Developmental Regulation of lck Gene Expression in T Lymphocytes

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Summary

In the mouse and human, mRNA transcripts encoding the lymphocyte-specific protein tyrosine kinase p56\(^{\text{lck}}\) are derived from two separate promoters resulting in heterogeneity in the 5' untranslated region sequence. The proximal promoter lies just 5' to the coding region for the gene and is active only in thymocytes. In contrast, the distal promoter lies 34 kilobases (kb) 5' in the human, and is active both in thymocytes and mature peripheral T cells. As previously reported, transgenic mice bearing functional proximal promoter sequence juxtaposed with the SV40 large T antigen gene invariably develop lymphoid tumors confined to the thymus. In the current work, transgenic mice bearing a 2.6-kb fragment of the human distal promoter fused to the SV40 large T antigen gene express large T antigen in thymocytes and in peripheral lymphoid cells, and develop tumors of both the thymus and the peripheral lymphoid organs. The ability of the human distal promoter to function appropriately in transgenic mice is consistent with the strong similarity observed between the mouse and human distal promoter sequences. With the exception of a single short interval that serves as a target for binding of nuclear factors, significant sequence similarity is not seen when the distal and proximal promoter sequences are compared. Hence, developmentally regulated, lineage-specific transcription of the lck gene is mediated by distinct promoter sequences that appear to be capable of functioning independently.

The \(^{\text{lck}}\) gene encodes a membrane-associated protein tyrosine kinase (p56\(^{\text{lck}}\)) that is expressed in cells of the lymphoid lineage, primarily T cells, lymphoid tumor cell lines, and a few nonlymphoid tumors (1-3). While the precise function of p56\(^{\text{lck}}\) in T cells remains undefined, it physically associates with the cytoplasmic tails of CD4 and CD8 coreceptor molecules (4-6). Hence it is believed that p56\(^{\text{lck}}\) may interact with the T cell antigen recognition complex and participate in an antigen-stimulated signal transduction pathway (7).

We and others have shown that \(^{\text{lck}}\) transcripts derived from certain cultured cell lines are heterogeneous with respect to the nucleotide sequence of the 5' untranslated region (5' UTR) (2, 6, 8-10). The two major transcript types seen in the cell lines, type I and type II, are derived from two alternative promoters. Transcription from a conserved promoter region contiguous with the \(^{\text{lck}}\) coding sequence (the proximal promoter) results in type I transcripts. Type II transcripts are derived by splicing of a larger transcript originating at another promoter separated by many kb from the coding sequence (the distal promoter) (6, 8, 11). Since the splice acceptor site for the type II RNA precursor is just 5 bp 5' to the translation initiation codon, almost the entire 5' UTR sequence of type II \(^{\text{lck}}\) transcripts differs from that of type I.

We do not know whether these results in cultured, often transformed cell lines reflect the true state of transcription in normal cells. Garvin et al. (8) have shown that transcripts containing type I and type II 5' UTRs are both present in normal human thymus RNA, suggesting that alternative promoter usage also occurs in normal human T cells. With this exception, the expression patterns of type I and type II \(^{\text{lck}}\) mRNAs in normal lymphoid cells have not been examined.

To better define the regulation of \(^{\text{lck}}\) gene expression, we have cloned genomic DNA fragments containing the human and mouse distal \(^{\text{lck}}\) promoters, and have sequenced the regions flanking the transcription start sites. While these mouse and human sequences are very similar to one another, they share little similarity with the proximal promoter sequences from either species. Nonetheless, a short region with a high conservation between distal and proximal promoters was identified and found to be sufficient to bind nuclear proteins in a sequence- and tissue-specific manner. We demonstrate that type I and type II transcripts are differentially expressed in...
a developmentally regulated fashion in normal lymphoid cells, and that the proximal and distal promoter elements can function independently in transgenic mice.

Materials and Methods

Genomic Libraries and Library Screening. Human clone SP2 (Fig. 1 a) was isolated from a genomic cosmid library (8) by hybridization screening with the human lck cDNA clone HK28 (2) using established procedures (8). A map was generated by analysis of restriction fragments and overlapping cosmids. Mouse clone 32.1 (Fig. 1 b) containing a 15.5-kb insert was isolated from a Charon 4A phage library of AblI and HaeIII partially digested BALB/c mouse sperm DNA (12) by hybridization screening with a 51 bp synthetic double-stranded oligonucleotide (5'AGGTCTAGGACCATGTGAAATGCGAAGAGGCTCCCAGGCTGGCAGG-3') specific for the 5' UTR sequence of mouse type II transcripts (13). Plating of the phage, screening by hybridization, isolation, and large scale culture were performed as previously described (1).

DNA Sequencing and Sequence Analysis. Nucleotide sequence was determined by the Sanger dideoxy chain-termination method (14) using specific oligonucleotide primers applied to both strands of double stranded pUC18 or pUC19 plasmids containing the appropriate SP2 and 32.1 subclones. Dot matrix comparisons, repeat analysis, and small-scale similarity searches were performed on a microcomputer running Genepepro software (Riverside Scientific Enterprises, Seattle, WA). The parameters for dot matrix comparisons were chosen empirically so as to strictly limit the number of matches occurring randomly. Sequence alignments were performed piece-wise in the program Nucaln (D. J. Lipman, Bethesda, MD) using a K-tuple of 3, window size of 20, and gap penalty of 7, and then pieced together manually for best fit. The EMBL19 DNA sequence library was searched within PC-GENE (Intelligenetics, Inc., Mountain View, CA).

RNA Blotting and RNAsae Protection. Human peripheral blood T cells were isolated as previously described (15, 16). Human thymocytes were subjected to complement lysis after incubation with either anti-CD4 (66.1), anti-CD8 (OKT8), or both monoclonal antibodies, as described (15). The efficiency of the separation was checked by flow cytometry of the surviving cells stained with conjugated antibodies specific for CD3e, CD4, CD8, and ß2 TCR.

Total cellular RNA was prepared from the resulting cells by the guanidinium thiocyanate/cesium chloride method (17). 5 μg of RNA were analyzed in each lane by electrophoresis in 2.2 M formamide/1% agarose gels and blotted as described (18). Blots were hybridized at 42°C in Southern blot buffer with 50% formamide (19) with a denatured synthetic double-stranded 64 bp [32P]-labeled DNA probe specific for the 5' UTRs of type II human lck transcripts (bases +2 to +105). After autoradiography, the blot was stripped of probe by boiling and reanalyzed with a 64 bp probe specific for the human type 1 5' UTR (bases +41 to +104). The probes were constructed and labeled as described below. After each hybridization, the blot was washed to a final stringency of 50°C in 1× SSC, 0.1% SDS (19). Densitometry was performed on a digitized video image using Visage software (Bio Image, Ann Arbor, MI).

Whole cellular RNA samples from normal mouse thymocytes and LN cells were analyzed by RNAsae protection as described (9). The antisense lck RNA probe was transcribed from a subcloned SfA1-EcoRI mouse genomic fragment spanning the 5' UTR of type I lck transcripts, the first exon, and the flanking promoter (5') and intron (3') sequences (Fig. 2 a). The CD4 RNA probe was made in a similar fashion from a 145 bp XhoI-PstI fragment from the 5' end of the mouse CD4 cDNA (20).

Gel Mobility Shift Assay. Extracts of nuclear proteins were prepared from the organs of freshly sacrificed normal mice as described (21), except that protease inhibitors were not added. Labeled probe was run on a 0.4 mm thick, 16% polyacrylamide gel and eluted from the gel slice into water. Extracts were incubated with DNA essentially as described (21). Briefly, 2-5 μl of extract was incubated with 1.5 μg of poly dIdC (Sigma Chemical Co., St. Louis, MO) for 15 min at 4°C in a total volume of 8 μl. 10 ng (1 μl) of unlabeled competitor DNA, or water, was then added, and after 10 min at room temperature, 10^6 cpm (~60 pg) of labeled probe was added and the mixture incubated for an additional 25 min. Samples were run on a 0.8 × 100 × 170-mm, 6% polyacrylamide gel at 175 V for 2 h at 4°C with recirculation of the buffer (50 mM Tris-Cl, 1 mM EDTA, pH 8.5), the gel was dried, and autoradiographed.

Oligonucleotide Probes and Competitors. Short, double-stranded DNA fragments were prepared for use as probes or unlabeled competitors by annealing pairs of synthetic DNA oligonucleotides that are complementary at their 3' ends in 50 mM NaCl, 10 mM Tris-Cl, pH 7.5, 6 mM MgCl2, 3 mM dithiothreitol for 15 min at 37°C, then adding the four deoxynucleotide triphosphates to 30 μM and filling in the recessed ends with Klenow fragment at room temperature for 5-10 min. For radiolabeled probes, [32P]dATP was substituted for dATP in the Klenow step, and chased with unlabeled dATP for an additional 5 min. Competitors prepared for use in gel mobility shifts were phenol/chloroform extracted, precipitated with ethanol, and redissolved in water before use.

Transgenic Animals. The human genomic clone SP12 (Fig. 1 a) which includes 2.5 kb of the region 5' to the human type II transcription start site (i.e., the distal lck promoter) was juxtaposed with a StuI-BamHI fragment of the SV40 genome containing the gene for large and small T antigen (22) to construct a clone called SP12T. Mice transgenic for this construct were generated as previously described (22). Animals were sacrificed at various ages or when they appeared ill. Tumors were visualized upon gross dissection and confirmed in some cases by histology. Cells from tumors and normal appearing lymphoid organs were suspended in solution, stained with fluorescence-conjugated antibodies to mouse CD4, CD8, and CD3e, and analyzed for the presence of those molecules and for size (forward scatter) by flow cytometry as previously described (22). Northern blot of tissue RNA from a transgenic mouse was prepared as described above and probed with the StuI-BamHI SV40 T antigen gene fragment labeled by random priming (19).

Results

The Human Distal lck Promoter Resides 34 kb 5' of the Proximal Promoter. Though the presumed mechanism by which type II lck transcripts are generated is via splicing of a precursor mRNA derived from the distal promoter, physical linkage of these sequences has not been shown. By screening a human genomic cosmid library, we isolated a single cosmid clone (SP2) containing both type I and type II 5' UTR sequences. Mapping of the entire region by subcloning, restriction enzyme analysis, and sequencing demonstrated that the type II transcription start site is positioned 34 kb 5' to that of the type I lck transcripts in human DNA (Fig. 1 a). The fidelity of the cosmid was confirmed by genomic blotting analyses (data not shown). The long interval separating these tran-
Expression of Type I and Type II Transcripts In Human Thymocyte Subsets.

Since the proximal promoter is active in the thymus and inactive in mature peripheral T cells, we wondered whether this developmental switch occurred at a particular stage of T cell maturation in the thymus. We examined the expression of type I or type II lck transcripts among normal human thymocyte subpopulations defined on the basis of their expression of the T cell surface coreceptor molecules CD4 and CD8 (Fig. 3). Using oligonucleotide probes specific for the 5' UTR of each type of transcript, both type I and type II transcripts were detected in each of the CD4/CD8 thymocyte subsets studied. Although lck mRNA levels did not differ greatly among the subsets, there was a trend toward higher expression of type II transcripts in the more mature, CD4+/CD8- or CD4-/CD8+ (double-negative) thymocytes (Fig. 3, lane 5). Conversely, the levels of type I mRNA were lower in the more mature thymocyte subtypes, particularly the CD4+ cells (Fig. 3, lane 4). The technique used to isolate the subsets, complement lysis, did not allow isolation of intermediate maturity CD4+/CD8- (double-negative) thymocytes from those missing one or both of the CD4 or CD8 molecules. However, double-positive cells constitute the majority of thymocytes and are thus represented in Fig. 3, lanes 1 and 2, admixed with the other, less abundant subtypes. Furthermore, in other experiments, RNA from human CD4+/CD8+ thymocytes separated by FACS® (Becton Dickinson and Co., Mountain View, CA) or peanut agglutinin contained abundant lck mRNA (data not shown). As complement lysis could
not eliminate double-negative cells from the single-positives in Fig. 3, lanes 3 and 4, we calculated the maximum proportion of hybridization signal potentially attributable to double-negative cells using flow cytometry analysis of the contributing pools (see Fig. 3, legend), and densitometry analysis of the blots. Fig. 3, A and B, lanes 3 and 4, respectively, contain, at most, 2%, 31%, 0.5%, and 2%, respectively, signal from double-negative cells. Hence, single-positive cells in the thymus do express some type I, as well as type II, Ick mRNA. We conclude that type I and type II Ick mRNA is present in each of the human thymocyte subpopulations defined by CD4 and CD8 expression, although we detected only small amounts of type I mRNA in the CD4−/CD8− compartment. These results are consistent with the recently reported findings of Reynolds et al. (25), who showed a similar distribution of differential promoter usage in flow cytometry-separated mouse thymocytes and peripheral T cells.

Virtually all peripheral T cells are derived from bone marrow progenitors that mature in the thymus (26). These immature thymocytes pass through a CD4+/CD8− stage that gives rise to more differentiated single-positive cells that are the immediate precursors of peripheral T cells (27). Since single-positive thymocytes contain type I Ick transcripts while peripheral T cells do not (Fig. 2 b and Fig. 3), the accumulation of these transcripts must cease in the short interval immediately preceding thymocyte emigration.

Sequence Elements of the Distal Ick Promoter Are Highly Conserved. Since the pattern of expression of type I and type II Ick transcripts is closely conserved in human and mouse lymphoid cells, one might expect to observe conservation in cis-linked sequences involved in distal and proximal promoter regulation. Indeed, we have previously reported that the human and mouse proximal promoter sequences contain regions of very high sequence identity (8). To examine the distal promoters for such conserved regions, we sequenced portions of the SP12 cosmid and the phage clone 32.1 containing the human and mouse distal promoters, respectively (Fig. 4).

Alignment of the mouse and human distal Ick promoter sequences reveals a high degree of linear conservation within the first 200 bases 5' to the transcription start site (Fig. 4). Further aligned similarities are seen 5' to a 257 bp segment that appears in the mouse but not in the human. Notably, there is also a high degree of sequence conservation in the 5' UTR for type II Ick transcripts. Significant similarities between the mouse and human sequences disappear 3' to the splice donor site (data not shown), indicating that at least the 5' end of this intron is not conserved.

The Proximal and Distal Ick Promoters Share Few Sequence Elements. While a dot matrix comparison of the mouse and human distal Ick promoter sequences demonstrates their high degree of similarity (Fig. 5 a), very little similarity is noted when the mouse distal promoter is compared to the mouse
Figure 3. Steady state levels of human type I and type II *kk* mRNA in thymocyte subsets and peripheral T cells (PTC). RNA samples from whole thymus (lanes 1 and 2), subpopulations of thymocytes isolated by complement lysis with the indicated reagents (lanes 3-5), and PTC (lane 6) were analyzed by Northern blotting and sequential probing for the unique 5' UTRs of human type I (A) and type II (B) *kk* transcripts. The composition of the lysis survivors was assessed by flow cytometry: lanes 1 and 2, 74% CD4+, 75% CD8+, 76% CD3+; lane 3, 95% CD4+, 8% CD8+, 96% CD3+; lane 4 3% CD4+, 91% CD8+, 99% CD3+; lane 5, 10% CD4 dull, 19% CD8 dull, 51% CD3+, 37% γδ TCR+.

proximal promoter (Fig. 5 b). Nevertheless, careful comparison revealed a sequence element in the distal promoter regions of both the mouse and human that is also present in the proximal promoter sequences of both species (Fig. 6 a). This element lacks similarities to the core consensus sequences of any previously described cis-regulatory elements of which we are aware, but its "core" (5'-TGCTGGGG-3') does have some similarities to sequences within regions known to bind the murine MHC class I transcription factor H2TF-1 (includes 5'-GGCTGGGG-3') (28), as well as a chicken lysozyme silence (includes 5'-AGCTGGGG-3') (29). A computer search for the "core" 8-mer of the element within lymphoid-specific promoters in the EMBL 19 DNA database failed to find an entry whose sequence conformed to all of the conserved regions of the entire element. We also noted that the sequence surrounding the element in the mouse distal promoter shows a curiously symmetric series of nested direct repeats (Fig. 6 b). Most of these repeats are also found in the same positions in the human distal promoter sequence. These observations are provocative and serve to focus attention on this region as a putative binding site for factors regulating expression of the *kk* promoter (see below). Several other short sequence segments (8-11 bp in length) are shared between the proximal and distal promoters within a species, but unlike the element in Fig. 6 a are not shared by both species (data not shown).

We further examined the human and mouse proximal and distal *kk* promoter sequences for the presence of a variety of previously identified eukaryotic cis–regulatory sequences. We found that the human and mouse proximal promoters contain a consensus sequence (5'-GGAAAGTG-3') for E4TF-1, a transcriptional regulator of adenovirus type 5 early region 4 (30, 31), located some 50 bases 5' to the IgH core enhancer sequence previously noted by both Takadera et al. (6) and Adler et al. (13). A target sequence (5'-GTCGTCA-3') for the cAMP response element binding protein was found in the inverse orientation in the mouse distal promoter (bases -318 to -324). The so-called PU-box (5'-GAGGAA-3'), an enhancer bound by the PU.1 protein (the protooncogene Spi-1, reference 32) in B lymphocytes and macrophages (33) is found three times in the mouse distal promoter (bases -189 to -194, -436 to -431, and -812 to -817) but not in the human distal promoter. CCAAT and TATAA elements are present in the human distal promoter sequence, but these are positioned far from the transcription start sites (bases -516 and -536, respectively), and thus probably represent chance occurrences.

The Conserved Sequence Element Is a Target for Nuclear Protein Binding. To examine the possibility that the conserved distal/proximal sequence element depicted in Fig. 6 a is an important functional element, we tested its ability to specifically bind components of nuclear protein extracts that presumably contain DNA-binding transcription factors, in a gel mobility shift assay. Fig. 6 c demonstrates that a protein (or protein complex) in lymphoid cell nuclei binds specifically to a 29 bp probe spanning this region of the mouse distal promoter (Fig. 6 a, MD). This binding (Fig. 6 c, lanes 1, 8, and 15) is specifically competed by an excess of unlabeled probe (the MD competitor; Fig. 6 c, lanes 2, 9, and 16) but not by the unrelated sequence OCT (Fig. 6 c, lanes 2, 9, and 16) that includes Oct-1 binding motifs (34) and also binds nuclear proteins in this assay (data not shown). Binding is also not competed by a longer DNA fragment that contains sequences flanking the mouse distal promoter transcription start site (bases -38 to +44, data not shown). Hence, this nuclear extract–induced gel mobility shift is specific for the probe.

The sequence dependence of the binding was further examined by competing with an excess of unlabeled probes (the MD competitor; Fig. 6 c, lanes 3, 10, and 17), but not by the unrelated sequence OCT (Fig. 6 c, lanes 2, 9, and 16) that includes Oct-1 binding motifs (34) and also binds nuclear proteins in this assay (data not shown). Binding is also not competed by a longer DNA fragment that contains sequences flanking the mouse distal promoter transcription start site (bases -38 to +44, data not shown). Hence, this nuclear extract–induced gel mobility shift is specific for the probe.

The sequence dependence of the binding was further examined by competing with an excess of cold competitors representing the similar sequences found in the human distal (HD, differing by four bases from the MD probe), the mouse proximal (MP, differing by eight bases), and two sequences from the human proximal (HPa and HPb, differing by 14 and 21 bases, respectively) *kk* promoters (Fig. 6 a). (HPb is quite divergent from MD but has more similarity to MP.) As seen in Fig. 6 c (lanes 4, 11, and 18), the very similar sequence from the human distal promoter, HD, inhibited MD probe binding as effectively as unlabeled MD sequence. The MP competitor failed to compete significantly in the experi-
ment in Fig. 6 c (lanes 5 and 12) but did compete successfully at much higher molar ratio (data not shown) in a sequence-specific manner. This observation suggests that the DNA-binding protein complex has some specificity for the MPse-sequence, but binds with a much lower affinity. The two sequences from the human proximal promoter were unable to compete effectively (Fig. 6 c, lanes 5, 6, 13, and 14).

The gel mobility shifts in thymus have a slower mobility than the otherwise similar shifts seen in spleen or liver, though their sequence-specificity is the same (Fig. 6 c). Hence, the nature of the complex found in thymus is different than that in total spleen, or in liver. By varying the conditions of the gel shift assay, the mobility differences noted between thymus and spleen or liver extracts are much more pronounced, indicating that this is a real and consistent difference (data not shown).

We conclude that both lymphoid and nonlymphoid organs contained nuclear proteins or complexes that specifically bind the sequence element highlighted by the sequence comparisons, that the nature of the complexes varies by organ type, and that the binding affinity in the gel mobility shift assay is sensitive to increasing variations from the MD target sequence.

The Human Distal Promoter Functions Independently in Transgenic Mice. Sequence analysis showed that cis promoter elements of the lck distal promoter have been conserved in evolution and suggested that the corresponding trans-acting factors responsible for tissue-specific and developmental regulation of the distal promoter may also have been conserved. We tested this hypothesis by expressing the human distal promoter to function in a heterologous background, i.e., by using it to drive a reporter gene in transgenic mice. Animals bearing a transgene (SP12T) consisting of a human genomic segment containing 2.5 kb of sequence 5' to the type II lck mRNA transcription start site (subclone SP12, Fig. 1) driving the SV40 large T antigen gene expressed SV40 T mRNA in thymus and spleen, and faintly in heart, but not in liver or kidney (Fig. 7). The very small amount of T antigen transcript detected in heart may represent that derived from the blood lymphocytes contained within the heart at the time of dissection rather than from the myocardium. Starting at 21 wk of age, mice bearing this transgene developed hemorrhagic tumors of the LN and spleen and gross enlargement of the thymus. Tumors were not noted in the heart or in any other non-lymphoid organs. Thus, unlike previously described transgenic mice bearing a similar T antigen trans-
gene driven by the mouse proximal lck promoter, which develop only thymic tumors (22), the human distal promoter directed T antigen mRNA expression and T antigen-induced transformation to the peripheral lymphoid organs as well as to the thymus. The behavior of the distal and proximal promoters in transgenic mice thus conforms to the expression patterns of type I and type II lck transcripts demonstrated in Figs. 2 and 3.

Discussion

We have demonstrated that the lck gene can be transcribed via two separate promoters that behave in a developmentally regulated fashion: while both promoters are active in thymocytes, only the distal promoter is active in mature T cells. One may conceive of three explanations for the presence of dual lck promoters. First, the simple requirement for p56" in both thymocytes and peripheral T cells may have driven conservation of a "constitutive" promoter (the distal promoter), while the presence and activity of the proximal promoter is genetically accidental and superfluous. The high degree of sequence conservation noted between the human and mouse, the persistence of a functional proximal promoter in both species, and the lack of major sequence similarities between the distal and proximal promoter sequences argue against this hypothesis.
The dual promoter system might also reflect the need for continued expression of *lk* in the context of a changing transcriptional milieu that could not support activation from a single set of regulatory elements under all conditions. In this model, the changing milieu would be driven not by the need for modified *lk* expression but by the regulation of other genes with related transcriptional control elements that must be modulated in the process of thymocyte maturation. Contradicting this hypothesis, we have shown that thymocytes of all developmental stages as well as peripheral lymphocytes transcribe effectively from the distal promoter.

It seems much more likely that the dual *lk* promoter system evolved as a mechanism for allowing differential regulation under different developmental conditions: i.e., there is an advantage to be gained from controlling *lk* expression in peripheral lymphocytes in a manner different from that in thymocytes undergoing selection and maturation.

While the nature of this advantage is obscure, it is impor-
Expression of the SP12T transgene in lymphoid tissues. Shown is an RNA blot performed using samples derived from the indicated tissues of a 6.5-wk-old SP12T transgene mouse. The blot was hybridized with a probe specific for SV40T antigen sequences (see Materials and Methods).

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