm filters (Millipore, Bedford, MA). MBL-5 ES cells were grown in 24-well trays (15 mm well diameter; Becton Dickinson Europe, Meylan, France) seeded at 500 cells per well in 500 μl of various dilutions of Cos 1 cell-conditioned medium. LIF activity was determined by assaying for ES cell differentiation, after 5–6 days in culture, using an alkaline phosphatase detection kit (Sigma).

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mLIF-M, and mLIF-T transcripts (Fig. 1C). It also demonstrated that mLIF-T was expressed in ES cells at similar levels to mLIF-D, arguing against the possibility that this transcript represented a low abundance transcriptional mis-start. Discrimination of the mLIF-T transcript from the mouse D and M transcripts by this probe indicated that mLIF-T diverged from the previously described transcripts at the 5′ end of the message. The size of the protected bands established that the position of divergence was ~134 nucleotides upstream of the HindIII restriction site in the mLIF cDNA (33), near the exon 1/exon 2 boundary.

The novel 5′ sequences of the mLIF-T transcript were cloned from Cpi ES cell RNA by RACE-PCR using the protocol described by Rathjen et al. (20). The cDNA diverged in sequence from the characterized mLIF-D and mLIF-M cDNAs precisely at the exon 1/exon 2 boundary and contained a novel 91-bp first exon spliced to the common second and third exons of mLIF (Fig. 2A).

The LIF-T first exon, designated exon 1T, was located in the mLIF genomic sequence between the first exon of the mLIF-M and mLIF-T transcripts (Fig. 1C). Alignments of the mLIF-M and mLIF-T sequences with the mLIF-D sequence indicated that mLIF-T was expressed in ES cells at similar levels to mLIF-D, arguing against the possibility that this transcript represented a low abundance transcriptional mis-start. Discrimination of the mLIF-T transcript from the mouse D and M transcripts by this probe indicated that mLIF-T diverged from the previously described transcripts at the 5′ end of the message. The size of the protected bands established that the position of divergence was ~134 nucleotides upstream of the HindIII restriction site in the mLIF cDNA (33), near the exon 1/exon 2 boundary.

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The LIF-T first exon, designated exon 1T, was located in the mLIF genomic sequence between the first exon of the mLIF-M transcript and the second exon (nucleotides 1791–1881; Ref. 34). This exon contained a conserved splice donor site (see Fig. 7C). In contrast with the previously described mLIF first exons, exon 1T contained no potential AUG initiation codons in-frame with the mLIF open reading frame (Fig. 2A). This observation, together with the presence of an in-frame stop codon at the 5′ end of the cDNA (boxed in Fig. 2A), indicates that translation of the mLIF open reading frame could not initiate within the LIF-T first exon.

The mLIF-T cDNA was validated by RNase protection and RT-PCR analysis of ES cell RNA. A riboprobe synthesized from the LIF-T cDNA (LIF-THI, Fig. 2B) protected two mRNA species. A fragment of ~134 bp (Fig. 2C), the distance between the 3′ end of the probe and the exon 1/exon 2 boundary, corresponded to the mLIF-D and mLIF-M transcripts. An additional protected fragment of 225 bp in ES cell RNA (Fig. 2C) confirmed that a mLIF transcript with complete sequence homology to the mLIF-T cDNA was expressed at levels similar to the mLIF-D transcript in ES cells. RT-PCR analysis of ES cell cDNA was conducted using primers specific for the mLIF-T first exon (2360) and mLIF exon 3 (583G) (Fig. 2B). Amplification of a single PCR product of 443 bp (Fig. 2D) confirmed the authenticity of the mLIF-T cDNA.

The LIF-M and LIF-T transcripts are conserved among eutherian mammals

Two previously unidentified hLIF transcripts, cloned from GCT 27/C4 human embryonal carcinoma cell cDNA by RACE-PCR (71), were found to contain alternate first exons spliced to the common second and third exons of the hLIF gene. The first exon sequences demonstrated significant homology to the mLIF-T and mLIF-M sequences (Fig. 3, A and B) and corresponded in their genomic positions to the mLIF-M and mLIF-T first exons (Fig. 3C). These data suggested that the alternate hLIF transcripts were the human equivalents of mLIF-M and mLIF-T. The LIF-T cDNA contained a 2-bp substitution and a 9-bp insertion (Fig. 3A) that were not present in the reported genomic sequence for hLIF (34). Both the substitution and the insertion were found in a genomic PCR product spanning this region and in an RT-PCR product obtained from GCT 27/C4 cells (data not shown).

Alignment of human and mouse mLIF-M and LIF-T sequences with other reported mammalian LIF genes (22, 34, 41) suggested a more widespread conservation of the LIF-M and LIF-T transcripts (see Fig. 7, B and C). RT-PCR was used to investigate the expression of these predicted transcripts in porcine primary fibroblasts. Primers directed against the predicted pLIF-T and pLIF-M first exons, in combination with a primer located in the 3′ untranslated region of the pLIF gene, directed amplification of single LIF products that were cloned and sequenced. Both sequences comprised alternate first exons spliced to the second and third exons of the pLIF gene. These first exons showed the predicted homology with the mouse and human LIF-M and LIF-T exons, respectively (Fig. 3, A and B), and identical sequences were found at appropriate positions within the pLIF genomic sequence (Fig. 3C).

Molecular identification and cloning of the LIF-M and LIF-T transcripts from human, mouse, and porcine cells indicates that the complex organization of the LIF gene, in which three alternate first exons can be spliced to common second and third exons, is widely conserved among eutherian mammals. This clarifies previous confusion regarding the conservation of alternate LIF transcripts in species other than the mouse (22, 34).

Alternate LIF transcripts are regulated independently in human and mouse

Independent regulation of the mLIF-D and mLIF-M transcripts has been described previously in vitro and in vivo (18–20). Expression...
transcripts were not detected (HeLa) or were expressed at levels of hLIF-D and hLIF-T (Fig. 4A). In some cell lines, such as 293T, hLIF-M was the dominant transcript, being expressed at higher levels than the combined levels of mLIF-T and mLIF-D (Fig. 4C). In other lines, mLIF-M transcripts were not detected (HeLa) or were expressed at levels similar to hLIF-D (GCT 27/C4). hLIF-T transcripts were expressed at variable but relatively low levels in diverse cultured cell lines, including 293T and GCT 27/C4 (Fig. 4D). However, other cell lines, including HeLa, did not express detectable hLIF-T transcripts.

These results demonstrate that expression of the three alternate LIF transcripts is differentially regulated in two mammalian species, indicating that each transcript may serve a biologically distinct and important role.

A novel, N-terminally truncated 17-kDa LIF protein

Translation of the secreted LIF cytokine initiates at an AUG initiation codon in exon 1D. The mLIF-T, hLIF-T and hLIF-M, pLIF-T and pLIF-M transcripts lack this AUG initiation codon and contain no other candidate initiation sequences in-frame with the LIF open reading frame (Fig. 3A). In the case of hLIF-T, pLIF-T, and pLIF-M, the presence of an in-frame termination codon in exon 1 indicated that translation could not initiate upstream of this position. In the mLIF-T transcript, three potential translational initiation sites in-frame with the mLIF open reading frame were identified downstream of exon 1: a grouping of CUGs and GUGs at the 5′ end of exon 2 and in-frame AUG codons in exon 2 and at the 5′ end of exon 3 (Fig. 5A). The first but not the second in-frame AUG, and several CUGs and GUGs, are conserved in all other reported LIF sequences (22, 34, 41).

To examine proteins encoded by mLIF transcripts, Cos 1 cells were transfected with plasmids directing expression of the mLIF-D (pDR10) or mLIF-T (pmLIF-TX) cDNAs. Cell lysates from transfected cells were examined for expression of mLIF proteins by Western blot (Fig. 5B) using an anti-mLIF polyclonal antiserum. No LIF protein was detected in lysates from cells transfected with pXMT2 (not shown but see Fig. 5B, mLIF-269). Translation of the mLIF-D transcript resulted in a ladder of LIF proteins ranging in size from 20 kDa, the size of mature, unglycosylated LIF (43), to ~40 kDa. These represent alternate glycosylation variants of the secreted LIF protein (71). Translation of the mLIF-T transcript produced a protein of ~17 kDa, consistent with translation of a 158-amino acid protein from the first in-frame AUG located within exon 2. This protein would be N-terminally truncated by 22 amino acids relative to the 20-kDa mLIF-D protein that results from cleavage of the signal peptide.

The site of translation initiation for the novel mLIF protein was refined by analysis of LIF expression from 5′ deletion mutants of the mLIF-T cDNA. The endpoints of the mLIF-T cDNAs are indicated in Fig. 5A. Clone pmLIF-91 was a 5′ deletion of the mLIF-T cDNA to position 91 (33), between the potential CUG/GUG initiation codons (42) and the first in-frame AUG. pmLIF-269 was a 5′ deletion of the mLIF-T cDNA to position 269 (33), between the first and second in-frame AUG codons. Deleted cDNAs cloned into pXMT2 (pmLIF-91X and pmLIF-269X) were overexpressed in Cos 1 cells, and cell lysates were analyzed by Western blot. Translation of mLIF-T and mLIF-91 resulted in expression of the 17-kDa mLIF protein, while no mLIF protein was detected in cells transfected with the mLIF-269 cDNA (Fig. 5B). Therefore, translation of the 17-kDa protein from the mLIF-T transcript was initiated in exon 2 between residues 91 and 269. This region includes the first in-frame AUG in the mLIF-T transcript.

The 17-kDa LIF protein is an agonist for the LIF receptor and localized within the cell

Secretion of the LIF protein translated from the LIF-D transcript is directed by a signal sequence encoded by the 5′ end of exon 2 (33). Translation of this sequence is dependent upon the presence of an upstream initiation codon, which is not present in any cloned...
LIF-T transcript, or the pLIF-M and hLIF-M transcripts. Because translation of the 17-kDa LIF protein identified in this work is initiated downstream of the secretion signal sequence, this N-terminally truncated LIF protein would lack a conventional mechanism for secretion. Therefore, it was of interest to determine the cellular localization of this protein and whether it is capable of signaling through cell surface receptors.

To examine the cellular localization of the 17-kDa LIF protein, Cos 1 cells were transfected with expression plasmids pXMT2, pDR10, and pmLIF-TX, and cell lysates and conditioned media were immunoprecipitated using an mLIF-specific Ab (Fig. 6, A and B). Translation of the mLIF-D transcript yielded the previously described ladder of glycosylation variants, which were found at high levels in cell lysates and conditioned medium (Fig. 6, A and B), as expected for a secreted protein. Translation of the mLIF-T cDNA resulted in expression of high levels of the 17-kDa LIF protein in cell lysates with negligible levels in medium conditioned by the transfected cells (Fig. 6, A and B), indicating that the 17-kDa LIF protein is localized within the cell. This has been confirmed by immunolocalization6 and demonstrated for proteins encoded by equivalent hLIF transcripts (71). The extremely low levels of 17-kDa protein detected in conditioned medium are thought to result from limited cell lysis.

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Murine ES cells can be used as an assay for LIF biological activity because they depend on signaling by LIF or other IL-6

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**FIGURE 4.** Differentially regulated expression of alternate mLIF and hLIF transcripts. A, RNase protection analysis of mLIF transcripts expressed in mouse embryonic and adult tissues. A total of 10 μg of day 16 embryonic and adult tissue total RNA was protected with the LIF riboprobe-derived from pmlIF-THI (Fig. 2B) and the rat GAP riboprobe (37) as a loading control. B, Graphical representation of mLIF-T expression levels as a percentage of mLIF-D and mLIF-M expression levels. C, RNase protection of hLIF-M transcripts in human cell lines. A total of 20 μg of cytoplasmic RNA was protected with hLIF-M- and hGAP-specific riboprobes. tRNA, yeast transfer RNA. Species protected by the hLIF riboprobes arise in a manner similar to that depicted for mLIF protected species in Fig. 1A. The hLIF-M riboprobe protected two species: one containing exons 1 plus 2 plus 3 (hLIF-D plus hLIF-T) and one containing exons 1 plus 2 plus 3 (hLIF-M). D, RNase protection of hLIF-T transcripts in human cell lines. A total of 20 μg of cytoplasmic RNA was protected with hLIF-T- and hGAP-specific riboprobes. The hLIF-T riboprobe protected two species: one containing exons 1 plus 2 plus 3 (hLIF-D plus hLIF-M) and one containing exons 1 plus 2 plus 3 (hLIF-T). C and D show lanes merged to form panels derived from single phosphorimages of the same gel.

**FIGURE 5.** Translation of the mLIF-T cDNA. A, Diagrammatic representation of the mLIF-T cDNA and deletion clones used to analyze translational initiation site selection. mLIF-91 contains sequences downstream of nucleotide 91 of the mLIF sequence (33). mLIF-269 contains sequences downstream of nucleotide 269 of the mLIF sequence. The relative positions of three potential translational initiation sites are shown. B, Western blot analysis of protein extracts from Cos 1 cells, transiently transfected with the expression vectors pDR10 (mLIF-D), pmLIF-TX (mLIF-T), pmLIF-91X (mLIF-91), and pmLIF-269X (mLIF-269). Bacterially expressed recombinant mLIF (20 kDa; Ref. 43) was obtained from Amrad (Melbourne, Australia).
family cytokines for continued maintenance in an undifferentiated state (44). Therefore, medium conditioned by Cos 1 cells transfectected with pXMT2 was unable to support the growth of undifferentiated ES cells; however, media conditioned by cells transfected with pDR10 (mLIF-D) supported the growth of undifferentiated ES cells at dilutions of 1/20,000 (Fig. 6D). By comparison, medium conditioned by cells transfected with mLIF-TX (mLIF-T) could not support the growth of undifferentiated ES cells past a 1/10–1/50 dilution, consistent with the negligible levels of LIF protein detected by immunoprecipitation and the intracellular localization of this protein.

Secretion of the normally intracellular 17-kDa mLIF protein was enforced by construction of a chimeric cDNA in which the open reading frame encoding the 17-kDa intracellular protein was fused at the N terminus with the secretion signal and proteolytic cleavage site encoded by the mLIF-M transcript (pmLIF-T EXTRA ). Medium conditioned by cells transfected with pmLIF-T EXTRA supported the growth of undifferentiated ES cells to a dilution of 1/10,000, comparable to the levels produced by transfection of cells with pDR10. The 1000-fold increase in biological activity resulting from secretion of the 17-kDa protein indicates that this protein acts as an agonist and is capable of productive interaction with cell surface LIF receptors.

Discussion

A complex organization of the LIF gene is conserved among eutherian mammals

In this paper, we report the identification and cloning of novel LIF transcripts from mouse ES cells, human embryonal carcinoma cells, and porcine primary fibroblasts. These transcripts, which comprise alternative first exon splice variants of the reported LIF transcripts, fall into two classes based on sequence homology and genomic location of the alternative first exons. Cloning of LIF-M transcripts from human and porcine cells and demonstration of independently regulated expression in human cells dispels skepticism about the existence of this transcript in species other than the mouse (22, 34). Cloning of LIF-T transcripts from three species identifies a novel, conserved LIF transcript. LIF-D transcripts, predicted to encode secreted glycoproteins, have previously been identified in each of the reported mammalian genes by cDNA cloning or predicted to exist on the basis of sequence homology (22, 34, 41, 45). Therefore, the present data point to a complex conserved organization of the mammalian LIF gene in which three alternative first exons can be spliced to common second and third exons to generate three distinct transcripts (Fig. 7).

The reported mammalian LIF gene sequences in the regions surrounding the LIF-M and LIF-T first exons are aligned in Fig. 7. The substantial homology in these regions suggests that ovine and bovine cells also express LIF-M and LIF-T transcripts. A region of homology ~140 bp in length, which includes the cloned LIF-T first exons, was found to be conserved between the mouse, human, porcine, bovine, and ovine LIF genes. The highest levels of sequence conservation were seen upstream of the cloned first exons, a region predicted to be the proximal promoter region for the LIF-T transcript. Only modest conservation of the transcribed sequences was observed, with the mLIF-T first exon sequence being the most divergent. The position of the splice donor site in the cloned transcripts also varied, with the mLIF-T exon showing the most divergent splice donor site with regard to position (Fig. 7C). These divergences presumably reflect the fact that these exons do not contain AUG initiation sites or encode protein.

Alignment of the known mammalian LIF gene sequences around the LIF-M first exons is shown in Fig. 7B. Once again, modest conservation of the noncoding transcribed regions was observed but there was substantial sequence homology upstream of the cloned LIF-M first exons, in the predicted proximal promoter regions. This homology has been identified previously by others (22, 34). The positions of splice donor sites for all the LIF-M transcripts except mLIF-M were strictly conserved. The unique in-frame AUG codon of the mLIF-M transcript aligns with the splice donor site in the other LIF-M transcripts, with its own splice donor site being positioned 11 bp downstream (Fig. 7B).

The genomic sequences encoding both the LIF-T and LIF-M first exons showed homology extending both upstream and downstream of the cloned regions (Fig. 7C) with surrounding sequences of much less similarity. Therefore, these regions represent blocks of conserved sequence located between exon 1D and exon 2.

Regulation of alternate LIF transcripts

Expression studies indicated that the three LIF transcripts are regulated independently in vitro and in vivo. This was manifest both in absolute levels of expression and in the relative levels of each
transcript and is consistent with alternative promoter usage for each transcript. The most abundant LIF transcript was normally either LIF-D or LIF-M in both human and mouse cells. While LIF-T was generally expressed at low levels, these were similar to the levels of LIF-D and LIF-M in several cell types in vitro and in vivo, pointing to probable physiological relevance. The relative levels of each transcript varied widely between cell types, and cells in which each of the transcripts were not detectable could be identified. This independent transcriptional regulation indicates that the alternative transcripts are unlikely to represent transcriptional “mis-starts” and suggests that the proteins encoded by these transcripts may have distinct biological roles.

In contrast to the promoter for the LIF-D transcript (34, 46), the predicted proximal promoters for the LIF-M and LIF-T transcripts lack TATA box consensus sequences (Fig. 7, A and B). However, both the LIF-M and LIF-T putative promoter sequences show conserved consensus binding sites for the transcription factors Sp1 and Ets-1. Sp1 has been implicated in the activation of many TATA-less promoters (47–49), while Ets-1 has been shown to interact with Sp1 on the megakaryocyte-specific dfb gene TATA-less promoter (50). The high levels of sequence conservation observed in the defined and predicted proximal promoter regions of all reported LIF genes suggests that the mechanisms regulating LIF-D, LIF-M, and LIF-T transcription are likely to be well conserved among eutherian mammals.

Experimental evidence from other workers has identified promoters downstream of the mLIF-D first exon. Transcriptional analysis of the mLIF gene (51) led to functional definition of two regulatory regions capable of independent transcriptional initiation, both located between exon 1D and exon 2. The first region, located between exon 1D and exon 1M, corresponds to part of a CpG island identified by Kaspar et al. (52) and encompasses the region of extensive homology upstream of exon 1M identified here and elsewhere (22, 34). This region appears to constitute the proximal promoter for mLIF-M. A second region capable of independent transcriptional initiation was identified between exon 1M and exon 2 and potentially corresponds to the LIF-T promoter. In addition to functional promoter definition, a conserved CpG island, found in association with promoters in many genes (53), was identified in the mLIF gene between exon 1D and exon 2 (52), encompassing the putative proximal promoter regions for the mLIF-M and mLIF-T transcripts. Two clusters of hypomethylated HpaII restriction sites, commonly associated with regulatory regions, were identified in this region. These mapped precisely to the predicted mLIF-M and mLIF-T proximal promoters between residues 1357 and 1421 and 1707 and 1785, respectively (Fig. 7, B and C; Ref. 34).
Functional promoter definition and mapping of DNA hypomethylation sites support the proposition that production of the alternative LIF transcripts is controlled by alternate transcriptional initiation processes. This is consistent with our observation that only a single species is amplified by RT-PCR using mouse, human, and porcine exon 1-specific primers in conjunction with exon 2 or exon 3 primers (Fig. 2D, not shown).

A mechanism for regulated expression of intracellular and extracellular cytokines

In the absence of an initiation codon within exon 1, we have shown that mLIF-T transcript translation is initiated downstream of the secretory signal sequence at the first in-frame AUG in exon 2. Although this AUG is not surrounded by a consensus translational initiation sequence (54), it is conserved among all reported LIF genes (22, 34, 41). Translation from this position results in production of a 17-kDa primary translation product that is N-terminally truncated by 22 amino acids relative to the mature LIF-D protein, but retains its extracellular bioactivity. This protein has no known mechanism for secretion and was shown to be retained within the cell by immunoprecipitation and bioassay. Therefore, expression of alternate LIF transcripts may provide a molecular explanation for the postulated existence of intracellular LIF protein in hepatocarcinoma cells (30). Intracellular LIF proteins may also be present at physiologically relevant levels in human embryonal carcinoma cells because transcripts encoding these proteins are the predominant LIF transcripts in these cells (71). However, detection of endogenous proteins is complicated by the fact that overexpression of intracellular LIF can cause cell apoptosis. 6

LIF may now be classed among the cytokines that are produced in intracellularly and extracellularly localized forms by translation of independent transcripts. This class of cytokines also includes the IL-1 receptor antagonist (55, 56) and IL-15 (57). Production of all of these intracellular cytokines is dependent upon alternate promoter activity driving differential splicing processes. However, in the cases of the IL-1 receptor antagonist and IL-15, the intracellular proteins are produced with leader peptides that are cleaved to produce a protein, but retains its extracellular bioactivity. This protein has no known mechanism for secretion and was shown to be retained within the cell by immunoprecipitation and bioassay. Therefore, expression of alternate LIF transcripts may provide a molecular explanation for the postulated existence of intracellular LIF protein in hepatocarcinoma cells (30). Intracellular LIF proteins may also be present at physiologically relevant levels in human embryonal carcinoma cells because transcripts encoding these proteins are the predominant LIF transcripts in these cells (71). However, detection of endogenous proteins is complicated by the fact that overexpression of intracellular LIF can cause cell apoptosis. 6

The likely production of the 17-kDa protein from LIF transcripts of many species, coupled with the observed differential regulation of these transcripts, points to an important biological role for the intracellular LIF proteins. The dramatic increase in levels of extracellular LIF activity seen when secretion of the truncated LIF protein was enforced (Fig. 6D) indicates that absence of the N-terminal 22 amino acids of the mature LIF-D protein is not critical for receptor signaling. This is consistent with the finding that the first 22 amino acids of the secreted LIF-D protein do not appear to be part of the LIF/gp190 or LIF/gp130 interaction domains (58–60). Thus, the 17-kDa LIF protein can act as an agonist in association with cell surface receptor complexes on a LIF-dependent cell population. These observations indicate that expression of the 17-kDa LIF protein can support LIF-dependent cells and suggest that the 17-kDa protein may be stored intracellularly for later release by cell lysis or by an unknown mechanism in response to environmental cues (61, 62), as shown for the intracellular IL-1R antagonist (63, 64). Alternatively, by analogy with fibroblast growth factor (26), intracellularly localized LIF proteins may be required for augmentation of cell surface receptor-based signal transduction. Finally, intracellular compartmentalization of LIF protein might allow autocrine, cell-autonomous LIF function. In this respect, it is intriguing that autocrine LIF action has been proposed in the regulation of embryo implantation (65), in deregulated pluripotent cell proliferation in teratocarcinomas (66), and in LIF-responsive transcription in hepatocarcinoma cells (30).

The absence of an in-frame initiation codon in the LIF-M first exons identified here distinguishes these transcripts from the reported mLIF-M transcript. Sequences within the mLIF-M first exon or the protein sequence encoded by this exon are thought to target secreted LIF protein translated from this transcript to the extracellular matrix (20). The deduced LIF-M proteins from other mammalian species are predicted to be translated from the in-frame AUG in exon 2 and localized within the cell. While this may represent a genuine difference in functionality between the murine and other mammalian LIF genes, it appears that translation of the hLIF-M transcript is complex and yields both the intracellular 17-kDa protein predicted from this work and a secreted protein (71).

The modular arrangement of the mammalian LIF gene provides a precise mechanism controlling alternative cytokine localizations. Moreover, independent regulation of transcripts encoding the alternatively localized LIF proteins may indicate important, non-equivalent biological functions for each protein. Comparison of the sites and mechanisms of action of the LIF proteins translated from the three distinct transcripts will further our understanding of the diversity of functions performed by the LIF gene. The ability to separate and manipulate experimentally expression of the secreted and intracellular LIF proteins should greatly simplify these studies and provide insight into the nature of intracellular cytokine action.

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References


