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Cloning and expression analysis of the murine lymphotoxin β gene

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ABSTRACT **Tumor necrosis factor α (TNF-α) and soluble lymphotoxin (LT) (also called LT-α or TNF-β) are cytokines with similar biological activities that are encoded by related and closely linked genes. TNF-α, a mediator of the inflammatory response, exists in soluble and transmembrane forms. LT-α can be secreted or retained at the cell surface by binding to a 33-kDa transmembrane subunit, LT-β. The recently cloned human LT-β gene encodes another TNF family member and is linked to the TNF/LT locus within the major histocompatibility complex locus. The cell surface LT is a heterotrimer consisting of LT-α and LT-β, whose physiological function is not yet clearly defined. We now report the sequence analysis of the genomic region and cDNA of murine LT-β gene, which is closely associated with the TNF-α and LT-α genes within the murine major histocompatibility complex locus. Unlike the TNF-α, LT-α, and human LT-β genes, which contain four exons, the murine LT-β contains three exons and encodes a 244-amino acid polypeptide with a 66-amino acid insert that is absent from the human homologue. *In situ* hybridization demonstrates constitutive expression of LT-β in lymphoid and hematopoietic tissues. LT-β transcription is maximal in the thymic medulla and in splenic white pulp. LT-β mRNA is also detected in the skin and in specific regions of the brain. The LT-β promoter region contains putative Ets-binding sites, suggesting that the expression of LT-β may be regulated in part by Ets transcription factors whose pattern of lymphoid expression overlaps that of LT-β.**

Lymphotoxin β (LT-β) is a transmembrane member of the growing family of tumor necrosis factor (TNF)-related ligands (1–3). TNF is a proinflammatory cytokine with multiple immunomodulatory activities, including a potent antitumor effect in mice (4, 5). LT-α (or TNF-β) was originally identified as a soluble cytotoxic factor involved in type I delayed hypersensitivity reactions (6, 7). The *in vivo* and *in vitro* activities of both cytokines overlap presumably due to their binding and signaling through the same two receptors (2). Disruption of these receptors by gene targeting (3, 8, 9) suggests that the primary pathophysiological role of the soluble forms of these two cytokines is to promote defense against infection.

Surface LT exists as a mixed trimer formed by LT-α and the cloned transmembrane subunit LT-β in a 1:2 ratio (1). This surface heterotrimeric complex recognizes a receptor distinct from the previously characterized receptors for soluble TNF and LT-α (10). The physiological function of the surface LT is suggested by a report (11) that demonstrates that mice deficient in LT-α have gross abnormalities in the development of lymphoid organs, including lymph nodes and the spleen. Since this phenotype is not observed in mice with disrupted genes for the two TNF/LT receptors (3, 8, 9), the interaction of the LT-β–LT-α complex with the third receptor (10) may be essential for this function of LT.

Genes encoding human and murine TNF and LT-α have been cloned (12–14) and mapped within the major histocompatibility complex (15, 16). The gene encoding human LT-β was also mapped to the same genetic locus (1).

Here we describe the cloning and characterization of the murine LT-β gene¶ as a step toward dissecting the function of LT in an animal model.

MATERIALS AND METHODS

DNA Clones and Probes. Murine genomic clones 4, 12, 13, and 14 have been described (13, 17). The 330-bp *Xho* I–*Hin*dIII fragment from clone 7.1 (14), containing a portion of the fourth exon of the human LT-β (1), was used to probe mouse genomic clones. Probes for Northern blot and *in situ* hybridizations were as follows: for LT-α, a *Bam*HI–*Kpn* I fragment (nt 11,880–12,992, GenBank accession no. U06950); for LT-β, a *Sau*3A fragment (nt 4535–5041).

DNA Sequencing. Sequencing was done using Sequenase (United States Biochemical) and the dideoxynucleotide chain-termination method as modified for supercoiled double-stranded templates (18). The dsDNA Cycle Sequencing system (GIBCO/BRL) was used for PCR sequencing.

Reverse Transcription–PCR (RT–PCR) and Primer Extension. PCR primers (synthesized on the Applied Biosystems model 392) were 5′-TCGGGTTGAGAAGATCATTGG (sense) and 5′-GCTCGTGTACCATAACGACC (antisense).

The reverse DNA strand was synthesized using 2–3 μg of total RNA and the Superscript Preamplification system (GIBCO/BRL). The PCR profile was denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 2 min for 20 cycles. Antisense primer LTBPE (5′-ACAGTGATAGGCACTGCCAACAACAGGGTC) was used for primer extension as described (18).

RNA Probes. [^{33}P]UTP- or ^{35}S-labeled UTP-labeled RNA was prepared on linearized templates using T7 RNA polymerase. Both antisense and sense probes were generated for LT-β and LT-α genes.

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Abbreviations: LT, lymphotoxin; RT–PCR, reverse transcription–PCR; TNF, tumor necrosis factor.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U12029).

Embryos, Tissue Sections, and *in Situ* Hybridization. FVB-N mice were used for analysis. Preparation of tissues and conditions for *in situ* hybridizations have been described (19).

Computer Analysis. Transmembrane domain predictions were made using the published algorithm (20) implemented in the SOAP program of PC/Gene software package (IntelliGenetics). Eukaryotic promoter elements were identified by using the FINDPATTERNS program of GCG software package (Genetics Computer Group, Madison, WI).

RESULTS

Cloning and Sequencing of Murine LT-β Gene. The murine LT-β gene was cloned from a murine genomic library by using a subclone of exon 4 of the human LT-β gene as probe. The same probe was used to hybridize to isolated genomic clones encompassing the murine TNF/LT genomic locus. Both types of analyses indicate that the murine LT-β gene is linked to the TNF and LT-α (TNF-β) genes in a manner similar to the organization of the human TNF/LT genetic locus. In particular, an LT-β-specific signal was observed with murine clones 12, 4, and 14, but not with clone 13 (data not shown), consistent with the localization of human LT-β gene in the class III region centromeric to TNF-α (1, 21, 22). Additional analysis placed the LT-β gene 2–3 kb downstream of the TNF gene within an 8-kb *Eco*RI fragment (Fig. 1). A detailed map of the 8-kb *Eco*RI fragment was established and the entire fragment was sequenced. The newly determined sequences overlap with the previously characterized portion of the murine TNF/LT locus (23).

The murine LT-β gene spans 1.7 kb and contains a nonconsensus TATA box, regions with high homology to exons of the human LT-β gene, and a consensus polyadenylylation signal (Fig. 2). The mRNA start site has been mapped using primer extension (data not shown), which reveals several initiation sites around nt 410–415 (Fig. 2) (i.e., 20–25 bp downstream of the TATA box and 5–10 bp upstream of the ATG codon), consistent with the organization of the human gene (1).

Approximately 200 bp of the sequence upstream of the putative transcription initiation sites are highly conserved between mouse and human sequences (1), suggesting a functional role in transcriptional regulation. Computer analysis of the upstream sequence (29) reveals a number of potential binding sites for known transcription factors, such as NF-κB/Rel, Ets, MyoD, and Egr-1 (25–28, 30) (Fig. 2), conserved between the mouse and human sequences.

In addition, the consensus donor splice site at the distal end of the presumptive exon 2 is absent in the murine gene (nt 993 and 994 in Fig. 2), suggesting that the human and murine LT-β genes may be differently spliced. To exclude cloning and/or sequencing artifacts, PCR sequencing of the same DNA region from two clones from a C57BL/6 genomic library and of two clones from a murine strain 129 genomic library was performed yielding identical results (data not shown). Genomic Southern blot analysis (24) and the results of the genomic library screening indicate that the cloned murine homologue of the human LT-β gene represents a unique sequence in the mouse genome, making the presence of a pseudogene in our clones unlikely.

Murine LT-β mRNA Is Spliced Differently Than Human LT-β mRNA. Total cDNA from several murine cell lines and from different mouse tissues was prepared and used for PCR amplifications across the presumptive introns 2 and 3 of the murine LT-β gene. The LTBP1 primer (Fig. 2) was designed to minimize the possibility of amplification from contaminating genomic DNA.

The results shown in Fig. 3*a* indicate that under stringent PCR conditions, a DNA fragment of 640 bp is the major PCR product obtained on total cDNA from three of four murine cell lines and on cDNA from embryonic thymus of C57BL/6 mice. The size of this fragment is consistent with the presence of the unspliced homologue of intron 2 of the human LT-β gene within the murine LT-β cDNA. The predicted size for the partially spliced RNA (without intron 1) was 940 bp, and this band was indeed detected, along with a 640-bp fragment, in some RNA preparations (Fig. 3*a*). Human Jurkat cells that produced large amounts of LT-β mRNA and the murine macrophage cell line ANA-1 (31) that did not transcribe LT-β gene (data not shown) gave no detectable amplification products, indicating that the 640-bp band was specific to murine LT-β.

This unexpected exon–intron organization was confirmed by direct sequencing of the corresponding segment of the murine LT-β cDNA, which was produced by RT–PCR (Fig. 3*b*). Total cDNA derived from a day 16 fetal thymus from C57BL mice was used to amplify the 640-bp fragment, which was then gel-purified and sequenced by PCR. The sequence analysis confirmed that the mouse gene contains a large second exon encoding 105 aa, also consistent with the sequence of the nearly full-length LT-β clones, isolated from a murine spleen cDNA library (J. Browning, personal communication). A partial cDNA sequence from the WEHI-231 murine B-cell line revealed at least two variations in the nucleotide and predicted amino acid sequences (Fig. 3*b*), suggesting the existence of structural polymorphism within the murine LT-β gene.

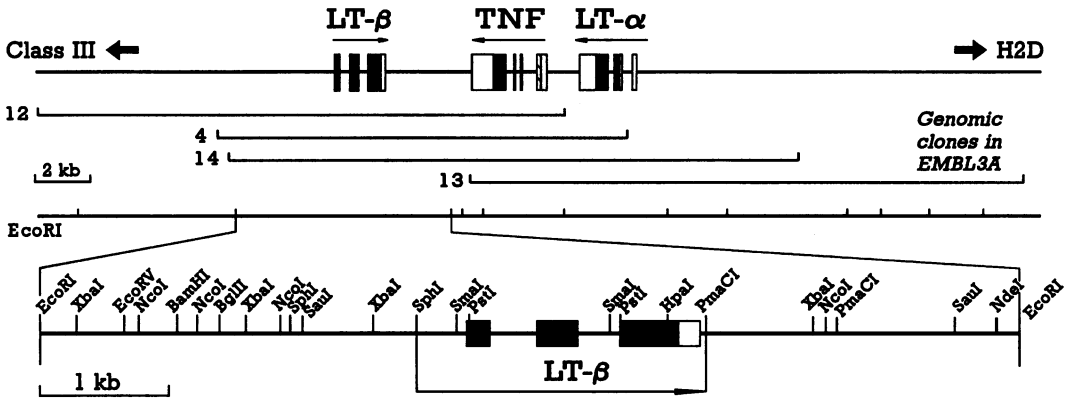

FIG. 1. Map of murine TNF/LT locus based on data from this work and from refs. 23 and 24. (*Top*) Region of mouse chromosome 17 containing TNF/LT locus. Locations of phage genomic clones (17) are shown. (*Middle*) *Eco*RI map. (*Bottom*) Organization of mouse LT-β gene within an 8-kb *Eco*RI fragment. The LT-β gene sequence corresponds to the arrow at the bottom (GenBank accession no. U06950, for the entire TNF/LT locus).

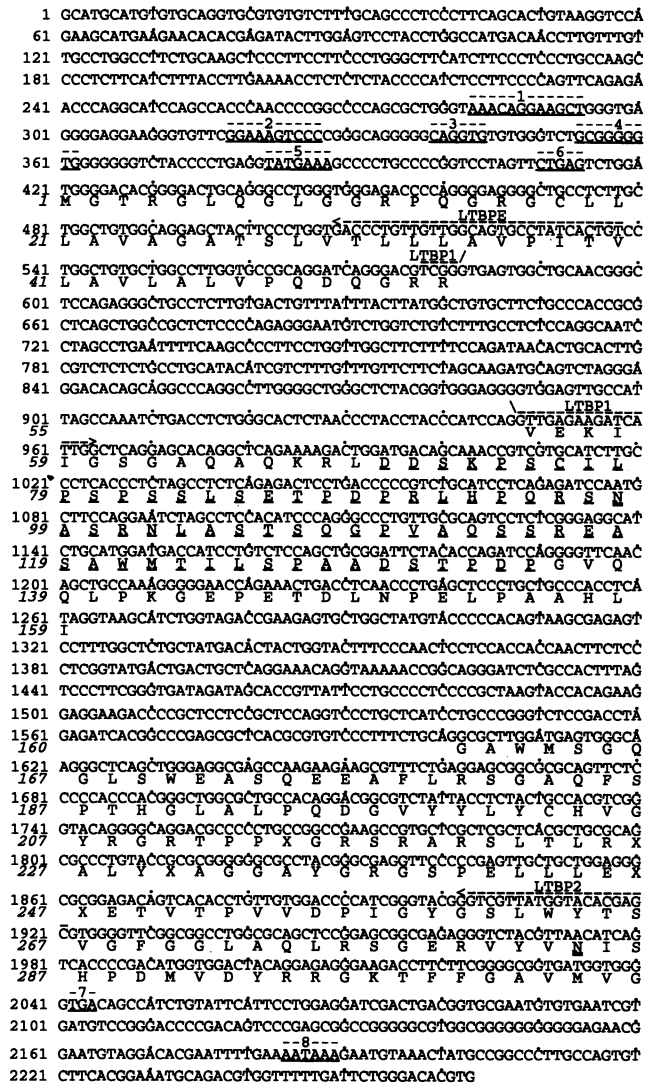

FIG. 2. Nucleotide sequence and landmarks of murine LT-β gene. Sequence corresponds to the *Sph* I–*Pma*CI fragment indicated in Fig. 1. Numbering corresponds to nucleotides (upper) and amino acids (lower) of LT-β. The following nucleotide sequences are underlined: 1–4, putative binding sites for transcription factors identified by computer analysis [1, Ets1/E74 (25); 2, Ets1/NF-κB (26); 3, MyoD (27); 4, Egr1 (28)]; 5, TATA box [identified by homology to the human gene (1)]; 6, position of mRNA start site; 7, stop codon; 8, polyadenylylation signal. LTBP1, LTBP2, and LTBPE correspond to oligonucleotide primers used for RT–PCR and primer extension, respectively. Protein sequence of murine LT-β is shown below the codons, the amino acids absent from human LT-β are underlined, and potential glycosylation sites at aa 98 and 284 are double underlined. A potential transmembrane domain is found between aa 16 and aa 50 with the probability of 0.99997.

Thus, murine LT-β is encoded by three exons as compared to four in human LT-β, and it contains a unique insert of 66 aa. Computer analysis of the presumptive LT-β protein indicates that this insert is located on the external part of the LT-β molecule, next to the transmembrane domain. The predicted molecular mass of unmodified murine LT-β is 33 kDa. Two consensus glycosylation sites are present in the mouse LT-β (Fig. 2), one within the unique 66-aa insert. Sequence variation in WEHI-231 cDNA resulted in the elimination of this additional modification site (Fig. 3*b*).

Expression of LT-β in Adult and Embryonic Murine Tissues. To determine the tissue-specific pattern of transcription of the LT-β gene, Northern blot analysis, RT–PCR, and *in situ* hybridizations were performed.

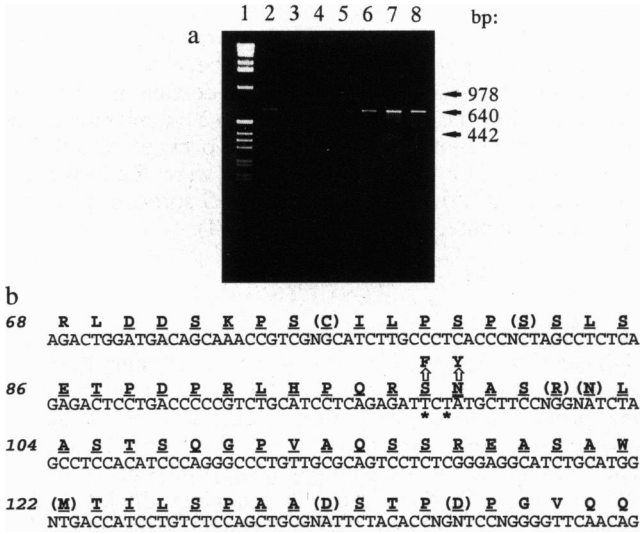

FIG. 3. RT–PCR analysis of LT-β mRNA. (*a*) Agarose gel analysis of RT–PCR products of various cDNAs amplified with primers LTBP1 and LTBP2. Lanes: 1, molecular size marker; 2, thymus from 16-day embryo; 3, thymus from 13-day embryo; 4, phorbol 12-myristate 13-acetate-activated human T-cell line Jurkat; 5, lipopolysaccharide-activated murine macrophage cell line ANA-1 (31); 6, murine B-cell line WEHI-231; 7, murine pre-B-cell line 70Z/3; 8, interleukin 6-activated murine promyelocytic cell line M1. (*b*) Partial nucleotide and amino acid sequence of LT-β mRNA from thymus of C57BL/6 mice. Amino acids absent from human LT-β are underlined; substitutions in mRNA from the WEHI-231 B-cell line are indicated by the arrows.

Northern blot analysis of several mouse tissues and cell lines determined that the highest level of LT-β transcription occurs in adult spleen and thymus, with detectable expression in bone marrow, lung, liver, and skeletal muscle and with no or little detectable signal in brain, heart, and testis (data not shown). In addition, the RT–PCR (Fig. 3*a*) detected LT-β expression in the thymus and activated B-cell lines but not in the lipopolysaccharide-activated macrophage cell line ANA-1 (31), known to produce TNF but not LT-α (data not shown). On the contrary, activated murine promyelocytic cells M1 did express LT-β mRNA (Fig. 3*a*).

In situ hybridization analysis for both LT-β and LT-α was performed on frozen sections of mouse embryos at 14–18 days of gestation, 1-day-old mice, and selected organs from adult mice. At day 18 of gestation and in 1-day-old mice, the thymus was the principal organ of LT-β transcription (Fig. 4*a* and *b*). At this and later stages, LT-β expression was highest in the thymic medulla, although significant levels of expression were detected in the cortex (Fig. 4*g*). Other lymphoid and hematopoietic organs, such as the spleen (Fig. 4*j*) and embryonic liver and bone marrow (data not shown), also contained cells expressing LT-β. In the spleen, LT-β was predominantly expressed in the white pulp (Fig. 4*j*). The pattern of LT-α expression in the thymus (Fig. 4*f*) and spleen (Fig. 4*i*) was similar to that of LT-β, although the level of LT-α signal was severalfold lower than that of LT-β. No significant background pattern of expression was observed with the sense RNA probes as controls (data not shown).

Interestingly, LT-β expression was also detected by *in situ* hybridization in the skin (Fig. 4*b*) and in specific cortical areas of the brain and Purkinje cells of the cerebellum (Fig. 4*d*).

DISCUSSION

We have cloned and characterized the murine LT-β gene, encoding the membrane-bound subunit of LT (1, 32). Based on the localization of the LT-β gene within previously characterized genomic clones encompassing the TNF/LT locus, LT-β is

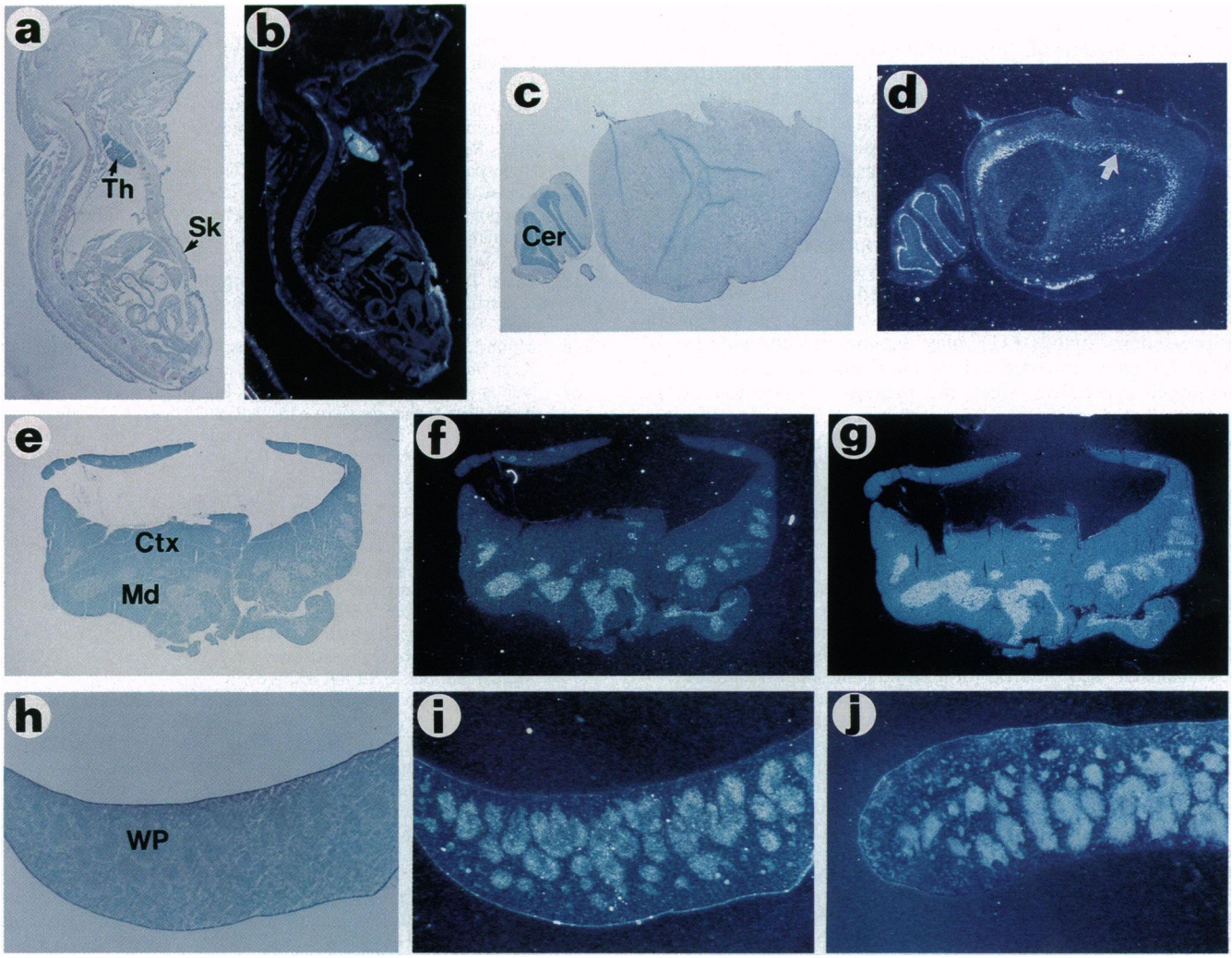

FIG. 4. *In situ* transcriptional analysis of LT-β and LT-α expression in mouse tissues. (*a* and *b*) One-day-old mouse. (*c* and *d*) Three-week-old brain, sagittal views. (*e–g*) Adult thymus. (*h–j*) Adult spleen. (*a*, *c*, *e*, and *h*) Bright-field views. (*b*, *d*, *f*, *g*, *i*, and *j*) Dark-field views. (*b*) Antisense LT-β probe, expression in thymus (Th), and skin (Sk). (*d*) Antisense LT-β probe, expression in Purkinje cells in cerebellum (Cer) and cerebral cortex (arrow). (*f* and *l*) Antisense LT-α probe. (*g* and *j*) Antisense LT-β probe, expression in cortex (Ctx) and medulla (Md) of thymus and splenic white pulp (Wp).

clearly linked to the major histocompatibility complex locus on the centromeric side of the TNF/LT locus and is ≈70 kb centromeric to H2D (16). The three functional genes are located within a 12-kb genomic region, and LT-β and LT-α genes flank the TNF gene (Fig. 1).

Several features make the LT-β gene different from the other two genes in the TNF/LT locus. The LT-β gene is transcribed in the opposite direction of TNF and LT-α, suggesting more complex genomic evolutionary events than tandem gene duplications. The 3′ untranslated region of the LT-β gene, unlike those of the two other genes, does not contain the TATT repeats, which are known to play an important role in mRNA stability (33, 34). This suggests that LT-β and LT-α may be regulated differently at the post-transcriptional level, consistent with a recent report (35). The murine LT-β gene contains only three exons, unlike the TNF and LT-α genes, which contain four exons, but this feature is not conserved between humans and mice. The presence of the additional amino acid sequence unique to murine LT-β may be useful in developing species-specific reagents.

RT–PCR and Northern blot analyses indicate that the LT-β gene is predominantly transcribed in the thymus and spleen (Fig. 3*a* and data not shown), consistent with the results of *in situ* hybridization analyses of embryos and adult tissues (Fig. 4). Our results indicate constitutively high levels of LT-β transcription in the lymphoid and hematopoietic tissues, especially in the thymic medulla (Fig. 4 *b* and *g*), suggesting a role for the LT-β in the development and function of T cells. The overall pattern of LT-β transcription during embryogenesis and postnatal life suggests that LT-β may serve multiple functions during development.

LT-α has been recently implicated in playing an important role in the development of the lymphoid organs, including lymph nodes and the spleen (11). LT-α and LT-β have similar patterns of expression in the embryonic and adult thymus and the adult spleen (Fig. 4), suggesting that constitutive expression of the two genes in some tissues may be coregulated. TNF-α has been reported (36) to be transcribed in the embryonic thymus, implying that there may be common transcriptional regulation mechanisms shared by all three genes.

The promoter regions of each of the three TNF/LT genes, however, show unique combinations of regulatory elements, implying that they may be differentially regulated in some tissues and can be activated through several different signaling pathways.

The LT-β promoter lacks an Sp1 site that is present in each of the two other genes; it is replaced by a related Egr-1 site (28)

that may be involved in inducible expression. The presence of two Ets sites in the promoter of the LT-β gene (Fig. 2) is interesting in view of the similarity in constitutive expression patterns between LT-β (Fig. 4) and that recently reported for the Ets-1 protooncogene (19), a transcriptional activator of lymphoid genes (37, 38). Ets-binding sites that are conserved between human and mouse genes are also located upstream to TNF and LT-α promoters (data not shown). The significance of the conserved MyoD site and its relation to expression of LT-β in muscle cells remains to be determined.

The promoters of all three genes contain consensus NF-κB sites, shown to be involved in the activation of TNF and LT-α genes (refs. 39 and 40 and unpublished data). Involvement of NF-κB/Rel proteins (26, 30), some of them predominantly expressed in thymus (41), may explain the coregulation of TNF/LT genes in certain tissues and the distinct patterns of inducible expression, since the protein composition of the transactivating complexes may depend on the nucleotide context of a particular κB site.

The cloning and characterization of the murine LT-β gene should lead to the development of specific molecular tools to study LT-β and allow the direct assessment of its function by gene targeting.

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