Journal of Cellular Biochemistry 29:171–181 (1985)
Perspectives in Inflammation, Neoplasia, and Vascular Cell Biology 25–35

Structure and Chromosomal Localization of the Human Lymphotoxin Gene

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We have isolated, sequenced, and determined the chromosomal localization of the gene encoding human lymphotoxin (LT). The single copy gene was isolated from a human genomic library using a ^{32}P-labeled 116 bp synthetic DNA fragment whose sequence was based on the NH_2-terminal amino acid sequence of LT. The gene spans 3 kb of DNA and is interrupted by three intervening sequences. The LT gene is located on human chromosome 6, as determined by Southern blot analysis of human-murine hybrid DNA. Putative transcriptional control regions and areas of homology with the promoters of interferon and other genes are identified.

Key words: lymphotoxin, cytotoxic factor, gene structure, chromosome 6

Human lymphotoxin (LT) is one of a group of cytotoxic factors produced by mitogen-activated peripheral blood mononuclear cells (PBMC) [1–4] and cell lines of hematopoietic origin [5]. LT derived from activated T lymphocytes and B lymphoblastoid cell line supernatants has been shown to have cytostatic or cytolytic activity *in vitro* [6–8] on cells transformed by viruses and carcinogens [9,10]. Experiments with impure LT have suggested that this lymphokine may exhibit antitumor activity *in vivo* [11–13]. Other cytotoxic factors secreted from PBMC with similar and/or identical biological activities as LT, such as tumor necrosis factor (TNF) [14–16] and natural killer cell cytotoxic factor (NKCF) [17,18], have also been shown to be cytostatic or cytolytic for transformed cell lines. We recently reported the isolation

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Received February 2, 1985; revised and accepted July 9, 1985.

and nucleotide sequence of cloned cDNAs for human LT [19] and human tumor necrosis factor [20], proving that they are distinct molecules with similar cytotoxic activities *in vitro* and *in vivo*. The relationship of NKCF to LT and TNF is currently under investigation.

This report describes the isolation, characterization, and DNA sequence of the human LT gene. In addition, chromosomal mapping using Southern blots of human-murine hybrid DNAs demonstrate that the LT gene is located on human chromosome 6. Homologous regions have been observed between the LT gene and interferon genes in the putative promoter region.

MATERIALS AND METHODS

Screening the Human Genomic DNA Library

The construction of the human 4X genomic DNA library has been described in detail elsewhere [21] and was a gift of William I. Wood. Phage (7.5×10^5) from the 4X recombinant human Charon 4A DNA library were plated on 40 15-cm plates and screened as previously described [22,23] with 10^8 cpm of a ^{32}P-labeled 116 bp N-terminal LT DNA probe [19,24]. Filters were washed in 1 × SSC (1 × SSC = 0.15 M NaCl, 0.15 M Na_3 Citrate), 0.1% SDS, twice for 40 min each at 40°C prior to autoradiography. Positive plaques were purified by standard methods [22]. On the second round of screening duplicate filters were hybridized with ^{32}P-labeled probes derived from the second and third segments of the synthetic LT gene [19,24].

Restriction Mapping and Genomic Southern Blots

Phage DNA was prepared [22] from individual hybridizing plaques. Human genomic DNA was prepared from peripheral blood lymphocytes [25]. Each phage DNA or genomic DNA was digested with a variety of restriction endonucleases (either singly or in combination) and then electrophoresed on a 1% agarose gel. DNAs were transferred from the gels to nitrocellulose [26] and hybridized with a ^{32}P-labeled LT cDNA probe [19]. *Hind*III-digested DNA λ was utilized as size markers.

DNA Sequence Analysis

A 2.4 kb *Eco*RI fragment and a 950 bp *Pst*I fragment which hybridized with the cDNA were separated by electrophoresis through 1% agarose, electroeluted, and ligated into pBR322 cleaved with *Eco*RI and *Pst*I, respectively. The entire gene was sequenced by the M13-dideoxy chain termination technique [27]. Sequencing templates were generated by subcloning smaller restriction fragments into the M13 cloning vectors mp8 and mp9 [28].

Chromosomal Localization

Chromosomal localization of the human LT gene was performed by Southern hybridization of DNA from human-murine somatic cell hybrids with the human LT cDNA probe. Somatic cell hybrids were constructed as previously described [29,30] and were characterized for their human chromosome content by direct karyotyping [31] and by assaying previously assigned marker enzymes [32,33]. The human and murine LT gene hybridization signals were easily distinguishable (the *Eco*RI fragments that hybridized were 2.4 kbp for the human gene and 3.7 kbp for the murine gene).

RESULTS

Isolation and Nucleotide Sequence of the Human LT Gene

A single 116 bp synthetic oligonucleotide probe [19,24] was used to screen approximately 10^6 plaques of a recombinant human-λ library. The DNA probe sequence was based on the NH_2-terminal amino acid sequence of LT derived from the RPMI 1788 cell line [34]. Five hybridizing plaques were identified. Two other chemically synthesized oligonucleotide probes (145, 210 bp) which span the entire LT coding region [19,24] were used on a second round of plaque screening to determine the extent of LT related sequences in each of the five clones. All five clones hybridized with all three LT synthetic probes and were shown by restriction endonuclease mapping to be overlapping DNA fragments spanning 17 kilobases (kb) of the human genome (data not shown).

A 2.4 kb *Eco*RI fragment from clone λXB13 and a 950 bp *Pst*I fragment containing the 3′ end of the LT gene were subcloned into the *Eco*RI and *Pst*I sites of pBR322, respectively, for further characterization. Figure 1a shows the resulting restriction map of the entire LT gene. Smaller restriction fragments were subcloned into the M13 vectors mp8 and mp9 and the DNA inserts were sequenced by the dideoxy chain termination method of Sanger et al [27]. (Fig. 1b).

The nucleotide sequence of the LT gene and the amino acid sequence of the LT protein, deduced from the cDNA [19,24], are shown in Figure 2. We sequenced

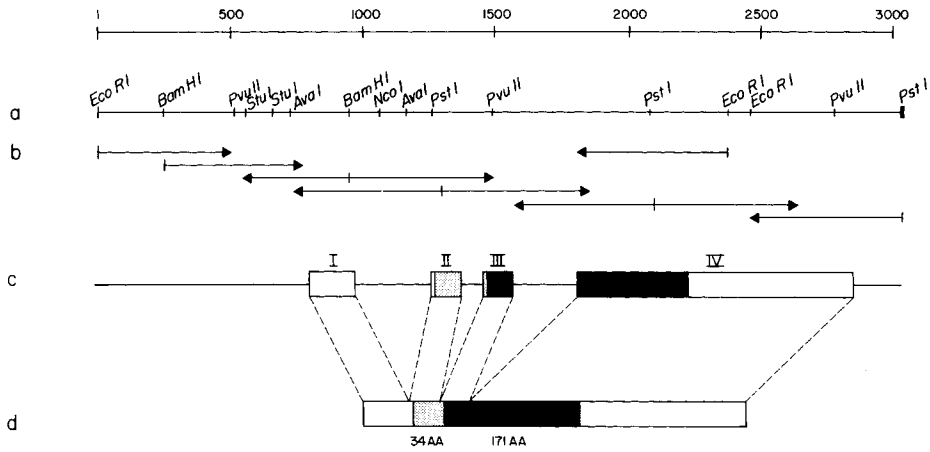

Fig. 1. Structure of the human LT gene. a) Restriction endonuclease sites for *Ava*I, *Bam*HI, *Eco*RI, *Nco*I, *Pst*I, *Pvu*II, and *Stu*I were determined by digestions of a recombinant bacteriophage λ clone (λXB13) DNA. b) Strategy used for DNA sequence determination of the LT gene. Arrows above the gene structure indicate the length and direction of sequences obtained by the chain termination method [27]. Single-stranded templates were obtained by subcloning the various fragments in M13 vectors mp8 and mp9. c) Schematic representation of the LT gene. The regions encoding the primary translation product are represented by closed bars. The signal sequence is marked by stippling and the region encoding the 5′ and 3′ untranslated sequences are marked by open bars. d) Schematic diagram of LT mRNA showing regions present in each of the four exons. The translated portion of the mRNA is shown as closed bars. The putative signal sequence is represented by stippling, and 5′ and 3′ untranslated regions are represented by open bars. The number of amino acids in the putative signal sequence and mature coding region are noted.

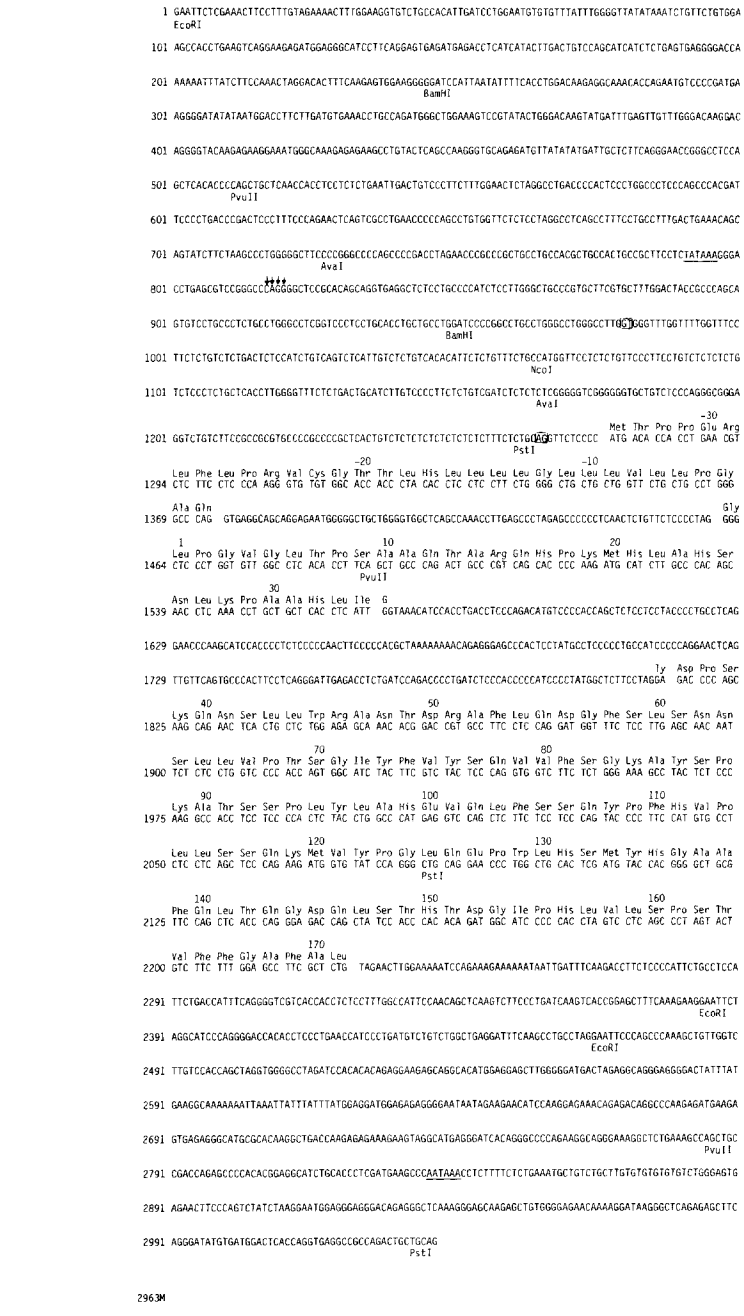

Fig. 2. Sequence of the human LT gene. The sequence was determined from the *Eco*RI site lying approximately 800 bp upstream from the initiation of transcription to the *Pst*I site about 250 bp downstream of the polyadenylation signal (AATAAA). The exon-intron junctions and reading frames for translation were assigned by comparison with the LT cDNA sequence [19,24]. The exon sequences are identical to the cDNA sequence. The sequence is numbered from the 5′ *Eco*RI site. Amino acids are numbered positively for the mature LT coding region and negatively for the putative signal sequence. The "TATA" box and AATAAA sequences are underlined. The sites of initiation of transcription (cap) are indicated by vertical arrows. The polyA$^+$ site is located at position 2854. Restriction endonuclease sites used in sequencing and additional sites are also shown. The first exon-intron junction sites, occurring in the 5′ untranslated region, are enclosed in boxes.

3,036 base pairs of DNA which show that the gene contains a total of three introns. As depicted in Figure 1, one intervening sequence exists in the 5′ untranslated region and two intervening sequences exist in the coding region of the gene. The first exon beginning from the cap site contains almost the entire 5′ untranslated region. The second exon contains 9 bp of 5′ untranslated DNA and the first 33 codons of the putative signal peptide. The third exon encodes the last amino acid of the signal peptide and the first 34 amino acids of the mature coding region. The fourth exon encodes the last 137 amino acids of the protein and the entire 3′ untranslated region containing the AATAAA polyadenylation signal [35]. The three intervening sequences are 287, 86, and 247 bp long, respectively. The exon-intron junctions contain the consensus GT donor and AG acceptor splice site sequences [36,37]. No differences were found in the coding region between the LT gene and cDNA.

The cap site nucleotide(s) (Fig. 2, arrows), not present in the LT cDNA clones, was identified by S1 nuclease mapping [38]. Oligo-dT selected RNA from stimulated PBMC was annealed to a ^{32}P-labeled, denatured 285 bp *Bam*HI/*Stu*I fragment, which contains the start of the LT gene (see Fig. 2). The annealed mixture was treated with S1 nuclease and then sized on a sequencing gel (data not shown). The RNA protected approximately 130 bases of the 285 base pair *Stu*I/*Bam*HI LT gene fragment, suggesting that transcription begins approximately at nucleotide 817. Four fragments were protected by the RNA, differing in length by just a few bases, indicating that the 5′ end of the LT mRNA may be heterogeneous and that the 5′-untranslated region is approximately 170 bases in length.

Genomic Blots

The results obtained from Southern blots of restriction digests of human genomic DNA probed with the ^{32}P-labeled LT cDNA are shown in Figure 3. Southern hybridization and washing conditions at either high or low stringency indicates that the coding region of LT consists of a unique single gene.

Two enzymes, MspI and TaqI, used to detect hotspots for mutations in mammalian DNA [39], were used to examine for restriction fragment length polymorphisms in the LT gene. Genomic DNA from nine individuals of various ethnic backgrounds was analyzed by Southern blotting using the LT cDNA probe. There were no discernible differences in the mobility of hybridizing fragments among the individual DNAs, suggesting that the LT gene is not highly polymorphic (data not shown).

Chromosomal Localization

DNA from 15 independently derived human-murine somatic cell hybrids were digested with the restriction endonuclease *Eco*RI and the resulting DNA fragments were separated by electrophoresis on 1% agarose slab gels. The DNA in the gels was then analyzed by hybridization using a ^{32}P-labeled LT cDNA probe after transfer to nitrocellulose. As shown in Table I, the LT probe only hybridized with somatic cell hybrids containing human chromosome 6. The LT probe weakly hybridized to one DNA which did not contain human chromosome 6 by karyotype analysis; however, the overall discordance for chromosome 6 is much lower than all other chromosomes (6.7% compared with 20–80%). This suggests that the LT gene is indeed located on chromosome 6.

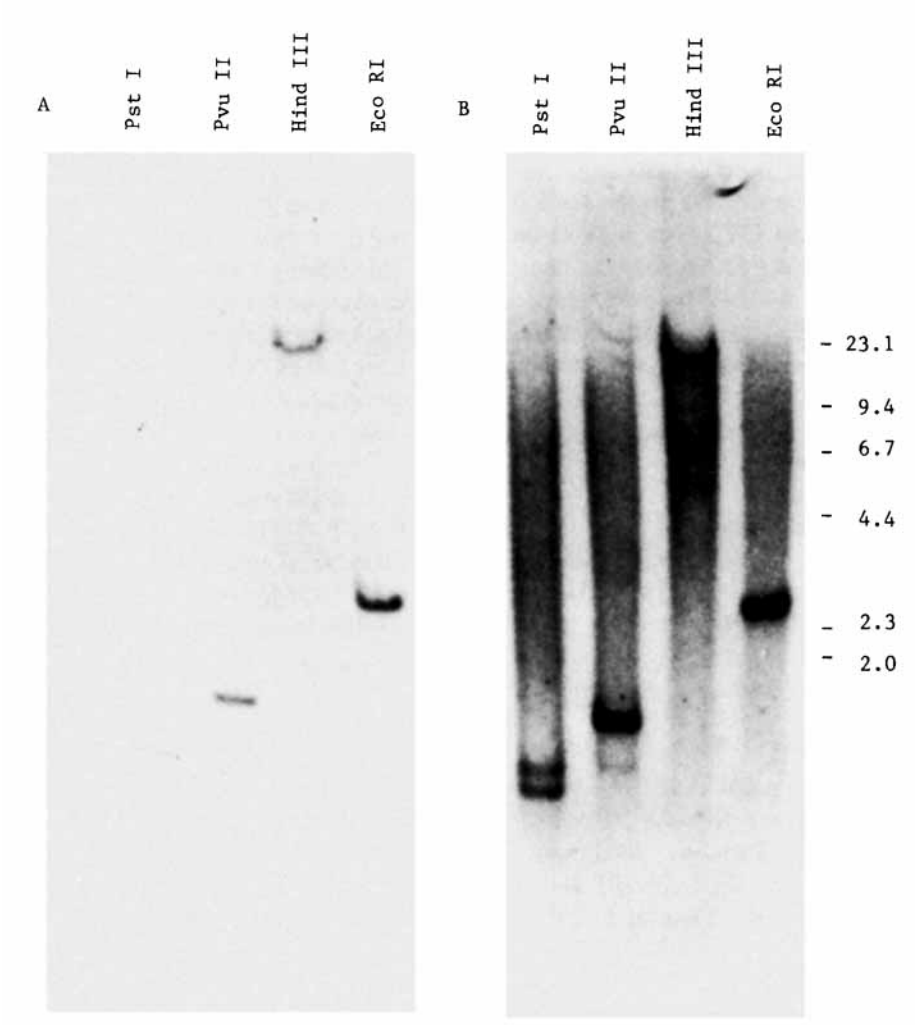

Fig. 3. Genomic blot of the human LT gene. A,B) Southern hybridization of human genomic DNA digested with restriction endonucleases. Restriction endonucleases used are noted. A) Hybridization at "high stringency" using 50% formamide, 42°C hybridization, followed by 0.2 × SSC, 0.1% SDS wash at 50°C. B) Hybridization at "low stringency" using 20% formamide, 42°C hybridization, followed by 2 × SSC, 0.1% SDS wash at 37°C. Numbers on the right indicate mobilities of *Hind*III λ DNA fragments in kilobases (kb).

DISCUSSION

We have isolated the human LT gene using a single 116 bp long synthetic deoxyoligonucleotide as a hybridization probe. The design of this DNA fragment was based on the NH_2-terminal amino acid sequence of human LT [19,24] and published human codon usage frequencies [40]. Several other cloned genomic DNA sequences [21,41–43] and cDNAs [19,20,44,45] have been previously identified using this "long probe" approach.

TABLE I. Segregation of *LT* With Human Chromosomes in Somatic Cell Hybrids

Hybrid	LT cDNA hybrid-ization	Human chromosomes																							Trans-location
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
TSL-2	+	−	+	−	−	+	+	−	−	−	+	−	+	−	−	−	−	−	+	−	+	+	−	+	
ATR-13	+	+	+	+	+	−	+	+	+	−	+	−	+	−	+	+	−	+	+	−	−	−	−	−	5X
NSL-9	−	−	−	−	−	+	−	−	+	−	+	−	+	+	+	+	+	+	−	−	+	+	+	−	17/9
NSL-7	+	−	−	−	−	−	+	−	−	+	−	−	+	+	+	−	−	−	−	+	−	+	−	−	12P−
NSL-15	+	−	+	−	+	+	−	+	+	−	−	−	+	+	+	+	−	+	+	+	−	+	+	+	
JSR-17S	−	+	−	+	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	7Q−
EXR-5CSAZ	+	+	−	+	+	+	+	+	+	−	+	−	+	+	+	+	−	+	+	+	+	+	+	+	X/11
WIL-6	+	−	+	−	+	+	+	+	+	−	+	+	−	−	+	−	−	+	−	+	+	+	−	+	
WIL-8X	−	−	−	+	+	+	−	+	−	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+	
WIL-7	+	−	+	+	−	+	+	−	+	−	+	+	−	+	+	−	−	+	+	−	−	+	−	+	
JWR-26C	+	−	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	+	+	−	+	+	−	+	1P−
JWR-22H	+	−	−	−	+	−	+	−	−	−	+	+	−	−	+	−	−	+	+	−	+	+	−	−	2/1
REW-11	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−	−	+	−	−	−	+	+	−	+	
XER-11	+	+	−	+	+	−	+	+	+	−	+	−	+	−	−	+	+	+	+	+	+	+	+	−	11/X X/11
XTR-22	+	−	+	−	+	+	+	−	+	−	+	+	−	−	−	−	−	−	+	+	+	+	+	−	X/3

Upon analysis of the nucleotide sequence, several points can be made concerning transcriptional signals found in the 5′ flanking regions of the coding segments. A well-conserved Goldberg-Hogness [46,47] sequence (TATAAA) thought to be important in promoting transcription initiation in mammalian cells is present at position 791 (Fig. 2). This sequence, which is found approximately 30 base pairs upstream of the cap site in most genes, is located 26 base pairs 5′ of the cap site in the LT gene. The LT "TATA" sequence (5′-TATAAA-3′, position 791 to 796) bears perfect homology with other lymphokine genes, ie, IFN-γ [48], IFN-β [49], and IL-2 [50] (Table II), and has striking homology with the "TATA" and adjacent sequences of both adenovirus and conalbumin genes (5′-CTATAAAAGGG-3′) [51]. Another consensus sequence, GG$^{C}_{T}$CAATCT [52], usually located approximately 75 base pairs upstream of the cap site and thought to be important in modulating RNA polymerase II transcription [52], is not readily identifiable in the LT gene.

Two other segments having homology with sequences occurring in the interferon genes have been found in the putative promoter region (Table II). Although their exact roles remain to be established, these homologous regions may be involved in gene expression and regulation. A consensus sequence (TTTTCACTGC) found just 3′ of the AATAAA polyadenylation signal in the 3′ untranslated region of several genes [52] is quite similar to such a sequence located at position 2846 in the LT gene (Table II). This sequence may be important in transcription termination and/or polyadenylation.

The intervening sequences and flanking regions of the LT gene contain many homopolymeric sequences. The first intervening sequence contains 11 copies of the hexanucleotide, TCTCTG, bordered on each side by TC-rich dinucleotide regions. At the 3′ end of this intron there is an 18 bp repeat of the dinucleotide TC. Following the poly A^+ addition site (position 858) there is a 12 bp repeat of the dinucleotide TG. Intervening sequences and flanking regions of other genes have been shown to contain repeating dinucleotides [53–55]. Pyrimidine-purine stretches can form left-

TABLE II. Homologous Sequences Occurring in the Promoter Region and Downstream From the Polyadenylation Site of the LT Gene and Other Genes*

A	TATAAA	LT	C	TGAAACA	LT
	−26			−125	
	TATAAA	IFN-γ		AGAAACA	IFN-α
	−31			−136	
	TATAAA	IL-2		AGAAACT	IFN-β
	−32			−120	
	TATAAA	Adenovirus, conalbumin		ACAAACT	IFN-γ
				−116	
				TAAAACT	Ovalbumin
				−127	
B	TCCTC	LT	D	TTTTCTCTGA	LT
	−31				
	TCCTC	IFN-γ		TTTTCACTGC	Consensus
	−53				
	TCCTC	IFN-β			
	−59				

*Sequences in A, B, and C are numbered starting from the cap site, position 817 in Figure 2.

handed double helices *in vitro* and may form similar structures (Z DNA) *in vivo*. Such structures may play a role in the regulation of gene expression [54,56]. Other noted homopolymeric sequences are intron 1 $(GGTTT)_4$; intron 2 $(AGCC))_3$; intron 3 $[CN(^{A}_{T}CCCC^{T}_{A})]_{11}$, $(CTTCCT)_2$, $(GGAAC)_2$. In addition, the 5′ untranslated region contains several duplications: $(CTGCCTGGGCCT)_2$, $(CGTGCTT)_2$, $(TCTCCT)_2$, $(TGCC)_6$. There are no data to suggest that these sequences have biological significance. However, it will be of interest to compare the genomic structures of other cytotoxic factors whose biological functions appear similar and/or identical, ie, tumor necrosis factor (TNF) and natural killer cell cytotoxic factor (NKCF). These sequences might play a role in the expression of the LT gene in mitogen- or antigen-activated lymphocytes.

Southern hybridization of a ^{32}P-labeled LT cDNA probe to genomic human DNA at high stringency indicates that LT is encoded by a single gene (Fig. 3A), which has been localized to chromosome 6. Utilizing low stringency conditions of hybridization and washing, it is possible to detect related genes of the same species [57,58] or related genes of different species [58,59]. In this regard we used low stringency conditions on Southern blots of human genomic DNA to detect any possible LT related genes. No additional hybridization signals were detected when compared to those hybridizing at the high stringency conditions (Fig. 3B), again indicating that this form of cytolytic molecule is encoded by a single unique gene. In addition, since Northern analysis of LT mRNA from PBMC and the RPMI 1788 cell line shows only one hybridizing band [19], it is likely that the reports of LT ranging in MW from 15 to 2100K [60–62] are due to aggregation phenomena and not to multiple LT proteins. LT has been isolated from the RPMI 1788 cell line in two forms, 25K (171 amino acids) and 20K (148 amino acids) [5]. The 25K form has an additional 23 amino acids at the NH_2-terminus from the 20K form. The intron position in the LT gene does not appear to account for the two forms seen via an alternative splicing mechanism. The 20K LT form may be either a processed or degradative form of 25K LT.

TNF is a cytotoxic protein that has been shown to be distinct from LT [20] yet shares 30% amino acid homology with LT. Other unique cytotoxic proteins may exist but have not yet been characterized well enough to determine their relationship to LT.

ACKNOWLEDGMENTS

We thank Jeanne Arch for the preparation of the manuscript.

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