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Functional Dissection of the *lck* Proximal Promoter

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The *lck* gene encodes a protein tyrosine kinase that participates in lymphocyte-specific signal transduction pathways. Previous studies have established that *lck* transcription is regulated by two distinct promoter elements termed proximal (or 3′) and distal (or 5′). The proximal promoter is active almost exclusively in thymocytes and becomes inactive later during T-cell maturation. To dissect the mechanisms responsible for *lck* gene regulation, we generated transgenic animals bearing 5′ truncations in the proximal promoter element. Sequences between −584 and +37 with respect to the proximal promoter transcription start site act to direct tissue-specific and temporally correct transcription of either a tagged version of the *lck* gene itself or a heterologous reporter sequence (*lacZ*). This region contains binding sites for at least five distinct nuclear proteins, of which one is found only in cells that support proximal *lck* promoter activity and a second appears only in nonexpressing cells. Interestingly, the transcribed region of the *lck* gene contains positive control elements that can substantially boost expression from minimal (−130 bp) proximal promoter constructs. These results provide a basis for the biochemical dissection of transcriptional regulators that act at defined points during T-cell development.

The *lck* gene encodes a lymphocyte-specific, membrane-associated protein tyrosine kinase ($p56^{lck}$) that is a member of the *src* family (35). Although its exact mechanisms of action remain unclear, previous studies suggest that $p56^{lck}$ participates in T-cell signalling. Coimmunoprecipitation experiments reveal that $p56^{lck}$ associates with the cytoplasmic tails of the CD4 and CD8 coreceptors of the T-cell antigen recognition complex (3), with the acidic region of the interleukin-2 receptor β chain (23), and with some other T-cell surface structures (47). Moreover, antibody-mediated cross-linking of CD4 molecules stimulates $p56^{lck}$ activity, supporting the hypothesis that $p56^{lck}$ mediates signal transduction from the ligand-occupied T-cell receptor (40, 52). Treatment of normal T lymphocytes with interleukin-2 also activates $p56^{lck}$ (23).

These experiments hint at possible functions of $p56^{lck}$ in mature T lymphocytes; however, there is reason to believe that this kinase also participates in regulation of thymocyte development. Transcripts from the *lck* locus are detectable as soon as hematopoietic progenitors first colonize the thymic anlage (42) and are present in all thymocyte subpopulations defined by CD4 and CD8 expression (55). Moreover, overexpression of $p56^{lck}$ in developing thymocytes results in a severe maturational disturbance characterized by failure to generate cells bearing a recognizable T-cell receptor complex. Indeed, a simple doubling of $p56^{lck}$ levels in such cells was sufficient to block thymocyte development completely (1). Since the CD4 molecule appears on at least a fraction of immature lymphoid progenitor cells (56), there is reason to believe that $p56^{lck}$ expression can contribute to signal transduction events in these cells as well.

To understand the regulation of *lck* gene expression better, we have pursued the characterization of *cis*-acting transcriptional control elements in the *lck* locus. Previous studies have demonstrated that two distinct, developmentally regulated promoter elements direct *lck* transcription in both human and murine lymphocytes. The human 5′ or distal *lck* promoter resides 34 kb 5′ to the translation initiation codon and directs the expression of *lck* transcripts in both thymocytes and mature T cells (55). In contrast, the proximal *lck* promoter, positioned immediately adjacent to the body of the gene, is active principally in the thymus (17). Studies with transgenic animals using the mouse *lck* (m*lck*) gene, which displays a similar organization, demonstrate that the proximal and distal promoter elements can function independently (16, 55). For example, expression of sequences that encode the S1 subunit of pertussis toxin and the murine $p59^{fyn}$ protein, when placed under the control of the proximal *lck* promoter, became thymocyte specific (9, 13).

Since proximal promoter-driven *lck* expression occurs early during lymphopoiesis, and since the level of $p56^{lck}$ can hugely influence lymphocyte maturation, we sought to dissect those mechanisms responsible for developmental regulation of *lck* transcription. Molecular characterization of critical regions in the *lck* proximal promoter could be achieved only by using transgenic methods, as no satisfactory in vitro model of lymphocyte development exists. Here we report that sequences between −37 and −130 with respect to the proximal transcription start site are absolutely required for tissue-specific and temporally correct expression of the murine *lck* gene. Remarkably, this same region is completely ineffective in directing expression of a heterologous reporter element. These studies provide a basis for the definition of *trans*-acting factors that regulate *lck* transcription, factors that by implication contribute importantly to the control of T-cell development.

MATERIALS AND METHODS

Preparation of tagged genomic m*lck* constructs. A tagged m*lck* genomic reporter construct was prepared by blunt-end insertion of a *Stu*I fragment containing the mouse genomic *lck* transcribed region (from position 160 of the transcript in exon 1 to position 9600 in exon 12) fused with 3′ untranslated region sequences and the polyadenylation signal from the human growth hormone (hGH) gene (2) into a vector (p1017) containing 3.2 kb of the m*lck* 5′ proximal sequence (9). In

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this way, the normal *lck* gene sequence was reconstructed with a deletion in the 5′ untranslated region that simplified subsequent analysis of transgene expression (lck^{-3200} LGY; see Fig. 1A). The p1017 plasmid has a unique *Bam*HI site at +37 that is not present in the endogenous *lck* sequence, allowing detection of transgene-derived transcripts (9). The lck^{-584} and lck^{-240} LGY constructs were prepared by partial digestion of lck^{-3200} LGY with *Asp*718, permitting assembly with the tagged m*lck* genomic fragment in separate vectors to ensure that contaminating sequences were not present in fragments to be injected.

The lck^{-130} and lck^{-37} LGY expression vectors were prepared by sequential ligation of overlapping oligonucleotides into an LGY vector. To prepare a promoterless LGY vector, lck^{-3200} LGY was partially digested with *Sma*I, cut with *Not*I, gel purified, and subcloned into *Sma*I-*Not*I-cut pBluescript KS (Stratagene). The resulting construct, lck^{-2200} LGY, has a unique *Xho*I site (from pBluescript KS) 5′ to the m*lck* regulatory sequence. lck^{-2200} LGY was then partially digested with *Bam*HI (at +37 of the *lck* sequence), completely digested with *Xho*I, and isolated on a low-melting-point preparative gel to remove the *lck* regulatory sequences. By using this basic construct, potential regulatory regions were then assembled. A first pair of oligonucleotides (*lck* 1 and 2), when annealed, contains a 5′ *Xho*I overhang and a 3′ *Bam*HI overhang to enable its directional ligation into the prepared promoterless vector, resulting in lck^{-7} LGY. All subsequently added oligonucleotides (*lck* 3 and 4, 5 and 6, and 7 and 8; see sequences below), when annealed and ligated correctly into the unique *Xho*I site of the expression vectors, starting with lck^{-7} LGY, recreate the unique *Xho*I site at the 5′ end of the integration and destroy the 3′ *Xho*I site. The structures of the recombinant plasmids were verified by DNA sequencing.

Oligonucleotides for promoter synthesis. The sequences of the oligonucleotides used for promoter synthesis were as follows:

lck 1, 5′-TCGAGGGCTCAGAGGGAACCCAGTCAGGAG CTTGAATCCCACGATTCGGG;
lck 2, 5′-GATCCCCGAATCGTGGGATTCAAGCTCCTG ACTGGGTTCCCTCTGAGCCC-3′;
lck 3, 5′-TCGAGCCTGGGCCTCCTGTGAACTTGGTGG3′;
lck 4, 5′-TCGACCACCAAGTTCACAGGAGGCCCAGGC-3′;
lck 5, 5′-TCGAGGGCGGTTTGCCCATCCCAGGTGGGA GGGTGGGACTAGG-3′;
lck 6, 5′-TCGACCTAGTCCCACCCTCCCACCTGGGATG GGCAAACCGCCC-3′;
lck 7, 5′-TCGAGCTGGGAGGCAGGAAGTGGGTAACTA GACTAACAAAGATGCCTG-3′;
lck 8, 5′-TCGACAGGCATCTTTGTTAGTCTAGTTACCC ACTTCCTGCCTCCCAGC-3.

Preparation of β-galactosidase–hGH expression constructs. The lck^{-3200} *lacZ*-hGH construct was prepared by insertion of a *Bgl*II-digested, gel-purified fragment containing a modified β-galactosidase gene (7) into the *Bam*HI site of the p1017 vector, which contains the hGH gene (see Fig. 2A). The lck^{-584} and lck^{-240} *lacZ*-hGH expression vectors were prepared by conventional methods, and each was subcloned separately.

Generation of transgenic mice. Purified linear DNA molecules (2 ng/μl) were injected into the pronuclei of (C57BL/6J × DBA/2J) F2 hybrid mouse zygotes. Subsequent generation and detection of transgenic mice were done as previously described (16, 26).

RNA isolation. Total RNA was isolated from mouse organs as described by Cathala et al. (8).

S1 nuclease analysis. S1 nuclease analysis was performed essentially as described by Searle et al. (45), with the following modifications. The probe used was an end-labeled oligonucleotide complementary to −9 to +36 of the m*lck* transcript, including the engineered *Bam*HI site at its 5′ end and a string of 12 A residues at its 3′ end, yielding the sequence 5′-GATCCCCGAATCGTGGGATTCAAGCTCC TGACTGGGTTCCCTCTGAGCCCTCAAAAAAAAAAA A-3′. The end-labeled probe was purified on an 8 M urea sequencing gel, eluted, annealed to 20 to 50 μg of total RNA (or RNA standard), brought to 50 μg with *Saccharomyces cerevisiae* tRNA in a volume of 50 μl, and incubated overnight in 60% formamide at 50°C. The hybridization reaction was then brought to 400 μl in 1× S1 salts, and 100 U S1 nuclease (Boehringer) was added. The samples were incubated at 50°C for 1 h. S1 nuclease-resistant products were precipitated with 1 ml of ethanol and separated on an 8 M urea–8% acrylamide thin gel. Following autoradiographic exposure of the dried gel, the S1 nuclease-resistant products were localized and counted. The absolute level of expression of the transgene was determined by comparison to the values obtained from the known standard. These standards were also used to quantitate the level of endogenous *lck* mRNA levels in control mice by using a slightly smaller S1 probe complementary to the *lck* sequence from +36 to −9, which includes 12 A residues but lacks the *Bam*HI site (5′-GA ATCGTGGGATTCAAGCTCCTGACTGGGTTCCCTCTG AGCCCTCAAAAAAAAAAAA-3′).

RNA standards. RNA standards corresponding to −11 to +36 of the m*lck* gene with the engineered *Bam*HI site included at +37 were prepared to quantitate the level of *lck* transgene expression. Briefly, two oligomers (5′-TCGAGG GCTCAGAGGGAACCCAGTCAGGAGCTTGAATCCCA CGATTCGGG-3′ and 5′-GATCCCCGAATCGTGGGATTC AAGCTCCTGACTGGGTTCCCTCTGAGCCC-3′) were treated with polynucleotide kinase, annealed, and ligated into the *Bam*HI-*Xho*I-cut pBluescript KS plasmid. Recombinant plasmids were screened by colony hybridization and sequenced to confirm the single-copy insert. This clone was then linearized with *Not*I and transcribed with T3 RNA polymerase by using an in vitro RNA synthesis kit (Stratagene). The product was quantitated by optical density at 260 nm and diluted into 5 mg of yeast tRNA per ml.

RNA blot analysis. RNA (20 μg) was denatured in 50% formamide–2.2 M formaldehyde at 68°C for 5 min and electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde (30). The gel was blotted onto nitrocellulose and hybridized with an α-^{32}P-labeled 2.0-kb *Sst*I fragment isolated from within the β-galactosidase gene (7), a similarly labeled *Eco*RI fragment from the mouse NT18 *lck* cDNA (35), or mouse elongation factor 1α cDNA.

Cell culture and nuclear extract preparation. T-cell lines (EL4, BW5147, Jurkat, 180, 186, and 2048) (2, 16) were grown in RPMI with 10% fetal calf serum to a density of approximately 10^6/ml. Nuclear extracts were made essentially by the method of Dignam et al. (14). Thymocytes and splenocytes were teased from their respective organs, and extracts were prepared in a similar manner. Tissue extracts were made from mouse organs as described by Buskin and Hauschka (6).

DNA probes and competitors. *lck* fragments were derived from subclones of the proximal *lck* regulatory sequence by digestion at unique sites within the sequence or in the polylinker. Probes were labeled with [α-^{32}P]dATP and Kle-

now DNA polymerase at one or both ends by using standard conditions (33) and purified on native thin acrylamide gels. Competition fragments were separated on low-melting-point agarose gels and eluted by standard procedures (33). Oligonucleotide probes or competitors were kinase treated with either [γ-^{32}P]ATP or cold ATP, hybridized in appropriate salt concentrations, and in some cases treated with Klenow DNA polymerase to ensure that the fragments were fully double stranded. Labeled probes were purified on native thin acrylamide gels.

Gel mobility shift assays. Binding reactions and analysis on native acrylamide gels were performed essentially as previously described (6). Briefly, 1 to 5 μg of extract was incubated with approximately 5 fmol of probe and 1 to 5 μg of poly(dI-dC) for DNA fragment probes or 0.2 to 2.0 μg of poly(dI-dC) for oligonucleotide probes. Excess cold competitors were added with the probe as indicated in the figure legends.

Oligonucleotide sequences used in gel mobility shift experiments. Oligonucleotides with the following sequences were used in gel mobility shift experiments.

−365/−328: 5′-GTACTGTGGTTGAGTGGTGGGGGTAG GGGTGCTGGG-3′
5′-GTACCCCAGCACCCCTACCCCCAC-CACTCAACCACA-3′;

−510/−460: 5′-GGTTCTGCTTGATTTCATTTGACAA-3′
5′-TTGCCTGGTTTAGCTGTG-GAAACTTTTTGTCAAATG-3′;

−510/−477: 5′-GGTTCTGCTTGATTTCATTTGACAA-3′
5′-GGAAACTTTTTGTCAAATGAAATC-3′;

−520/−477: 5′-GTGTCTATGAGGTTCTGCTTGATTTCA TTTGACAA-3′
5′-GGAAACTTTTTGTCAAATGAAATC-3′

−511/−482: 5′-GTACAGGTTCTGCTTGATTTCATTTGA CAAAAAG-3′
5′-GTACCTTTTTGTCAAATGAAATCAAG CAGAACCT-3′;

TCF-1 (51): 5′-GGGAGACTGAGAACAAAGCGCTCTCA CAC-3′
5′-CCCGTGTGAGAGCGCTTTGTTCTCAG TCT-3′

TCF-1α/LEF-1 (50): 5′-TCGACGTAGGGCACCCTTTGA AGCTCTCCC-3
5′-TCGAGGGAGAGCTTCAAAGG GTGCCCTACG-3′

Methylation interference assays. The labeled probes were methylated as described by Maxam and Gilbert (36) and incubated with nuclear extracts, and the resulting complexes were resolved by preparative gel shift. The protein-DNA complexes and free probe were excised from the gel and purified, and the nucleic acids were cleaved at G residues (36). Samples were fractionated on 8 M urea sequencing gels along with reference sequencing lanes.

RESULTS

Analysis of transcriptional activity in transgenic mice. The developmental and tissue-specific expression of a tagged *lck* genomic reporter gene (pLGY) fused to the proximal *lck* 5′ regulatory sequence was monitored in transgenic mice. Figure 1A is a diagram of the constructs tested, indicating the sites of 5′ truncation beginning at −3.2 kb with respect to the proximal transcription start site. Total RNA from founder animals was isolated from a variety of tissues, and transgene expression was assessed by S1 nuclease analysis. We chose an mRNA mapping assay to detect transgene expression, since changes in the 5′ regulatory region might have resulted in the generation of novel nonspecific transcriptional start sites. Although no new sites were observed, we did detect the presence of weak upstream transcription start sites in the endogenous mouse proximal promoter and the transgenes placed under its control, similar to observations made with the human *lck* promoter (48), which had not been identified in our previous study (17). The absolute level of transgene expression was determined by comparison to an *lck* RNA standard generated by in vitro transcription (Fig. 1B, inset). In all, 25 independent founder animals were examined. Deletion analysis revealed that as little as 130 bp of the 5′ sequence, relative to the start of transcription, was sufficient to direct the expression of the tagged transgene in thymocytes. Figure 2 demonstrates that transgene transcripts, detected as a more rapidly migrating species in RNA blots probed with the *lck* cDNA, accumulated faithfully in the thymus and were not observed in kidney or liver tissue (Fig. 2) or in heart, lung, or skeletal muscle tissue (data not shown). Very little transgene expression was observed in the spleen or other peripheral tissues, recapitulating the expression profile of the endogenous *lck* transcript. The level of transgene expression increased modestly as the 5′ sequence was deleted from −3200 to −240, an effect that was especially prominent following removal of sequences from −584 to −433, although a good deal of variability was observed (Fig. 1B).

Levels of expression comparable to or greater than those of the endogenous gene were easily achieved. Indeed, seven of nine animals bearing the −433 construct and six of six animals bearing the −240 construct exhibited these high levels of transgene expression. Deletion of all sequences 5′ to −37 abolished transgene transcription in four of four founder animals, a result made all the more significant by the fact that thymocytes from all of the other pLGY transgene-bearing animals contained measurable levels of the transgene transcript. Hence, sequences between −37 and −130 are necessary for proximal promoter function. In no case did 5′ truncation of *lck* gene sequences alter the expression pattern of the transgene. That is, transcripts did not accumulate in nonlymphoid tissues and were detected almost exclusively in thymus RNA (Fig. 2). These observations indicated that a very short DNA sequence, representing at most 240 bp, might contain all of the information necessary to direct thymus-specific expression of heterologous elements.

To test this conjecture, the β-galactosidase gene (*lacZ*) was inserted into the hGH structural gene, included only to provide an intron-containing genomic structure that in many cases improves expression in transgenic mice (5, 11, 39), and the entire reporter construct was placed 3′ to *lck* regulatory sequences (Fig. 3A). In all, 38 transgenic founder animals were generated by using these constructs. Accumulation of the *lacZ*-hGH transcript was uniformly lower than that observed for the tagged genomic *lck* construct as monitored by RNA blot analysis, which proved to be the most sensitive assay for these transcripts. Furthermore, in contrast to the results obtained by using the tagged genomic reporter gene, only a fraction of the thymuses from *lacZ*-hGH transgenic founders contained detectable transgene-derived transcripts.

Figure 3B shows a representative RNA blot analysis

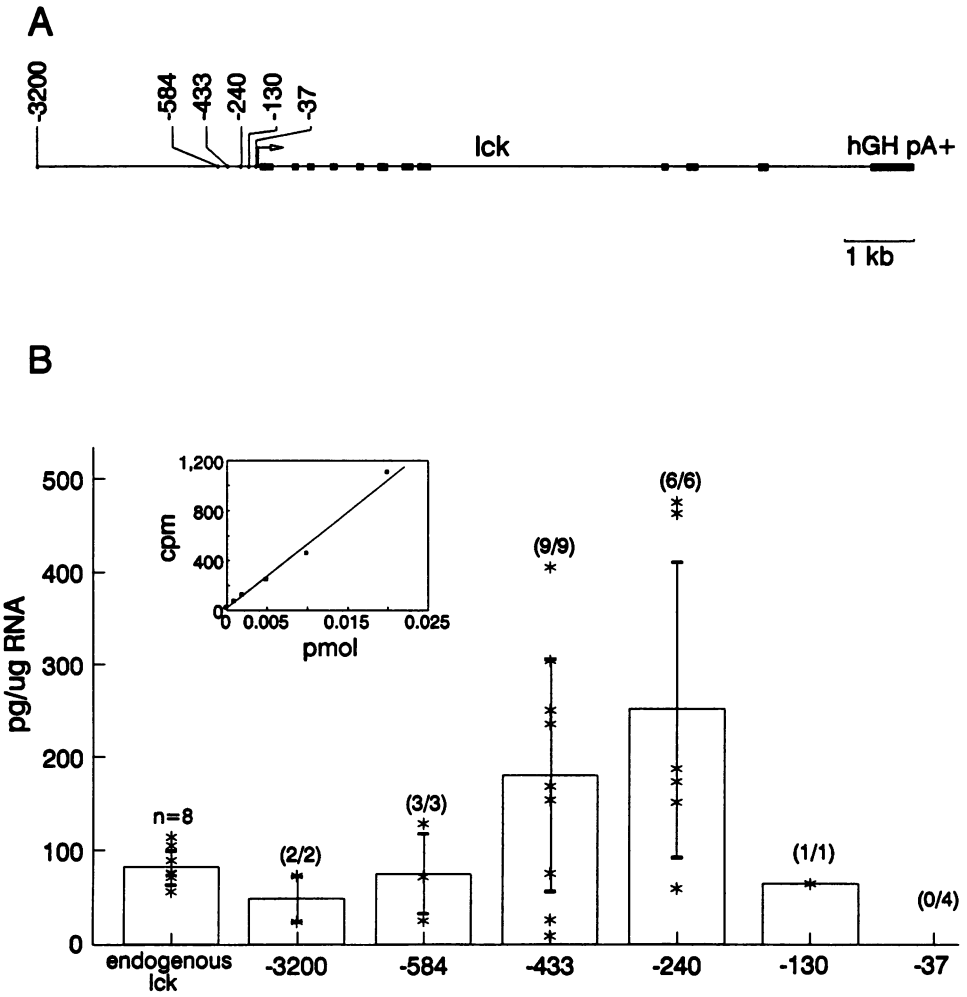

FIG. 1. Expression of a tagged genomic *lck* reporter gene in transgenic mice. (A) Genomic *lck* constructs used to generate transgenic mice. Shown is a map of the mouse *lck* gene reporter with exons indicated as filled boxes. The endpoints of 5′ truncations are indicated by numbered lines. A polyadenylation signal and 3′ untranslated region sequences derived from the hGH gene were added at the 3′ end. See the text for details. (B) Results of S1 nuclease analysis on genomic *lck* constructs or endogenous *lck* mRNA (picograms of *lck* mRNA per microgram of total RNA). Each point represents an individual founder animal. Each open box denotes the average expression level for each construct, with 1-standard-deviation limits (where $n > 2$) indicated by vertical lines. Values in parentheses represent the number of expressing animals divided by the number of founders for each construct. The inset shows a typical standard curve generated with in vitro-synthesized RNA (see Materials and Methods).

obtained by using thymus RNA from *lacZ*-hGH founder mice. Transcript accumulation was observed at similar levels for both the −3200 and the −584 deletion constructs. Figure 4 demonstrates that, as in the case of the tagged *lck* genomic reporter gene, *lacZ* transcripts under the control of the *lck* promoter appeared exclusively in thymocytes. Surprisingly, the −240 deletion construct failed to support transcription of the *lacZ*-hGH reporter gene (barely detectable expression was observed in 1 of 18 founder animals). This result contrasts with the high-level expression of the tagged *lck* reporter when driven by the −240 promoter sequence. We conclude that although sequences capable of directing thymocyte-specific transcription are present within the −584 to +37 region, additional sequences within the transcribed region of the *lck* gene improve the activity of the basal promoter region to a significant extent. Indeed, these sequences are absolutely required for effective expression in thymocytes when a −240 deletion construct is employed.

Gel mobility shift and methylation interference analysis of the proximal *lck* regulatory sequence from −584 to +37. The observed transcriptional activity associated with the proximal *lck* promoter presumably results from interactions between nuclear transcription factors and specific DNA-binding sites in the −584 to +37 region of the *lck* gene. To identify protein-DNA complexes potentially involved in regulating *lck* transcription, fragments derived from −584 to +37 were radiolabeled and incubated with nuclear extracts derived from cells in which the *lck* proximal promoter is active. Included among this group were normal murine thymocytes, four widely studied transformed T-cell lines (LSTRA, EL4, BW5147, and human Jurkat cells), and three T-cell lines derived from transgenic mice that express either the simian virus 40 large T antigen (lines 180 and 186) or the *lck* gene itself (2048) under the control of the proximal *lck* promoter. In the latter three cases, continued in vitro growth of the cells presumably requires expression of the transgene

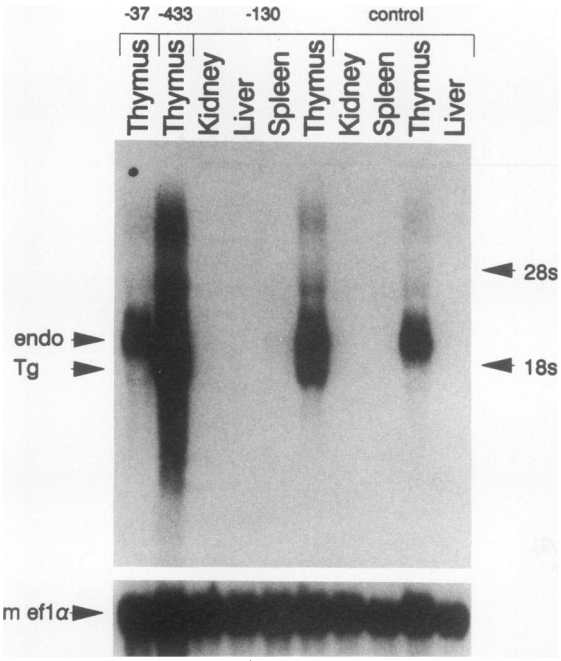

FIG. 2. Fidelity of expression of a tagged *lck* reporter gene in transgenic mice. Shown is a representative RNA blot obtained by using animals bearing −433 or −130 expression constructs. The source of RNA is indicated above each lane, and the positions of 28S and 18S ribosomal markers are noted at the right. The transgene (Tg)-encoded mRNA is slightly smaller than that encoded by the endogenous *lck* gene (endo); both were detected by using an *lck* cDNA probe, and their positions are shown at the left. The bottom panel reveals the results obtained when the blot was reanalyzed with a probe for an abundant transcript present in all cells, mouse elongation factor 1α, providing a control for RNA loading and transfer.

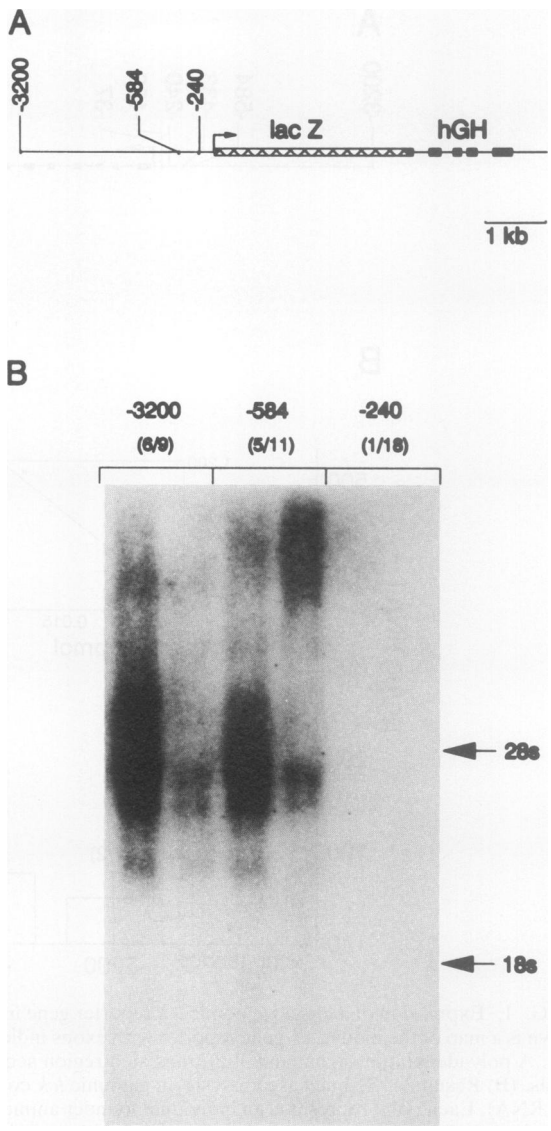

FIG. 3. Expression of the *lacZ*-hGH reporter gene in transgenic mice. (A) *lacZ*-hGH constructs used to generate transgenic mice. The positions of the *lacZ* gene, the hGH exons, and the ends of the 5′ deletion constructs are noted. (B) Representative RNA blot analysis of total thymus RNAs from lck^{-3200}, lck^{-584}, and lck^{-240} *lacZ*-hGH founder mice. The value in parentheses represents the number of expressing animals divided by the number of founder mice for each construct.

and, hence, activity of the *lck* proximal promoter. Control extracts were derived from tissues lacking proximal promoter-driven *lck* transcripts, including liver, kidney, testis, and spleen tissues.

An initial evaluation of regions containing sites for nuclear factor binding was achieved by using probes from −584 to −433, from −433 to −240, and from −240 to +37. Distinct banding patterns were seen with each fragment. Figure 5 demonstrates that the region from −584 to −433 was capable of forming a complex (A1) with factors present in LSTRA cell nuclear extracts. In contrast, a different complex (A2) was observed with extracts derived from spleen and most other cells that lack proximal *lck* transcripts (Fig. 5). By scanning this 150-bp region with oligonucleotides, we learned that the A1 complex represents binding to sequences contained within the region from −520 to −477. Indeed, A1 complex formation was completely inhibited by an excess of this unlabeled oligonucleotide (Fig. 5). Additional insight into the characteristics of the A1 factor was obtained by methylation interference analysis, which identified a single G residue at position −506 that blocked protein binding when methylated (Fig. 6). It is worth noting that A1 complexes were never visualized in normal cells that support *lck* proximal promoter activity.

The A2 complex behaved quite differently. Its formation was competitively inhibited by an oligonucleotide comprising positions −510 to −460 of the *lck* gene (Fig. 5). Two G residues at positions −477 and −478 were identified by methylation interference analysis of this complex (Fig. 6), which explains why the 34-mer −510/−477 did not block formation of the A2 complex (Fig. 5). Hence at least two nuclear factors, one of which is specifically found in cell types that do not support transcription from the proximal *lck* promoter, interact with sequences in the region between −584 and −433 of the *lck* gene.

Analysis of the region from −433 to −240 revealed two sets of complexes (B1 and B2), each consisting of several distinct bands, both of which appeared when nuclear extracts from *lck*-expressing cells were used (Fig. 7). No complexes formed when extracts from nonexpressing cells

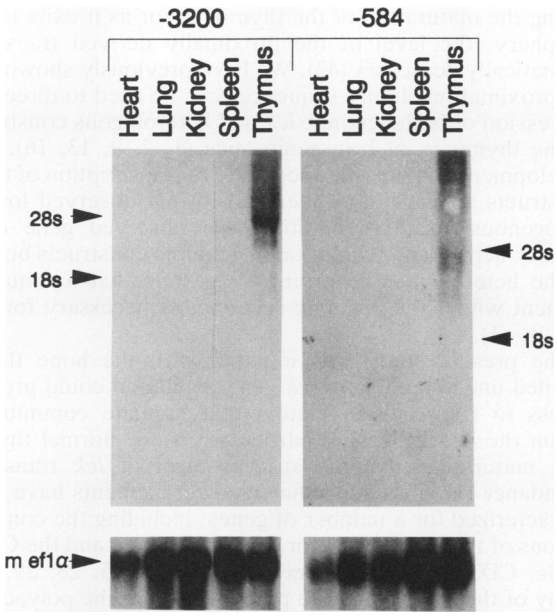

FIG. 4. Fidelity of expression of the *lacZ*-hGH reporter gene under the control of the *lck* proximal promoter. Shown are representative RNA blot analyses for two animals bearing either the −3200-bp construct (left panel) or the −584-bp construct (right panel). The source of RNA is noted above each lane, and the positions of 28S and 18S rRNAs are indicated. The bottom panel provides a control for RNA loading using the elongation factor 1α probe as described in the legend to Fig. 2.

were incubated with this probe (summarized in Table 1). Formation of both complexes was inhibited by addition of an oligonucleotide, −365 to −328, which when used as a probe permitted better resolution of the B1 and B2 complexes (Fig. 7). Methylation of a number of G residues from −335 to

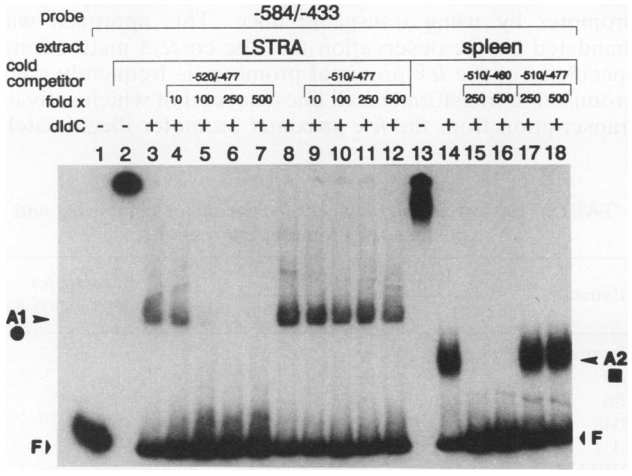

FIG. 5. Gel mobility shift analysis of the −584 to −433 region of the m*lck* gene. Shown are the patterns observed when a −584 to −433 probe was incubated with the indicated nuclear extracts in the presence of cold competitor sequences in the amounts shown. The positions of the A1 and A2 complexes are indicated by arrowheads, as is the position of the free probe (F). The filled-circle and filled-square symbols denote the A1 and A2 complexes in this and subsequent figures. dIdC, poly(dI-dC).

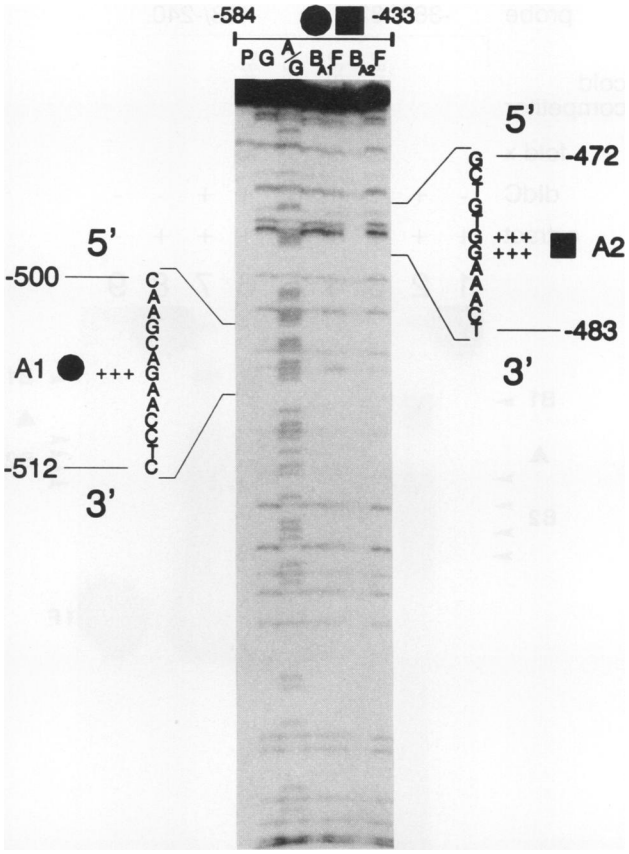

FIG. 6. Binding sites for the A1 and A2 complexes. Shown are the results of methylation interference analysis of protein-DNA complexes and the sequences immediately surrounding identified G residues. Lanes: P, −584 to −433 probe; G, Maxam-and-Gilbert G reaction; A/G, A+G reaction; B_{A1}, bound A1 which is to be compared with the free probe in the adjacent lane F; B_{A2}, bound A2 which is to be compared with the free probe in the extreme right-hand lane F. Sequences at the sites where methylation interferes with complex formation are displayed directly for each complex.

−350 inhibited the formation of this complex. Identical patterns of methylation interference were obtained when either the B1 or B2 complex was used, suggesting that one or more components involved in the formation of these complexes are identical (Fig. 8). Indeed, we cannot exclude the possibility that these two sets of bands result from proteolysis of a single binding species. The close correlation between transcriptional activities of the *lck* proximal promoter in cells and tissues, as well as the presence of the B1 and B2 complexes, makes the factor(s) involved in binding this region an attractive candidate for an *lck*-regulatory molecule.

Inspection of the sequence immediately surrounding the proximal *lck* transcriptional start site (−240 to +37) revealed potential binding sites for three previously characterized lymphocyte-specific transcription factors: TCF-1, TCF-1α/LEF-1, and LYF-1, which interact with the CD3ε, TCRα, and TdT genes, respectively (31, 37, 50, 51, 53, 54). To test whether these *lck*-derived sequences could actually bind the previously described factors, oligonucleotide probes were synthesized on the basis of the published binding sites for TCF-1 and TCF-1α/LEF-1. Incubation of the TCF-1 probe

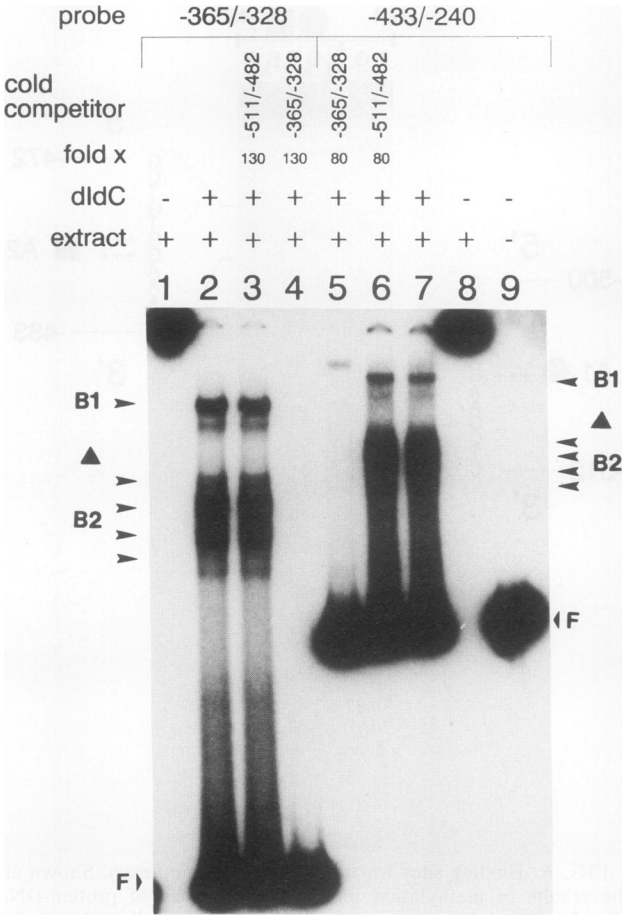

FIG. 7. Gel mobility shift analysis of the −433 to −240 region of the m*lck* gene. Shown are the patterns observed when probes from the regions from −433 to −240 and −365 to −328 were incubated with nuclear extracts from the 180 *lck*-simian virus 40 cell line in the presence or absence of the indicated competitors. The B1 and B2 complexes (henceforth denoted by a filled triangle) are denoted by arrowheads. F, free probe; dIdC, poly(dI-dC).

with EL4 nuclear extracts resulted in the formation of a specific complex which was sensitive to competition with the *lck* −584 to +37 sequence (Fig. 9, lane 7). A similar result was obtained when the labeled TCF-1α/LEF-1 probe was incubated with a BW5147 nuclear extract (Fig. 9, lane 15). Nonspecific competitors had no effect on binding; however, the TCF-1- and TCF-1α/LEF-1-binding sites exhibited cross-competition. Overlap between the TCF-1- and TCF-1α-binding sites has been previously reported by Oosterwegel et al. (37).

DISCUSSION

Specificity of the proximal *lck* regulatory sequence. In both mice and humans, transcription of the *lck* gene initiates at two distinct sites which have been designated the distal and proximal regulatory regions. The proximal site lies immediately 5′ to the coding sequence, while the distal site resides >10 kb 5′ of the proximal site (55). Transcription from the proximal promoter begins during the earliest stages of T-cell development and is observed in all thymocyte subsets as defined by CD4 and CD8 expression (55). At some point during the maturation of the thymocyte or as it exits to the periphery, the level of the proximally derived transcript dramatically decreases (42). We have previously shown that the proximal regulatory sequences can be used to direct the expression of both genomic *lck* and heterologous constructs to the thymuses of transgenic mice (1, 2, 9, 13, 16). The developmental and tissue specificity of transcription of these constructs appears to be identical to that observed for the endogenous gene. In addition, we observed gene copy number-dependent expression of genomic constructs but not of the heterologous reporters, suggesting that a sequence element within the genomic sequence is necessary for this activity (1).

The present study was initiated with the hope that a detailed understanding of *lck* gene regulation could provide access to transcription factors that regulate commitment within the T-cell lineage, particularly since normal thymocyte maturation requires strict control of *lck* transcript abundance (1). T-cell-specific enhancer elements have been characterized for a number of genes, including the constant regions of the T-cell receptor α, β, and γ loci, and the CD-4, CD3ε, CD3δ, and CD2 genes (12, 18, 20, 25, 28, 29, 44). Many of these elements are positioned 3′ to the polyadenylation site. The results presented here, coupled with our previous findings (16), demonstrate that a region spanning positions −584 to −37 with respect to the proximal *lck* transcription start site contains all of the information required for tissue-specific and developmentally appropriate expression of transgenes in a fashion that mimics normal proximal promoter activity. These data do not exclude the possibility of the presence of additional *cis*-acting sequences elsewhere in the *lck* locus. For example, sequences within the normal *lck* transcribed region appear to complement the function of regulatory elements located between −240 and −37 in the proximal promoter. This finding illustrates the complexity of regulatory influences that affect *lck* gene expression.

Simultaneous control of *lck* transcription with respect to time and place. Although numerous cell lines that support *lck* expression exist, we felt compelled to perform a primary dissection of the *cis*-acting regions in the *lck* proximal promoter by using transgenic mice. This approach was mandated by the observation that the correct maturational specificity of the *lck* proximal promoter is frequently compromised in transformed cell lines, several of which activate transcription from the *lck* proximal promoter illegitimately

TABLE 1. Correlation between nuclear factor complexes and proximal *lck* promoter expression

Extract	Presence of complex:			Proximal *lck* promoter expression
	A1	A2	B	
Thymus	−	−	+	+
180	−	−	+	+
186	ND[a]	ND	+	+
2048	−	−	+	+
EL4	+	−	+	+
BW5147	ND	ND	+	+
Jurkat	ND	ND	+	+
LSTRA	+	−	+	+
Spleen	−	+	−	−
Liver	−	+	−	−
Kidney	−	+	−	−
Testis	−	−	−	−

[a] ND, not done.

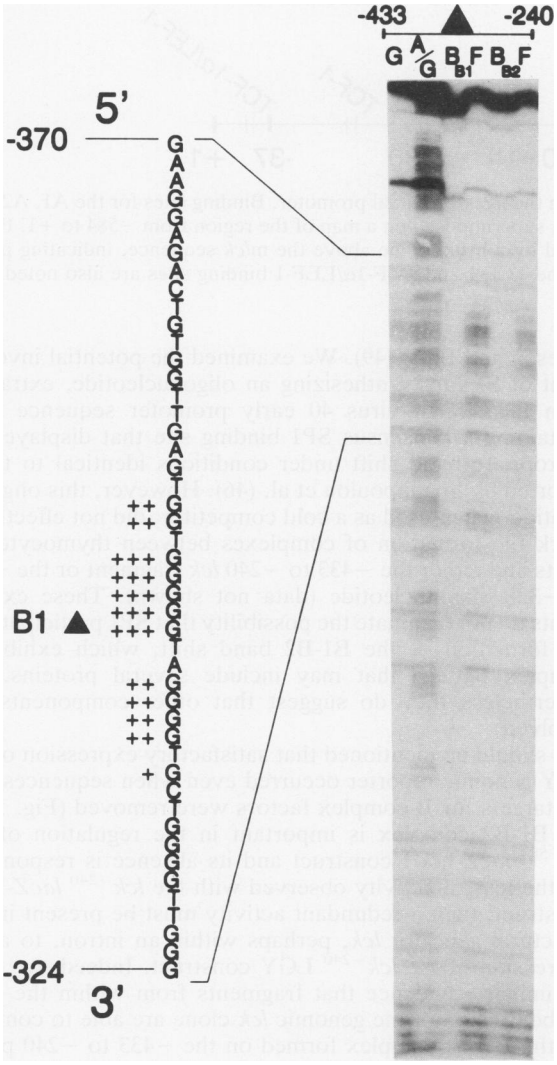

FIG. 8. Binding sites for the B1 and B2 protein-DNA complexes. Shown are the results of a methylation interference study analogous to that in Fig. 4. Lanes: G, Maxam-and-Gilbert G reaction; A/G, A+G reaction; B_{B1}, bound B1, which is to be compared with the free probe in the adjacent lane F; B_{B2}, bound B2 which is to be compared with the free probe in the extreme right-hand lane F. The sequence at the sites where methylation interferes with complex formation is displayed at the left.

(43). Moreover, in vivo dissection of proximal promoter function provided the opportunity to discriminate between factors that impose tissue specificity on *lck* expression and those that regulate transcription during the thymocyte maturation sequence. Such studies are laborious; 63 independent transgenic founder animals were generated to complete a 5′ deletion series. Remarkably, control of *lck* transgene expression was perfectly coupled in time and place. All of those constructs that retained transcriptional activity functioned in thymocytes rather than in peripheral T cells and were inactive in nonlymphoid tissues.

Systematic analysis of transcriptional control elements in transgenic mice has been pursued by using the protamine (41) and elastase (22) genes, among others. However studies of the β globin locus seem particularly germane to the present report. The embryonic, fetal, and adult globin genes each have distinct promoter elements but, in addition, are regulated by a locus control region that confers copy number-dependent expression on globin transgenes (21). Moreover, utilization of the different globin genes is developmentally regulated (38). Although the mechanism(s) responsible for hemoglobin switching remains incompletely dissected, studies of genetic lesions that yield hereditary persistence of fetal hemoglobin indicate that both positive and negative regulatory elements that function within the context of the broader locus control exist (15).

In much the same way, we suspect that while positive control mechanisms function to direct *lck* proximal promoter activity in thymocytes, a form of transcriptional silencing must occur in more mature peripheral T cells. Evidence favoring this view derives from study of the region from −584 to −37, which must, perforce, contain both kinds of regulatory elements. Our analysis demonstrates that at least five binding sites for nuclear factors exist within this small region that reliably directs expression of heterologous reporter sequences in transgenic mice. Table 1 summarizes the results of these electrophoretic mobility shift assays. Three different patterns of nuclear factor expression were observed in various tissues and cell lines; however, there was a strong correlation between proximal promoter activity and the presence of "B-type" binding factors. Similarly, the A2 complex was seen only when nuclear extracts derived from cells that fail to support proximal *lck* promoter activity were used. Figure 10 presents a schematic diagram of these proximal promoter-binding sites, placed in the context of the regions of high DNA sequence similarity between the murine

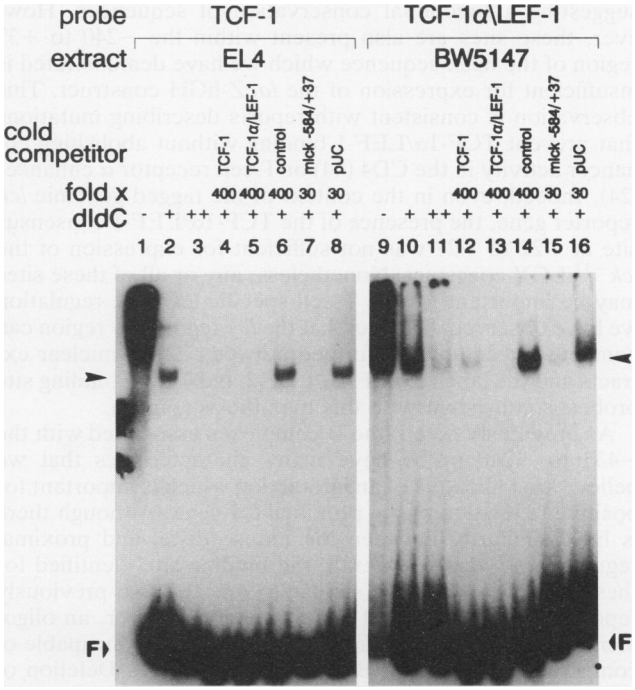

FIG. 9. Gel mobility shift analysis of labeled TCF-1 and TCF-1α/LEF-1 oligonucleotide binding site probes incubated with EL4 and BW5147 cell line nuclear extracts. Excess cold competitors were added in the indicated amounts. The control pUC inhibitor was a 230-bp *Apa*-L1-*Hin*dIII fragment of pUC18. Lanes 3 and 11 contained twice the amount of poly(dI-dC) as lanes 2 and 10, respectively. Arrowheads denote the positions of specific complexes. F, free probe.

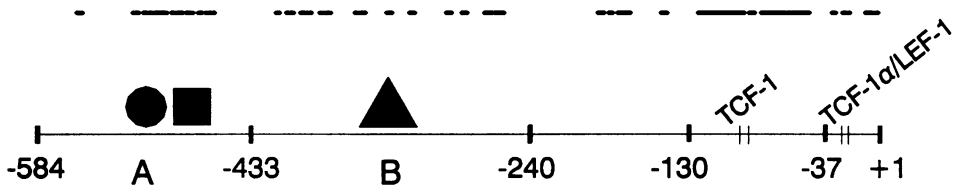

FIG. 10. Diagrammatic representation of potential regulatory sequences in the m*lck* proximal promoter. Binding sites for the A1, A2, and B complexes are denoted by a filled circle, square, and triangle, respectively, superimposed on a map of the region from −584 to +1. Points of identity with the analogous human proximal *lck* sequence are represented by a broken line above the m*lck* sequence, indicating points where there are five or more consecutive identical bases. The positions of the TCF-1 and TCF-1α/LEF-1 binding sites are also noted.

and human *lck* genes. It is apparent that the sites of factor binding fall within regions that are highly conserved. However, other areas of high overall similarity were not obvious sites of nuclear factor binding in our gel mobility shift studies. These regions may nevertheless prove to contain important regulatory information.

Identity of factors that regulate the *lck* proximal promoter. Inspection of the mouse *lck* proximal promoter sequence reveals a great deal of identity with the analogous human *lck* sequence (17) and several previously characterized transcription factor-binding sites (Fig. 10). Although no TATA or CAAT box-binding sites are present (4, 10), there are sites for E4TF-1 (5′-GGAAGTG-3′), a transcriptional regulator of the adenovirus type 5 early region (27); an immunoglobulin H core enhancer (5′-CCAGGTGG-3′; 32); and T-cell-specific nuclear factors TCF-1α/LEF-1 (5′-CANAG-3′; 50, 53, 54), TCF-1 (which binds the same site as TCF-1α/LEF-1; 37, 51), and LYF-1 (5′-PyPyTGGGAGPu-3′; 31). All of these sites reside in areas of close identity with the human *lck* sequence, suggesting a functional conservation of sequences. However, these sites are also present within the −240 to +37 region of the m*lck* sequence which we have demonstrated is insufficient for expression of the *lacZ*-hGH construct. This observation is consistent with reports describing mutations that prevent TCF-1α/LEF-1 binding without abolishing enhancer activity in the CD4 (44) or T-cell receptor α enhancer (24). Indeed, even in the context of the tagged genomic *lck* reporter gene, the presence of the TCF-1α/LEF-1 consensus site at −22 to −27 was not sufficient for expression of the lck^{-37} LGY construct. Nonetheless, any or all of these sites may be important for the T-cell-specific *lck* gene regulation we have observed. The fact that the *lck* regulatory region can compete for complexes formed between T-cell nuclear extracts and the labeled TCF-1α/LEF-1 and TCF-1 binding site probes is consistent with this hypothesis (Fig. 7).

As previously noted, the B complexes associated with the −433 to −240 probe have many characteristics that we believe are indicative of an interaction which is important for positive regulation of the proximal *lck* gene. Although there is little similarity between the mouse distal and proximal regulatory sequences overall, the binding site identified for these complexes is very similar to one that we previously reported (55) in the distal *lck* promoter (however, an oligomer used to define the distal promoter site was incapable of competitively inhibiting B complex formation). Deletion of this sequence in the *lacZ*-hGH construct was correlated with loss of expression in 17 of 18 founders tested, and the exception produced transcripts that were barely detectable on long exposures of RNA blots. The minimal sequence defined by methylation interference and oligonucleotide competition and binding studies reveals an extremely G residue-rich binding site. This sequence (5′-GGGTAGGG-3′) was identified as a possible binding site for SP1 by Thiesen and Bach (49). We examined the potential involvement of SP1 by synthesizing an oligonucleotide, extracted from the simian virus 40 early promoter sequence (19), containing a consensus SP1 binding site that displayed an appropriate band shift under conditions identical to those reported by Spanopoulou et al. (46). However, this oligonucleotide, when used as a cold competitor, did not effectively block the formation of complexes between thymocyte extracts and either the −433 to −240 *lck* fragment or the −365 to −328 oligonucleotide (data not shown). These experiments do not eliminate the possibility that SP1 participates in the formation of the B1-B2 band shift, which exhibits a complex pattern that may include several proteins, but nevertheless they do suggest that other components are involved.

It should be mentioned that satisfactory expression of the LGY genomic reporter occurred even when sequences that are targets for B complex factors were removed (Fig. 1). If the B1-B2 complex is important in the regulation of the lck^{-584} *lacZ*-hGH construct and its absence is responsible for the loss of activity observed with the lck^{-240} *lacZ*-hGH construct, then a redundant activity must be present in the structural gene for *lck*, perhaps within an intron, to allow expression of the lck^{-240} LGY construct. Indeed, we have preliminary evidence that fragments from within the transcribed region of the genomic *lck* clone are able to compete for the B1-B2 complex formed on the −433 to −240 probe (data not shown).

Molecular dissection of transcriptional control mechanisms in the *lck* gene. The results reported here, coupled with our previous studies (1, 2, 9, 13, 16, 55), demonstrate that the two promoters of the *lck* gene can function to a large extent independently of one another. Although the evolutionary pressure that has resulted in the establishment of this two-promoter regulatory system in mice and other mammals remains unelucidated, $p56^{lck}$ clearly functions in several different signalling pathways: as a component of the T-cell receptor complex (3), through interactions with the interleukin-2 receptor (23), and as a regulator of thymocyte development (1). Thymocytes contain appreciably more *lck* mRNA than do mature T lymphocytes (34), and we speculate that the *lck* proximal promoter may have evolved to permit satisfactory accumulation of the high levels of *lck* transcripts (about fivefold greater than in mature T cells; 34) necessary to maintain this differential expression pattern. Viewed in this context, there is no requirement that the *trans*-acting factors that interact with the proximal and distal *lck* promoters share any common structural features. The results reported here provide a basis for examining the changes in expression of transcriptional regulators that occur as thymocytes mature and differentiate into T cells. Moreover, this in vivo dissection of *cis*-acting regulatory regions in the *lck* proximal promoter should permit the

design of improved *lck*-based expression vectors and the biochemical resolution of factors that regulate both *lck* expression and thymocyte maturation.

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