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# Cloning, Expression, and Chromosomal Localization to 11p12–13 of a Human LIM/HOMEODOMAIN Gene, hLim-1

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## ABSTRACT

We have identified a putative transcription factor, designated hLim-1, from human fetal brain using degenerate polymerase chain reaction (PCR) and cDNA library screening. The deduced open reading frame, derived from sequencing a 3.0-kb hLim-1 cDNA, encodes a protein of 384 amino acids with two cysteine-rich LIM domains and one homeobox (HOX) DNA-binding domain. The nucleotide sequence of hLim-1 cDNA is 87% identical to mouse Lim-1 and the predicted amino acid sequence is greater than 97% conserved. Expression patterns of hLim-1 were evaluated by Northern analysis and reverse transcription (RT)-PCR coupled with Southern blotting. HLim-1 expression was observed in human brain, thymus, and tonsillar tissue. Expression of hLim-1 was also observed in 58% of acute myelogenous leukemia (AML) cell lines and in four of five primary samples from patients with chronic myeloid leukemia (CML) in myeloid blast transformation. The gene encoding hLim-1 was mapped using fluorescence *in situ* hybridization (FISH) to human chromosome 11p12–13. The expression pattern and structural characteristics of the hLim-1 gene suggest that it encodes a transcriptional regulatory protein involved in the control of differentiation and development of neural and lymphoid cells. Its expression in CML in blast crisis suggests that it may be involved with progression in this disease; a prospective study is required to confirm this.

## INTRODUCTION

GENES CONTAINING HOMEODOMAINS play an important role in the growth and differentiation of the developing embryo and the adult animal. The genes encoding homeodomain proteins may be broadly classed as either clustered or dispersed. This latter group of homeotic genes can be further divided based upon the presence of certain functional motifs in addition to the DNA-binding homeodomain; one such motif is the LIM domain, first recognized in the Lin 11 gene of *Caenorhabditis elegans* (Freyd *et al.*, 1990), *isl-1* of mammalian cells (Karlsson *et al.*, 1990), and *mec-3* of *C. elegans* (Way and Chalfie, 1988). The LIM motif consists of cysteine-rich regions capable of complexing zinc and forming finger-like structures. These zinc finger motifs have been shown to be involved in protein–protein interactions; to date, there is no convincing evidence of DNA-binding activity.

Members of the LIM-hox family of genes that have been identified include Lin 11, *isl-1*, *mec-3*, xLim-1 (Taira *et al.*,

1992), mLim-1 (Barnes *et al.*, 1994), rLH2 (Xu *et al.*, 1993), hLH2 (Wu *et al.*, 1996), and mLim-3 (Seidel *et al.*, 1994). Lin 11 and *mec3* are important in the determination of cell fate in mechanosensory neurons and vulval cells, respectively. mLH2 was first identified in developing B lymphocytes. The gene is expressed in early precursors and is absent from the mature progeny, suggesting a role in the differentiation of the cells (Xu *et al.*, 1993). LH2 is also expressed in pituitary cells and appears to have a role in the regulation of expression of pituitary glycoprotein hormone  $\alpha$ -subunit gene (Roberson *et al.*, 1994). mLIM3 is expressed in the neuroepithelium of embryos and in adult life it is expressed in the anterior and intermediate lobes of the pituitary. xLim-1 is predominantly expressed during embryogenesis in the gastrula stage and in the adult is expressed in the brain (Taira *et al.*, 1992). The murine homologue, mLim-1, is predominantly expressed in the brain and kidney in the embryo and adult (Barnes *et al.*, 1994).

Using degenerate oligonucleotides, we identified the expression of mLim-1 in mouse fetal brain, liver and spleen. We

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cloned mouse Lim-1 and used this as a probe to isolate the human homologue. The gene is expressed in brain, tonsil, and thymus of adult humans. In addition, we found aberrant expression of hLim-1 in a variety of human primary leukemias and human leukemic cell lines. Human Lim-1 was mapped by fluorescent *in situ* hybridization to chromosome band 11p12-13.

## MATERIALS AND METHODS

### Cell lines and patient samples

Peripheral blood or bone marrow was obtained from normal individuals and from patients with leukemia following informed consent. Mononuclear cells were isolated by Ficoll-Hypaque centrifugation. T lymphocytes were removed from peripheral blood samples by E-rosetting with sheep red blood cells followed by a second Ficoll-Hypaque centrifugation (Minden *et al.*, 1979). Cells were either used immediately to prepare RNA and DNA or were stored in liquid nitrogen in  $\alpha$ -minimal essential medium (MEM) containing 10% dimethylsulfoxide (DMSO) and 50% fetal calf serum (FCS). The cell lines evaluated are listed in Table 1.

To prepare a CD34<sup>+</sup> enriched cell population, mononuclear

bone marrow cells from a normal individual were depleted of adherent cells by plastic adherence. The cells were then incubated on ice with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 monoclonal antibody (Serotec, Quebec) for 1 hr. Five times 10<sup>5</sup> CD34<sup>+</sup> cells were then separated using fluorescence-activated cell sorting (FACStar, Becton Dickinson, San Jose, CA).

### RNA isolation

Total RNA was extracted from peripheral blood, bone marrow, and the cell lines and from several different fetal mouse tissues by a guanidinium thiocyanate-based method (Chirgwin *et al.*, 1979).

### cDNA synthesis

Total RNA (1  $\mu$ g) was added to a 20- $\mu$ l solution containing 40 mM Tris-HCl pH 8.3, 50 mM KCl, 5 mM dithiothreitol (DTT), 2 mM MgCl<sub>2</sub>, 10 pM of random hexamer (Pharmacia), 2  $\mu$ M of each deoxyribonucleotide triphosphate (dNTP), 1  $\mu$ l (40 units) of RNasin ribonuclease inhibitor (Promega Corp., Madison, WI) and 1  $\mu$ l (100 units) of Moloney murine leukemia virus reverse transcriptase (RT) (GIBCO-BRL, Canada). This mixture was incubated at 37°C for 45 min.

TABLE 1. EXPRESSION OF hLIM-1 IN NORMAL CELLS AND MALIGNANT CELL LINES

	Lineage	hLim-1
Normal tissue		
PB mononuclear cells	Mature T and B leukocytes	—
BM cells	Developing hematopoietic tissue	—
CD341 cells	BM progenitor cells	—
Brain	Nervous system	+++
Placenta	Fetal tissue	—
Tonsil	Lymphoid tissue	++
Testis	Reproductive tissue	—
Thymus	Precursor T-lymphoid	++
Cell line		
OCI/AML-1	Acute myelogenous leukemia	++
OCI/AML-2	Acute myelogenous leukemia	++
OCI/AML-3	Acute myelogenous leukemia	+++
OCI/AML-4	Acute myelogenous leukemia	+++
OCI/AML-5	Acute myelogenous leukemia	—
OCI/AML-6	Acute myelogenous leukemia	—
KG-1	Acute myelogenous leukemia	—
NB-4	Acute promyelocytic leukemia	—
U937	Acute monocytic leukemia	—
HEL	Erythroleukemia	—
TF-1	Erythroleukemia	+
MO7E	Megakaryoblastic leukemia	—
K562	Chronic myelogenous leukemia myeloid blast crisis	+++
ALL-Sil	T-cell lymphoblastic leukemia	—
KOPKT-1	T-cell lymphoblastic leukemia	+
MKB-1	T-cell lymphoblastic leukemia	—
MOLT-16	T-cell lymphoblastic leukemia	—
HUT-78	Cutaneous T-cell lymphoma	—
OCI/Ly-13.1	T-cell lymphoma at presentation	+++
OCI/Ly-13.2	T-cell lymphoma at relapse	+++
OCI/Ly-8	B-cell lymphoma	—
A-172	Glioblastoma	+
HeLa	Epithelioid carcinoma	—

(—) Not detectable; (+) weakly positive; (+++) strongly positive.

*Generation of mouse Lim-1 (mlim-1) probe by PCR cloning*

Total cell RNA extracted from fetal mouse brain, liver, spleen, and thymus was reverse-transcribed into single-strand cDNA as described above. cDNA was then mixed with two degenerate primers; 5'-(C/A)G(A/G)GGACC(T/A)(C/A)G(A/G)-ACNACNAT-3' (primer A, sense strand corresponding to RG-PRTTI and 5'-(A/G)TT(T/C)TGAAACCA(G/C)ACC(T/C)G-3' (primer B, antisense strand corresponding to QVWFQN); the location of the primers is shown in Fig. 1A. The final reaction volume was 100  $\mu$ l, containing 0.2 mM of each dNTP, 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl pH 8.3, and 2.5 units of *Taq* polymerase. The target domain was amplified using the following conditions: two cycles at 94°C for 1 min, 37°C for 2 min, and 72°C for 1 min, followed by 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. A final 10-min extension at 72°C was added after the last cycle. The amplified 0.16-kb band was purified from an agarose gel and was cloned into a TA cloning vector using blue/white selection (TA cloning kit, Invitrogen). White colonies were picked and sequenced using dideoxy sequencing. Sequence analysis and comparison were done using the University of Wisconsin Genetics Computer Group programs. The clone containing the polymerase chain reaction (PCR) fragment displaying the highest degree of DNA sequence homology with Xlim-1 was purified and the fragment was labeled to a high specific activity with [ $\alpha$ -<sup>32</sup>P]dCTP by random priming with hexanucleotides.

*Human cDNA library screening*

Plaques (10<sup>6</sup>) of a human fetal brain cDNA library in the *Eco* RI site of  $\lambda$ ZapII (Stratagene) were screened using the 0.16-kb PCR fragment of mLim-1 as a random-primed probe. Overnight hybridization was performed at 60°C in hybridization solution containing 4 $\times$  SET, 0.1% sodium pyrophosphate, 0.2% NaDodSO<sub>4</sub>, and 500  $\mu$ g/ml of heparin. The final washing condition was 0.2 $\times$  SSC, 0.1% NaDodSO<sub>4</sub> at 60°C for 20 min. *In vivo* excision of the positive clones was performed as per the instructions from the manufacturer (Stratagene).

*DNA sequencing of hLim-1 cDNA clone*

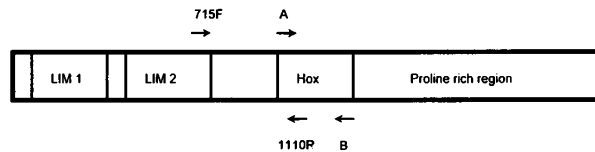
DNA sequencing of the hLim-1 clone was carried out using the Sanger dideoxy method with Sequenase, Version 2.0 (U.S. Biochemicals). The universal M13 primers and four oligonucleotides derived from sequences obtained from various subclones of the 3.0-kb cDNA were used as primers. DNA sequences and their corresponding amino acid sequences were analyzed using the University of Wisconsin Genetics Computer Group program. The sequence was deposited in GenBank (accession #U14755).

*Expression of hLim-1 detected by Northern blotting and by RT-PCR coupled with Southern blotting*

For Northern blotting, a commercially available human multitissue blot (Stratagene) was used as a source of RNA and hybridized with the full-length hLim-1 cDNA as a random-primed probe or with a random-labeled internal probe to  $\beta$ -actin. Autoradiography was performed at -70°C for 12-48 hr using Kodak XAR film.

For detection of hLim-1 by reverse transcription (RT)-PCR coupled with Southern blotting, 5 $\mu$ l of cDNA obtained from RNA prepared from a variety of human tissue sources was mixed in 100  $\mu$ l of solution containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.4  $\mu$ M of each dNTP, 10% dimethylsulfoxide (DMSO) and 2.5 units of *Taq* polymerase. The hLim-1-specific oligonucleotide primers hLM715F (5'-ACAAG-CAGCTCTCCACT-3', sense) and hLM1107R (5'-GACCTGATGACGCGCAT-3', antisense) were added at a final concentration of 50 pM. These primers correspond to the regions spanning nucleotides 715-732 (hLM715F) and 1,093-1,110 (hLM1107R) of hLim-1 cDNA. Reaction mixtures were ampli-

**A**



**B**

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GAATCCCGCGCTTCTCGCAACCGAGCTCGGGAGTCTGCTCTCTCTCTCCG 60
TTTTATTATTATTTCGGTTCGCCGCGCGGTCTCGCTGACCTPACTCCTCCGGGG 120
CTCTGAGCAGAAGGTCGCGATTCTCTCCGCGCTGAGACTCTTTTCTCGCCCGGAGC 181
TCAGCGCGCGCGCTCCAGCCCGGGCCCGGACTCCCGGCTGCACACTCTACTGAGAC 240
CCCCAGCGCGGATCAGCCTCGTTCTCCACCTACTTTGATTCTCTGGTGGAGTTTGG 300
CTTGACCGCGGAGTGTGTCTCTTTTGGAGAGACTGGGAGCTCGTGGCGGATTTGG 360
TTCAGGAGTATCCCTGGGCTCTACTTTGCCCTCTCTCTCTCTGGGCTCATCAGACC 420
AAACCAAGACCATGTTCACTGTGCCGGCTGCAAAAGGCCATCTCGGACCGCTTCTC 480
M V H C A G C K R P I L D R F L
TTGAACGTGTTGACAGGGCTGGCAGCTCAAGTGGCTCCAGTCTGTGAATGTAAATGC 540
L N V L D R A W H V K C V Q C C E C K Q
AACCTGACCGAGAAGTCTTCCAGGGAAGGCAAACTACTCAAGAAGCAGCTTCTC 600
N L T E K C F S R E G K L Y C K N D F F
CGGTGTTTCGTTACCAATGGCAGGCTGCCCTCAGGCGATCTCCCTAGGACCTGGTG 660
R C F G T K C A G C R Q G I S P S D L V
CGGAGCGCGGAGCAAACTTTCACTGACTGCTTCACTGCATGATGTGTAACAAG 720
R R A R S K V F H L N C F T C M M C N K
CAGCTTCCACTGGCGAGGAAGTCTACATCATCGAGAGAAAGTTCGTCTCGTCAAGAG 780
Q L S T G E E L Y I I D E N K F V C K E
GATTACCTAAGTAACAGCAGTGTGCCAAAGAGAACAGCTTCACTCGCCGACCGGGC 840
D Y L S N S S V A K E N S L H S A T T G
AGTGACCCAGTTTGTCTCCGATTCCCAAGACCGCTCCGAGGACGACCAAGGACTCG 900
S D P S L S P D S Q D P S Q D A C K D S
GAGAGCGCAAGTGTCCGACAAGGAGCGGTAGCAAGAGATGACGACCGAGACCTG 960
E S A N V S D K E A G S N E N D Q N L
GGCGCAAGCGCGGGGACCGCCACCACCAAGCAAGCAGCTGGAGACCTGAGG 1020
G A R K R G P G T T I K A K Q L S L D L V
GCCGCTTCGTGCTACCCCAAGCCACCGCCACATCCCGGAGGACTGGCGGAGGAG 1080
A A F A A T P K P T R H I R E Q L A T E
ACGGGCTCAACATCGCGCTCATTCAGTCTGGTTCCAGAACCGGGCTCCAGGAGCGG 1140
T G L N M R V I Q V W F Q N R R S K E R
AGGATGAAGAGCTGAGCGCCCTGGCCGCGCACGCTTCTTCCGAGTCCCGCGGATG 1200
R M K Q L S A L A G H A F F R S P R M
CGGCCGCTGGTGGACCGCTGGAGCGGGCGAGCTCATCCCAATGGTCCCTTCTCTC 1260
R P L V D R L E P G E L I P N G P F S F
TACGGAGATTACCAGAGCGAGTACTACGGCCCGGGCAACTACGACTTCTCCGCAA 1320
Y G D Y Q S E Y Y G P G G N Y D F F P Q
GGCCCCGCTCCTCGAGGCCAGACACAGTGGACCTACCTTCGTGGCCGCTATCTGGG 1380
G P P S S Q A Q T P V D L P F V P S S G
CCGTCGGGACCGCCCTGGGTGGCTGGAGCACCGCTGCCGGCCACCCCGTGGAGC 1440
P S G T P L G G L E H P L P G A C H H P S S
GAGGCGCAGCGTATACCGACATCTGGCGCACCCCGGGGACTCGCCAGCCCGGAG 1500
E A Q R F T D I L A H P P G D S P S P E
CCCAGCTGCCCGGCTCTGACTCCATGTGGCGGAGGCTTCGGAGCCAGCCCGCCC 1560
P S L P G P L H S M S A E V F G P S P P
TCTCGTCTGTCGTTCAACGGTGGGGGAGCTACGGAAACACCTGTCCACCCCCC 1620
F S S L S V N G G A S Y G N H L S H P P
GAAATGAACGAGCGCGCTGTGTAGCGGGCTCTCGCAGCTCTCGGAGTCTGTGGTT 1680
E M N E A A V W *
GTACAGAAATGAACCTTTATTTAAGAAAAATAG
    
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**FIG. 1.** A. Schematic representation of Lim-1 showing the sites of the degenerate primers to the Hox domain (primer A forward, primer B reverse). For the amino acids encoded by these primers see Materials and Methods. The sites of the LIM domains, homeodomain, and proline-rich region are shown. 715F and 1110R are the primers used for RT-PCR expression studies. B. Nucleotide sequence and deduced amino acid sequence of hLim-1 cDNA. The tandem copies of the LIM motifs followed by the homeobox domain are underlined. The insertion site of the domain encoding the alternative transcript is indicated by the bolded arrow.

fied by PCR in standard fashion. Parallel experiments, which included primers specific for the  $\beta$ -actin gene, were amplified in similar fashion and served as controls. PCR products were transferred onto a Hybond-N<sup>+</sup> nylon membrane (Amersham, Oakville, ON) using the procedure described by Southern (1975) and hybridized, where appropriate, with either the full-length hLim-1 cDNA as a random-primed probe or with a random-labeled internal probe to the  $\beta$ -actin PCR product. Autoradiography was performed at  $-70^{\circ}\text{C}$  for 12 hr using Kodak XAR film.

Samples were considered positive for hLim-1 expression if a band of appropriate size ( $\sim 0.4$  kb) was observed by hybridization with the full-length hLim-1 cDNA. Samples were considered to be negative if RT-PCR amplification of  $5\ \mu\text{l}$  of the cDNA reaction mixture did not yield a band by hybridization whereas a human  $\beta$ -actin band of appropriate size ( $\sim 0.5$  kb) was visible after gel electrophoresis and ethidium bromide staining.

#### *Chromosome localization by fluorescence in situ hybridization*

Plaques ( $10^6$ ) from a human genomic library made from a leukemic sample were screened with a hLim-1 cDNA probe. A hLim-1-specific phage clone containing a 16-kb insert was identified and confirmed by hybridization with several hLim-1 oligonucleotides.

Lymphocytes isolated from human cord blood were cultured in  $\alpha$ -MEM containing 10% FCS and phytohemagglutinin (PHA) at  $37^{\circ}\text{C}$  for 68–72 hr. Lymphocyte cultures were then treated with bromodeoxyuridine (BrdU) (0.18 mg/ml; Sigma, St. Louis, MO) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block and recultured for 6 hr in  $\alpha$ -MEM containing thymidine (2.5  $\mu\text{g}/\text{ml}$ ; Sigma). Cells were harvested, and metaphase spreads were prepared using standard procedures. The hLim-1-containing phage clone was labeled with biotinylated dATP (BioNick, GIBCO-BRL) at  $15^{\circ}\text{C}$  for 1 hr. Fluorescence *in situ* hybridization (FISH) was performed as previously described (Heng *et al.*, 1992). Briefly, slides were baked at  $55^{\circ}\text{C}$  for 1 hr, and following RNase A treatment, the DNA was denatured in 70% formamide in  $2\times$  SSC for 1 min at  $70^{\circ}\text{C}$  and then dehydrated with ethanol. The biotinylated hLim-1 genomic probe was denatured at  $75^{\circ}\text{C}$  for 5 min in a hybridization mix of 50% formamide,  $2\times$ SSC, and 10% dextran sulfate. After overnight hybridization, the slides were washed and the signals were revealed by fluorescein-labeled avidin (Oncor Inc., Canada). Chromosomes were identified by staining with 4',6-diamidino-2-phenylindole (DAPI). Assignment of FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with DAPI-banded chromosomes (Heng and Tsui, 1993).

## RESULTS

#### *Isolation and sequence of mLim-1 gene*

At the time of initiation of our studies, the murine homolog of XLim-1 had not been deduced. To screen a human cDNA library, we generated a mLim-1 probe using XLim-1 as a template. RNA extracted from fetal mouse brain, liver, and spleen was reverse transcribed and the cDNA was used for amplification by RT-PCR. Using the degenerate primers A and B, corre-

sponding to the RGPRTTI and QVWFQN amino acid sequences in the homeodomain of XLim-1, respectively, a predicted product of 0.16-kb was amplified reproducibly (Fig. 1A). The purified band was cloned into a plasmid vector and 20 clones sequenced. Analysis revealed that two classes of sequences were obtained. One sequence class, isolated from fetal brain and spleen, displayed 82% homology with the XLim-1 homeobox domain, and was therefore named mLim-1. The other sequence class, isolated from all three fetal tissues, showed homology with a human  $\alpha$ -fetoprotein gene enhancer binding protein gene,  $\alpha$ -PEB, which encodes a zinc finger homeobox protein. Because our primary interest is in identifying members of the LIM-Hox family, this result was not pursued further.

#### *Cloning, sequencing, and structure of hLim-1 cDNA*

The mLim-1 fragment was used as a probe to screen a human fetal brain cDNA library. A clone containing a 3.0-kb insert was identified and 1.712-kb of the 5'-end was sequenced (Fig. 1B). The sequence, termed hLim-1, was deposited in GenBank (accession no. U14755) and represents the human homolog of XLim-1. Figure 1 shows the nucleotide and deduced amino acid sequences of this 1.712-kb cDNA. Overall, the nucleotide sequence of the coding region displays 87% homology with mLim-1, 85% homology with chicken Lim-1 (cLim-1), and 79% homology with XLim-1. The 5' untranslated region of hLim-1 is 432 bp in length. It is of note that the first 220 bp of the 5' untranslated region is 70% identical with the mouse 5' untranslated region, whereas there is no homology between these two sequences over the remainder of the 5' untranslated region. This conservation of sequence suggests that there may be a regulatory function of the 5' untranslated sequences. The translated region extends from nucleotides 433 to 1,643. A comparison of the nucleic acid sequences of mLim-1 and hLim-1 was 87% similar; however, at the amino acid level, the sequence was 97% identical (Fig. 2). Like other Lim-hox genes, the Lim domain is ahead of the homeobox domain. The 3' untranslated region of hLim-1 is approximately 1.3-kb in length; this has not been sequenced.

#### *Gene expression*

Expression of hLim-1 was evaluated first by Northern blotting using a commercially available human multitissue blot and RNA extracted from two human leukemia cell lines. Expression of hLim-1 was not detected in normal tissue; however, a 4.0-kb band corresponding to the full-length transcript was detected in RNA of the K562 cell line, a line derived from the leukemic cells of a patient with chronic myeloid leukemia (CML) in myeloid blast transformation (Fig. 3).

The ability to detect hLim-1 expression was enhanced when evaluated by RT-PCR coupled with Southern blotting of RNA from normal tissues. RT-PCR products were observed in samples from human brain, tonsil, and thymus, but not peripheral blood mononuclear cells, normal bone marrow, or CD34<sup>+</sup> bone marrow cells, colon, testis, ovary, or placenta. In RNA from brain and tonsil, there were two bands of 405 bp and 498 bp, whereas in thymus only the smaller band was present (Fig. 4).

To identify the nature of the 405- and 498-bp bands, both were cloned and sequenced. The 405-bp band has a sequence identical to the cDNA isolated from the brain cDNA library. The 498-bp fragment was identical to the 405-bp band except

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human:  MVHCAGCKRPIIDRFLLNVLDRAWHVKVCQCECKCNLTE 40
mouse:  MVHCAGCKRPIIDRFLLNVLDRAWHVKVCQCECKCNLTE
Xenopus: MVHCAGCERPILDRFLLNVLDRAWHVKVCQCECKCNLTE

human:  KCFsREGKLYCKNDFFRFCGFKCAGCRQGISPSDLVRRAR 80
mouse:  KCFsREGKLYCKNDFFRFCGFKCAGCAQGISPSDLVRRAR
xenopus: KCFsREGKLYCKNDFFRFCGFKCAGCAQGISPSDLVRRAR

human:  SKVFHLNCFTCMNCNKQLSTGEELYIIDENKFVCKEDYLS 120
mouse:  SKVFHLNCFTCMNCNKQLSTGEELYIIDENKFVCKEDYLS
xenopus: SKVFHLNCFTCMNCNKQLSTGEELYIIDENKFVCKEDYLN

human:  NSSVAKENSLHSATTGSDPSLSPDSQDPSQDDAKDSESAN 160
mouse:  NSSVAKENSLHSATTGSDPSLSPDSQDPSQDDAKDSESAN
xenopus: NNNAAKENSFISVT. GSDPSLSPESQDPLQDDAKDSESAN

human:  VSDKEAGSNENDDQNLGAKRRGPGTTIKAKQLETLKAFA 200
mouse:  VSDKEGGSNENDDQNLGAKRRGPRTTIKAKQLETLKAFA
xenopus: VSDKEAGINENDDQNLGAKRRGPRTTIKAKQLETLKAFA

human:  ATPKPTRHIREQLAQETGLNMRVIQVWFQNRRSKERRMKQ 240
mouse:  ATPKPTRHIREQLAQETGLNMRVIQVWFQNRRSKEARMKQ
xenopus: ATPKPTRHIREQLAQETGLNMRVIQVWFQNRRSKERRMKQ

human:  LSALAG. .HAFFRSRRMRPLVDRLEPGELIPNGPFSFYG 280
mouse:  LSALGARRHAFFRSRRMRPLVDRLEPGELIPNGPFSFYG
xenopus: LSALGARRHAFFRSRRMRPLVDRLEPGELIPNGPFAFYG

human:  DYQSEYYGPGGNYDFFPQGPSSQAQTPVDLPFVPSGSPS 320
mouse:  DYQSEYYGPGGNYDFFPQGPSSQAQTPVDLPFVPSGSPS
xenopus: DYQSEYYGPGSNYDFFPQGPSSQAQTPVDLPFVPSVVA
human:  GTPLGLEHPLPGHH. PSSEAQRFTDILAHPPGDSPSPEP 360
mouse:  GTPLGGLDHLPLPGHHAPSSEAQRFTDILAHPPGDSPSPEP
xenopus: GTPLGAMDHPPIPGHH. PSSDAQRFDTIMSHPPGDSPSPEP

human:  SLPGPLHMSAEVFGSPFPFSSLSVNGGASYGNHLSHPPE 400
mouse:  SLPGPLHMSAEVFGSPFPFSSLSVNGGASYGNHLSHPPE
xenopus: NLPGSMHMSAEVFGSQSPFPFSSLSVNGG. .YGNHLSHPPE

human:  MNEAAVW
mouse:  MNEAAVW
xenopus: MNETAVW
    
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FIG. 2. Amino acid sequence alignment of XLim-1, mLim-1, and hLim-1. Vertical bars indicate identical residues.

for a 93-bp insert beginning at nucleotide 829; the insert maintains the reading frame (Fig. 5). To determine the genomic structure responsible for these two forms, we performed PCR on normal DNA using the same two primers. This resulted in the amplification of a 498-bp piece. Inspection of this sequence revealed potential splice donor sites of GGGT beginning at position 828 of the full-length cDNA and a potential splice acceptor of AGCC 93 bp downstream. Because the PCR primers used in the RT-PCR experiments are potentially in the same exon, we confirmed that the larger 498-bp RT-PCR product was derived from RNA and not genomic DNA by treating samples with RNase or DNase. Pretreatment of the samples with RNase eliminated both bands. Pretreatment of the samples with DNase did not alter the result, thus indicating that the RT-PCR product is derived from RNA and not DNA.

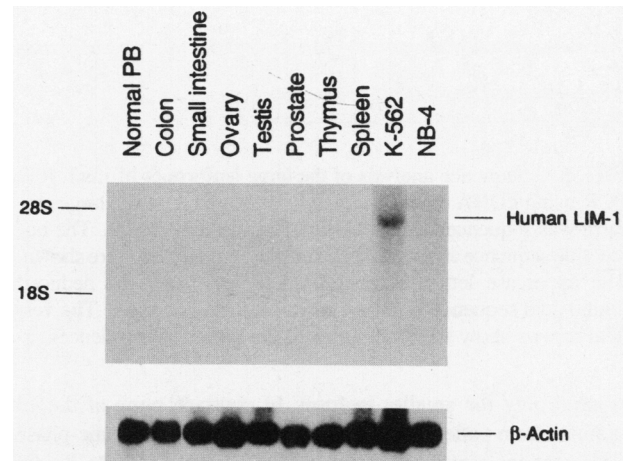


FIG. 3. Northern analysis of the hLim-1 gene transcript using a human multitissue blot (Clontech) and mRNA from two leukemia cell lines, K562 and NB-4. For the cell lines, 2  $\mu$ g of poly(A)<sup>+</sup> RNA was used. Size markers are indicated on the left. The sizes of the transcripts were determined by the relative position of the 28S and 18S ribosomal bands of co-electrophoresed total RNA.

The finding of hLim-1 expression in a human leukemia cell line but not in normal T cells or CD34<sup>+</sup> bone marrow cells prompted a more extensive evaluation of hLim-1 expression in a variety of human leukemias using established cell lines and primary patient samples (Tables 1 and 2). Variable expression was seen in the cell lines. Overall, 8 of the 21 (38%) different leukemia/lymphoma cell lines showed expression of hLim-1. Interestingly, 4 of 7 (57%) acute myelogenous leukemia (AML) cell lines evaluated displayed hLim-1 expression. Both isoforms were expressed in the cell lines, with the exception of KOPKT-1, a cell line derived from an acute T-cell leukemia, which ex-

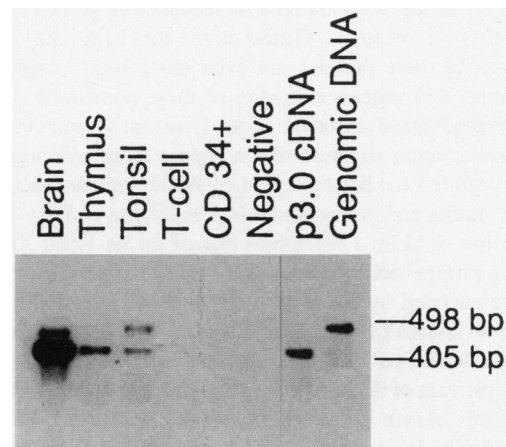


FIG. 4. RT-PCR coupled with Southern blotting to detect hLim-1 expression. cDNA prepared from the tissue and cell populations indicated were amplified as outlined in Materials and Methods. Specific amplification of hLim-1 was performed using the hLM715F and hLM1110R oligoprimers. A plasmid containing hLim-1 cDNA and normal human genomic DNA were used as positive control samples. The lane indicated as negative contained no template DNA.

```

      1
cttcactcggGTGAGGCCCAATTCTGGCTGGCTAGGTGCAAGCGGGTCTGGGGGAGGAAG
L H S G E A P I P G W L G A S G S W G R K
      |
GCTCGCCAAGGCCCGGCTCATCTGTCTTCCCTCTTAGccaccacgggc
A R Q G P G S S V L S L L A T T G
1713

```

**FIG. 5.** Sequence analysis of the large isoform of hLim-1. RT-PCR using cDNA from tonsil was performed, and the large isoform was sequenced after isolation from an agarose gel. The nucleotide sequence and its deduced amino acid sequence are shown. The uppercase letters represent the 93-bp insert; the deduced amino acid sequence is shown below and is underlined. The vertical arrows show the boundaries of the additional sequences.

pressed only the smaller isoform. In contrast, none of the 13 samples from patients with AML and only 1 of 9 chronic-phase CML patient samples expressed hLim-1 RNA (Table 2). Of note, however, was the finding of hLim-1 expression in four of five samples obtained from patients with CML in myeloid blast transformation.

#### Chromosome mapping of hLim-1 by FISH

FISH, using the 16-kb hLim-1 containing phage DNA clone as a labeled probe, revealed exclusive labeling to chromosome 11 (Fig. 6A,B). A total of 15 metaphase spreads were scored, of which 10 were photographed. Signals were assigned to 11p12-13 (Fig. 6C).

## DISCUSSION

In this study, we report the cloning, expression, and chromosomal localization of hLim-1, the proposed human homolog of mLim-1 and XLim-1. The evidence that hLim-1 is, in fact, the homolog of the above-mentioned genes is convincing. The cDNA nucleotide sequence of the coding region of hLim-1 is 87% and 79% identical with mLim-1 and XLim-1, respectively. Moreover, the deduced amino acid sequence of hLim-1 is 97% identical with mLim-1. Furthermore, the LIM and homeodomains of these proteins are even more highly conserved. The amino acid sequence identity of these domains is greater than 99% identical amongst hLim-1, mLim-1, and XLim-1. This conservation suggests that a precise structural organization is required for the execution of the biological function(s) of LIM motifs and homeodomains. Finally, the carboxy-terminal portion of hLim-1 resembles that of mLim-1 and XLim-1 in being proline-rich, whereas, this region is glutamine-rich in proteins encoded by many other LIM-class homeobox genes.

Like all known LIM-class homeobox genes, hLim-1 encodes a protein with two tandemly repeated cysteine-rich LIM domains upstream of the homeodomain. The unique structural feature of this human homolog, however, is that it encodes two isoforms. The larger isoform has an additional 93 bp inserted between the LIM domains and the homeodomain. These two different forms arise as a result of alternative splicing. The additional sequences in the RNA results in the in-frame addition of 31 amino acids in the region between the distal LIM domain and the homeobox domain. Whether this insert has any functional significance is not known.

Barnes *et al.* used RNase protection to evaluate the expression of mLim-1 in adult tissues. They found high levels of ex-

pression in cerebellum, medulla, and kidney and very low levels in cerebrum. They did not test thymus or tonsil, although spleen was negative by this method for mLim-1 expression. We used the more sensitive RT-PCR method to detect hLim-1 expression in adult human tissues. High levels of expression were found in brain, thymus, and tonsil; the other tested tissues were negative. We did not test expression in human kidney. It is of note that hLim-1 is expressed in a hierarchical manner in the T-cell lineage because cells from the thymus were positive, whereas peripheral blood mononuclear cells, composed primarily of mature T cells, were negative. This pattern of restricted expression is in keeping with other LIM-class homeobox genes thus far described. In the adult mouse, the restricted pattern of mLim-1 expression suggests a role in the maintenance of differentiated renal tissue and in the maintenance of differentiated cell populations within the central nervous system (CNS) (Barnes *et al.*, 1994). Likewise, the expression pattern of LH-2, another mammalian LIM/homeobox gene, suggests that this protein may serve as a transcription factor involved in the differentiation of distinct B- and T-cell subpopulations (Xu *et al.*, 1993).

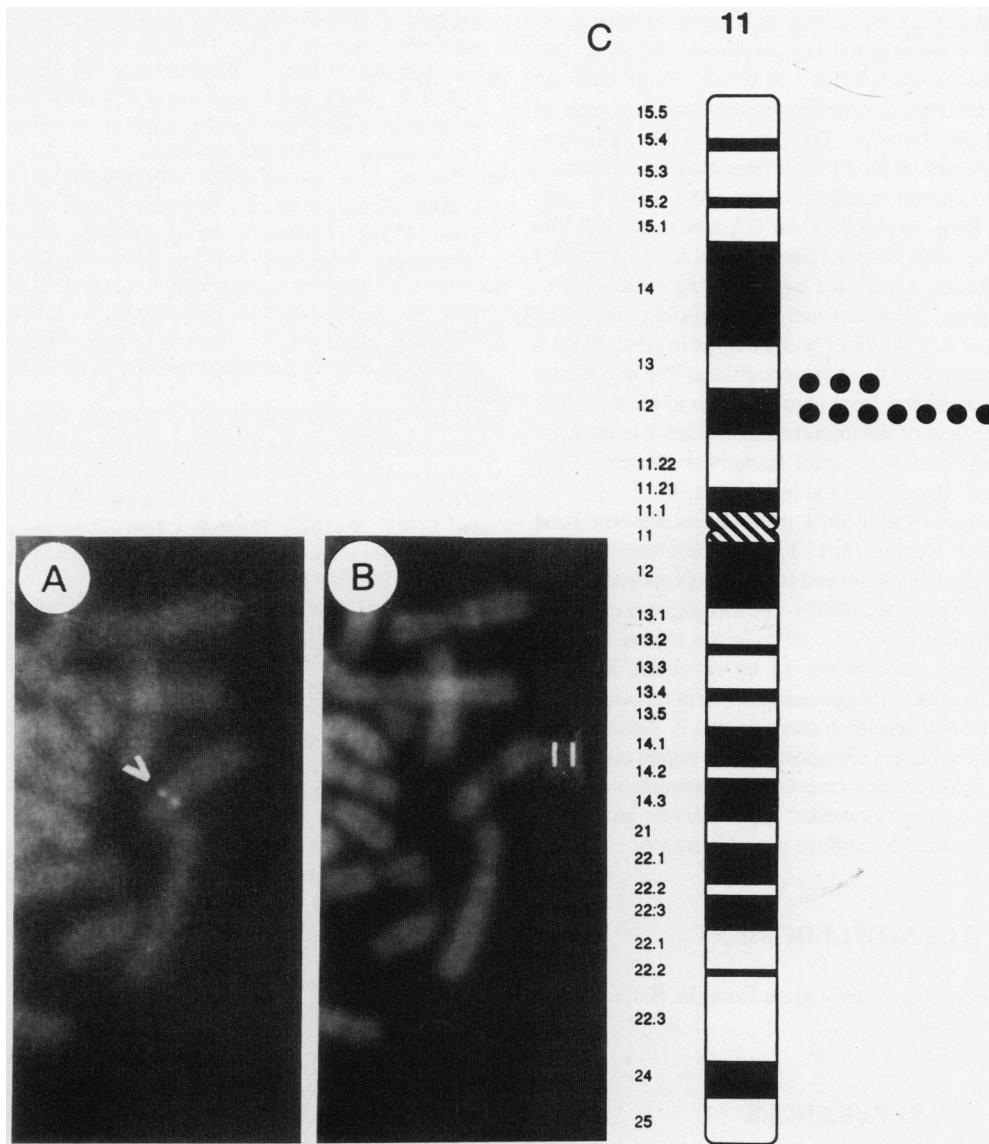
The inability to detect hLim-1 expression in normal tissue by Northern blotting, but its detection in selected tissue by RT-

TABLE 2. EXPRESSION OF hLIM-1 IN PRIMARY LEUKEMIA SAMPLES

Diagnosis	Clinical Status	hLim-1
AML-M1	Presentation	-
AML-M2	Presentation	-
AML-M2	Presentation	-
AML-M2	Presentation	-
AML-M2	Relapse	-
AML-M4	Presentation	-
AML-M4	Presentation	-
AML-M4	Presentation	-
AML-M4	Relapse	-
AML-M4	Relapse	-
AML-M4	Relapse	-
AML-M5	Presentation	-
AML-M7	Presentation	-
AML-M7	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	+
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-CP	Presentation	-
CML-BP	Presentation	-
CML-BP	Presentation	++
CML-BP	Presentation	++
CML-BP	Presentation	++
CML-BP	Presentation	++

AML is classified using the FAB (French-American-British) classification.

CML is classified using the NCI criteria: CP denotes first chronic phase and BC denotes blast crisis. The symbol - represents no detectable hLim-1 expression, + represents weak positive, and +++ represents strongly positive.



**FIG. 6.** A. hLIM-1 gene mapped to human chromosome band 11p12–13 using FISH biotinylated hLim-1 genomic phage DNA. The biotinylated probe was hybridized to metaphasic chromosomes of normal human lymphocytes and detected with fluorescein-labeled avidin. Fluorescent signals are indicated by arrows. B. Visualization of chromosome banding with staining with 4,6-diamidino-2-phenylindole (DAPI). C. Ideogram of human chromosome 11 showing the location of hLIM-1 gene. Each dot represents the location of a double-fluorescent signal on chromosome 11.

PCR indicates that hLim-1 is expressed at relatively low levels. In contrast, some malignant cells overexpress hLim-1, as shown by our detection using Northern blotting of hLim-1 transcripts in the K562 cell line. The aberrant expression of proteins containing LIM and/or Hox domains has been previously shown to be implicated in the genesis of leukemias. The RBTN-2 gene, which encodes a LIM-domain protein, was originally identified by its involvement in a recurrent chromosomal translocation in T-cell acute lymphoblastic leukemia (ALL) and subsequently its overexpression has been shown to have transforming activity in transgenic mice (Fisch *et al.*, 1992). Hox genes have also been implicated in the development of leukemia as a result of chromosomal translocations and activation by retroviral insertion (Perkins *et al.*, 1990; Kennedy *et al.*, 1991).

The finding of hLim-1 overexpression in K562, coupled with the observation of hLim-1 expression in lymphoid tissue and precursor T cells, led to a more extensive evaluation of its expression profile in a variety of hemato-lymphoid tumor cell lines and in primary leukemia samples. Overall, hLim-1 expression in human leukemia and lymphoma cell lines was variable, but with a preponderance in AML cell lines. We believe that the finding of hLim-1 expression in AML cell lines is significant, because a relatively pure population of CD34<sup>+</sup> normal bone marrow cells, the postulated normal counterpart of leukemia cells of myeloid origin, did not express hLim-1. Interestingly, hLim-1 expression was not detected in the evaluation of primary AML samples; this is similar to our findings of hLH2, another Lim/Hox gene (Wu *et al.*, 1996). The inability to detect



hLim-1 in primary AML samples may be because the gene is expressed at very low levels in a minor population of cells; it has been estimated that on the order of 1 in  $10^4$  to 1 in  $10^5$  cells are able to establish leukemia in immune-deficient severe combined immunodeficient (SCID) mice (Lapidot *et al.*, 1994). Alternatively, the activation of hLim-1 in cell lines may be an artefact, resulting from the growth in culture of the cells. A direct comparison of RNA from the sample used to establish the cell line is required to address this issue. Evidence that hLim-1 is involved in leukemia in patients is provided by the finding of hLim-1 expression in the blasts of patients with CML in blast crisis. The presence of hLim-1 in CML blast crisis, but not in AML, further confirms the uniqueness of these two conditions. The importance of hLim-1 in the transition from chronic phase to blast crisis is not known. Regardless of the importance of hLim-1 in transformation, it may prove to be a useful marker for identifying patients whose disease is entering the blast phase.

Finally, we mapped the hLim-1 gene to chromosome band 11p12-13 by FISH. Region 11p12 has not been linked to any diseases, but 11p13 contains several important genes such as the Wilms' tumor (WT) gene and RBTN-2 (Champagne *et al.*, 1989; Rose *et al.*, 1990; Boehm *et al.*, 1991). In the mouse, mLim-1 has been mapped to chromosome 11 by restriction fragment length polymorphism and linkage analysis, yet in this animal the WT gene and RBTN-2 reside on chromosome 2. This observation suggests that the human chromosome 11p region containing hLim-1 and the mouse chromosome domain that contains mLim-1 are regions of synteny. However, more markers around the hLim-1 locus are needed to confirm the relationship.

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