Journal of Cellular Biochemistry 38:117–126 (1988)
Growth Factors and Their Receptors: Genetic Control and Rational Application 1–10

Structure and Expression of *lck* Transcripts in Human Lymphoid Cells

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The murine *lck* gene encodes a membrane-associated protein tyrosine kinase that has been implicated in lymphocyte oncogenesis. Here we report the structure of normal human *lck* transcripts and the pattern of expression of these transcripts in developing thymus and in peripheral T cell subsets. The human *lck* gene encodes a 509 amino acid polypeptide that is closely related to the murine *lck*-encoded protein throughout its length. Analysis of the deduced amino acid sequence of human $p56^{lck}$ demonstrates that an amino-terminal domain, widely divergent among the seven known *src* family members, has been conserved between murine and human $p56^{lck}$, and thus probably includes sequences crucial to the lymphocyte-specific function of this molecule. Human *lck* transcripts were detected in $CD4^+$ and $CD8^+$ T cells, in partially purified B cells, and in Epstein-Barr virus-immortalized B cell lines, but not in monocytes, granulocytes, or in nonhematopoietic cell types. Human *lck* transcripts are readily detectable in fetal thymocytes at 70 days of gestation, but not at 57 days of gestation, indicating that *lck* expression appears coordinately with the appearance of lymphoid cells in the developing thymus. Thus *lck* gene expression is a marker for cells of the lymphocyte lineage in man. We conclude that the *lck* gene probably participates in a signal transduction pathway uniquely present in lymphoid cells.

Key words: lymphocyte signaling, *src* gene family, protein tyrosine kinases

Protein tyrosine kinases may be grouped into two distinct classes, both of which are implicated in the control of cell growth. The first class includes a series of transmembrane receptors that transduce signals supplied by growth factors, e.g., epidermal growth factor or platelet-derived growth factor [1–5]. A second class of protein tyrosine kinases includes molecules that while typically membrane associated appear to reside entirely intracellularly, lacking transmembrane or ligand-binding domains [6]. Many members of this second class are encoded by cellular homologues of retroviral oncogenes (e.g., c-*src*, c-*yes*, and c-*fgr*) and thus clearly may under certain circumstances provide a growth-promoting stimulus. The mechanism whereby tyrosine phosphorylation regu-

Received February 1, 1988; accepted April 5, 1988.

lates cellular proliferation is enigmatic, since relevant substrates remain for the most part undefined [7].

We have focused attention on a member of the *src*-like gene family, *lck*. Although structurally closely related to *src*, *lck* is specifically expressed in lymphocytes among normal cell types [8–10]. Moreover, the level of *lck* transcripts in T lymphocytes responds dramatically to combinations of signals that stimulate lymphokine secretion [9]. The murine *lck* gene encodes a 56-kd polypeptide that is membrane associated, presumably by virtue of covalently linked myristate [11]. Rearrangement and overexpression of the *lck* gene is a characteristic of some murine lymphomas, suggesting that $p56^{lck}$ may assist in controlling lymphocyte proliferation [8,10,12–14]. Since considerable information exists regarding the factors required for T cell growth [15,16], there is reason to hope that analysis of the regulation of the *lck* gene will provide insights into its possible functions. Here we describe the structure of normal human *lck* transcripts and the pattern of expression of these transcripts in normal blood cells and in developing thymus.

MATERIALS AND METHODS

cDNA Libraries and Library Screening

A mitogen-stimulated human peripheral blood leukocyte cDNA library was the kind gift of Drs. P. Concannon and L. Hood [17]. Dr. D. Littman kindly provided two additional human cDNA libraries, one generated using RNA from peripheral blood leukocytes [18] and a second generated using RNA from the PEER cell line [19]. All three libraries were propagated and screened as previously described using the 1.75-kb *Eco*RI fragment of the NT18 plasmid that contains the entire murine *lck* coding region sequence [8].

DNA Sequencing

Recombinant phage containing human *lck* sequences were plaque purified and phage DNA isolated using a plate-lysate technique [8]. The *Eco*RI inserts were subcloned into pUC18 and pUC19 plasmid vectors and into M13mp18 and M13mp19 phage vectors for sequence analysis using the dideoxynucleotide chain termination method [20,21]. All sequences were determined on both strands and on overlapping fragments. The majority of the sequence was obtained using *lck*-specific oligonucleotides, synthesized using an Applied Biosystems DNA synthesizer, as primers [22].

Isolation and Fractionation of Human Peripheral Blood Cells

Human mononuclear cells, T lymphocytes, granulocytes, and monocytes were purified from adult peripheral blood as previously described [9,28]. Isolation of T cell subsets was achieved by treatment of appropriate fractions with monoclonal antibodies (mAbs) and complement as follows: for $CD4^+$ cells, mAbs FC-2 (CD16) and 10.1 (anti-CD8), and for $CD8^+$ cells, mAbs FC-2 and 66.1 (anti-CD4). Viable cells were recovered by Ficoll-Hypaque centrifugation. By indirect immunofluorescence, $CD4^+$, and $CD8^+$ T cell subfractions were greater than 90% reactive with their appropriate mAbs. These mAbs were kindly provided by Genetic Systems Inc., Seattle, WA, with the exception of FC-2, which was the generous gift of Dr. E. Clark, University of Washington. B lymphocytes were partially purified (approximately 70%) using B lymphokwik (One Lambda, Los Angeles, CA) according to the manufacturer's instructions.

RNA Isolation and RNA Blotting

Total RNA from cell lines, purified cells, or from thymic tissue was obtained and analyzed as previously described [8].

RESULTS

Using the murine *lck* gene as a probe, we previously identified *lck* transcripts in human thymus RNA and localized the human *lck* gene to chromosome 1p32–35 at a site of frequent chromosomal abnormalities in human lymphomas and neuroblastomas [23]. To characterize normal human *lck* transcripts, we used the murine *lck* probe to screen cDNA libraries generated using RNA from human peripheral blood lymphocytes and a human T cell line [17–19].

Cloning of the Human *lck* Gene

Figure 1A presents restriction maps for three human *lck* cDNA clones. Clone HK28 was derived from a partially processed transcript and contains a 173-bp intron sequence that was not present in any of 22 other clones spanning this region. The entire sequence of the human *lck* cDNA, derived from normal peripheral blood lymphocytes, is compared with that of a nearly full-length murine *lck* cDNA in Figure 1B, along with the deduced amino acid sequences of each. Trevillyan et al. [24] have previously reported the 3′ 850 bp of this sequence, which they obtained by screening a cDNA library made using RNA from the Jurkat T cell line. Similarly, Koga et al. [25] have reported the sequence of what is clearly a human *lck* cDNA derived from the Jurkat T cell line. The HK28 human *lck* cDNA clone includes 56 bp of presumed 5′ untranslated region, an open reading frame of 509 codons, and a 365 bp 3′ untranslated region including a single prototypical recognition site for polyadenylation (underlined). Overall, the human and murine *lck* coding sequences are 91% identical at the nucleotide level and differ by only 17 of 509 amino acids (Figs. 1B, 2). Our sequence differs significantly (26 amino acid substitutions) from that reported by Koga et al. [25]. In particular, their sequence lacks a conserved tyrosine (codon 505), which is the site of in vivo phosphorylation in murine $p56^{lck}$ [26]. We have previously shown that mutations at this site activate the *lck* kinase and unmask its transforming potential [26], thus the differences between the Koga et al. sequence and our normal T cell sequence may be biologically important. At the same time, comparison of the Koga et al. sequence with that of Trevillyan et al. [24], both of which were derived from cDNAs from Jurkat cells, suggests that these differences represent sequencing errors [27]. The incomplete sequence of Trevillyan et al. [24] differs from our sequence by a single base deletion in the 3′ untranslated region but is identical within the 133 codons that they report (the 5′-most G of their sequence is presumably artifactual). Similarly, the incomplete sequence of Veillette et al. [27] is identical except for a typographical error that substitutes His (CAT) for Leu (CTC) at codon 472.

Evolutionary Conservation of the *lck* Gene

Human *lck* is more closely related to murine *lck* than it is to any of the other six *src*-family kinases. This is shown graphically in Figure 2 where the deduced amino acid sequences of the human *lck*- and *hck*-encoded proteins are aligned. Seven of the 17

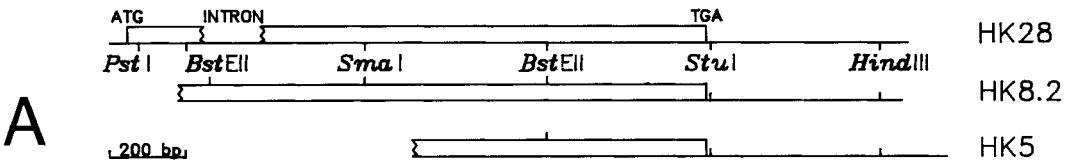

Fig. 1. Structure of human *lck* transcripts deduced from cDNA clones. **A**: Restriction maps of three overlapping human *lck* cDNA clones. The translation, initiation, and termination codons are indicated and the coding region is boxed. Clone HK28, from a normal peripheral blood lymphocyte cDNA library [17], represents a partially processed transcript and contains a 173-bp intron sequence at the indicated position. **B**: The complete nucleotide sequence encoded by the human *lck* gene (with the intron sequence of HK28 deleted) is compared with the full-length sequence encoded by the murine *lck* gene [8,10]. The coding region is presented as triplets with the deduced amino acid sequence for the human *lck*-encoded protein indicated in single-letter code. The consensus poly-A addition site is underlined.

amino acid substitutions separating the murine and human *lck* sequences are concentrated in the first 60 amino acids. Nevertheless, although sequence conservation in the amino-terminal domain may be slightly more relaxed than elsewhere in the molecule, the close relationship between murine and human *lck* sequences in this region is consistent with the view that this "domain" is specialized for unique, and in many cases lineage-specific, functions [28,29].

Expression of *lck* in Human Lymphocytes

Evidence for the conservation of *lck* function in man and mouse was sought through analysis of *lck* transcripts in human cells. Using a probe encompassing the entire *lck* coding region, cross-hybridizing transcripts were detected in human peripheral blood mononuclear cells and in fractionated T lymphocytes, but not in granulocytes or monocytes (Fig. 3). In a separate experiment, appropriately sized *lck* transcripts were also detected in partially purified peripheral blood B lymphocytes (Fig. 3).

Since our best B lymphocyte preparations were surely contaminated with other cell types, and since Koga et al. [25] failed to identify *lck* transcripts in three human B cell lines, we sought to confirm the presence of *lck* transcripts in B lymphocytes. To this end, we examined Epstein-Barr virus-immortalized human B lymphocytes for *lck* expression. To verify that the *lck* transcripts identified were in each case encoded by the *lck* gene and not by a closely related but distinct genetic element, we used a unique 288-bp *StuI/HindIII* probe derived from the 3′ untranslated region of the human *lck* cDNA (Fig. 1A). Since 3′ untranslated regions are, in most cases, under little selection pressure, it is unlikely that this sequence would be shared by other protein tyrosine kinase genes. Indeed, the murine and human *lck* transcripts are only 45% homologous within this region (Fig. 1B) and hence do not cross-hybridize using our experimental conditions. Figure 4 demonstrates that the 3′ untranslated region *lck* probe identifies transcripts in both $CD4^+$ and $CD8^+$ T lymphocytes and at a lower level in partially purified peripheral blood B lymphocytes as well. These results are consistent with previous studies of the expression of the *lck* gene in murine lymphoid cell lines [8]. Importantly, transcripts were also present in two Epstein-Barr virus (EBV)-transformed polyclonal B cell lines (e.g., line PG) but not in a human neuroblastoma cell line (SKN), a human melanoma cell line, or human fetal adrenal cells (Fig. 4). Veillette et al. [27] also identified *lck* transcripts in EBV-transformed peripheral blood cells and in 4/5 Burkitt

B

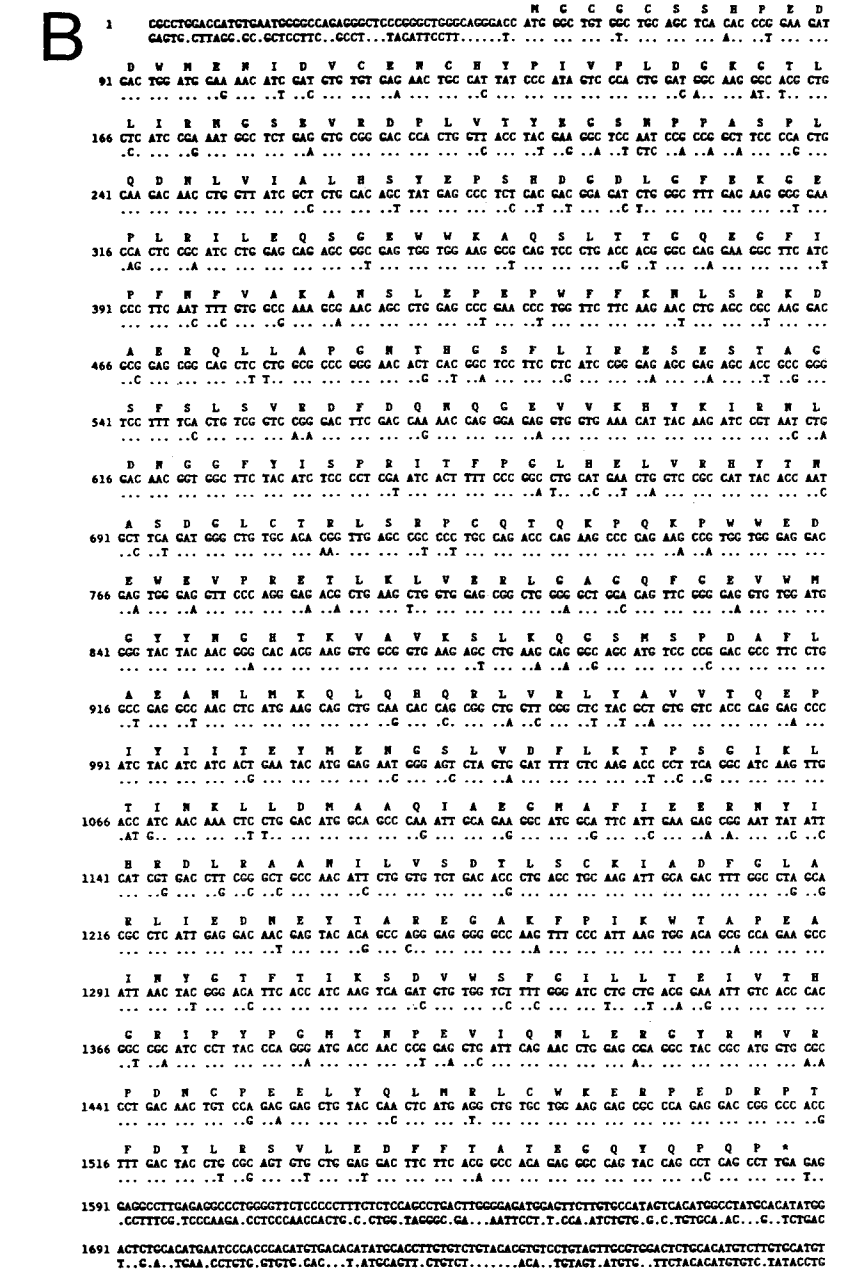

Figure 1B

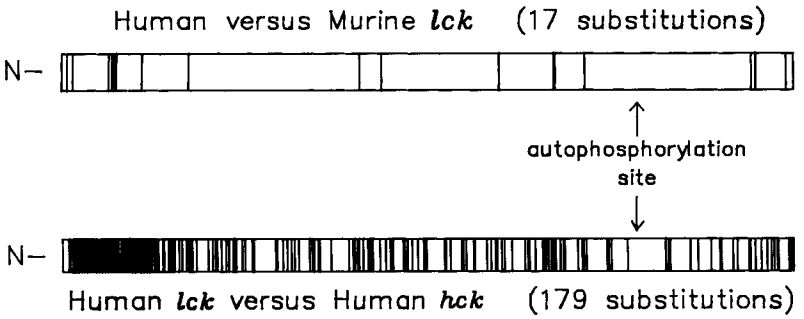

Fig. 2. Conservation of the *lck* amino-terminal region in man and mouse. Shown are two histograms derived by comparing the human (this paper) and murine [8,10] *lck* sequences (**top**) or the human *lck* sequence with its closest known *src*-family counterparty, the human *hck* [27,31] sequence (**bottom**). In each case, a vertical line has been drawn at each position where the two proteins compared differ in sequence. The site of in vitro autophosphorylation in $p56^{lck}$ is indicated.

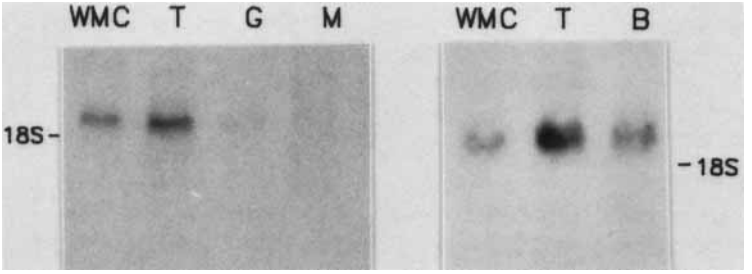

Fig. 3. *lck* expression in human peripheral blood leukocytes. Total RNA was isolated from whole mononuclear cells (**WMC**) and granulocyte (**G**), monocyte (**M**), T lymphocyte (**T**), and B lymphoctye (**B**) fractions and 10 μg of each RNA sample was subjected to RNA blot analysis using the 2.1-kb HK28 cDNA insert as a probe. The left and right panels represent two independent experiments. The position of 18S rRNA markers is indicated.

lymphoma cell lines. We conclude that the *lck* gene product is probably expressed in most (if not all) lymphocyte classes.

Expression of *lck* Is a Marker for the Appearance of Lymphoid Elements in the Developing Human Thymus

We have previously demonstrated that the murine *lck* gene is expressed at approximately fivefold higher levels in thymus as compared with spleen [8]. In man, adult spleen contains only about 1 pg *lck* mRNA per microgram total RNA as compared to 12 pg *lck* mRNA per μg total RNA in thymus [9]. To ascertain when in development *lck* expression is first observed, we probed RNA isolated from human fetal thymus tissue obtained at 57, 70, 100, and 110 days of gestation and from 2.5-month-, 2.5-year-, and 8.5-year-old individuals using the human *lck* cDNA. Figure 5 demonstrates that *lck* transcripts are identifiable at 70 but not at 57 days of gestation. Lymphoid elements first appear in the human thymus at 8 to 9 weeks of gestation [30]. Thus the development of *lck* expression correlates precisely with the arrival of bone marrow-derived immigrants in the developing thymus. In separate experiments, *lck* transcripts were detectable in

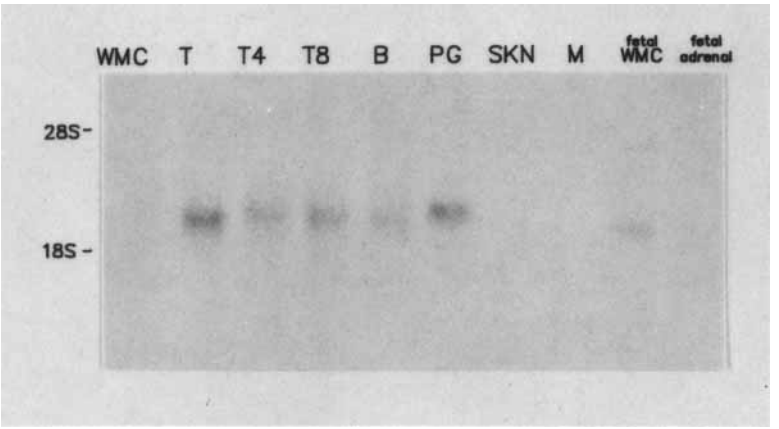

Fig. 4. Expression of *lck* mRNA in human T cell subsets and in cell lines. Total RNA (10 μg) was isolated from whole mononuclear cells (**WMC**), fractionated T lymphocytes (**T**), $CD4^+$ lymphocytes (**T4**), $CD8^+$ lymphocytes (**T8**), B lymphocytes (**B**), an Epstein-Barr virus-transformed cell line (**PG**), the human neuroblastoma cell line SKN-BE2 (**SKN**), a human melanoma cell line (**M**), and from fetal whole mononuclear cells and adrenal cells, and was subjected to RNA blot analysis using a probe derived from the 3′ untranslated region of the human *lck* cDNA by digestion with *Stu*I and *Hind*III (see Fig. 1A). The position of 18S and 28S rRNA markers is indicated.

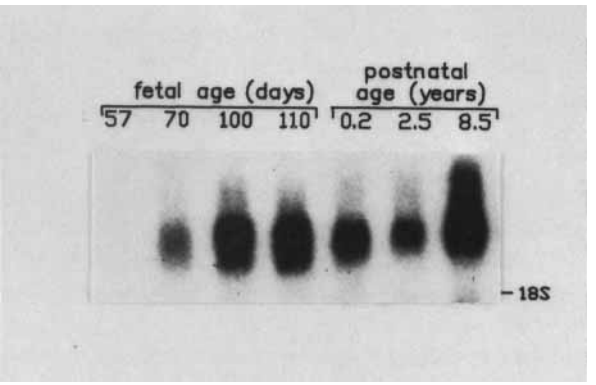

Fig. 5. Ontogeny of *lck* expression in human thymus. Total RNA was isolated from human fetal and postnatal thymic specimens and *lck* expression examined by RNA blot analysis of 10 μg of total RNA using the full-length HK28 cDNA as a probe. The approximate developmental age of each specimen is indicated at the top of the figure and the position of 18S rRNA markers is shown. The presence of RNA in each lane was verified by reprobing the blot with a labeled human elongation factor 1α cDNA (data not shown).

mature $CD4^+$ and $CD8^+$ thymocytes, in $CD4^+/CD8^+$ (double-positive) cells, and in thymocytes lacking both markers (data not shown).

DISCUSSION

Seven *src*-like protein tyrosine kinases have been described: c-*fgr* [31,32], *hck* [28,33], *lck* [8,120], *lyn* [34], c-*src* [35], *fyn* [36,37], and c-*yes* [38]. All are similar in size and contain a characteristic protein tyrosine kinase "domain" defined by sterotyped

sequence motifs believed to participate in catalytic function [2]. Three of the *src*-like kinases were originally identified as products of retroviral oncogenes, directly implicating these molecules in the control of replication. In addition, the *lck* gene is rearranged and overexpressed in some murine lymphomas [8,10] and both *lck* and *hck* are located at sites of frequent chromosomal aberrations in human malignant disease [23,33]. Nevertheless, high-level expression of *src*-like kinases is often observed in cells with little replicative potential. Thus c-*src* itself is expressed in neurons and platelets [39,40] and *hck* is maximally expressed in granulocytes [28]. These observations suggest that *src*-family members must either, under some circumstances, actively suppress proliferation, or must be primarily active in metabolic processes unrelated to cell growth.

Restricted Expression of *lck*

Additional perspective on the function of the *src*-like protein tyrosine kinases can be gained by comparing the expression patterns of each. For example, the human *hck* gene is expressed in myeloid cells [28], and *hck* expression appears to increase when immature myeloid cells become committed to the myeloid lineage ([33] and S.F. Ziegler and R.M. Perlmuter, unpublished observations). By analyzing murine tissues and transformed cell lines, we previously proposed that the murine *lck* gene is expressed primarily in lymphocytes [8]. Here we have used an *lck*-specific probe to document that the human *lck* gene is expressed predominantly in T lymphocytes of both the $CD4^+$ and the $CD8^+$ subclasses. Thus both the structure and the expression pattern of the *lck* gene have been conserved since the time that mouse and man diverged. Veillette et al. [27] have also demonstrated that the human *lck* gene is specifically expressed in lymphoid cells, although many colon carcinomas and some lung tumors also contain appreciable levels of *lck* transcripts.

Structural Variation Among *src*-Like Protein Tyrosine Kinases

Differences among *src*-family members are concentrated in the amino-terminal 70 residues of each, suggesting that the unique function(s) of *lck* as compared with *hck* is defined by this amino-terminal region. Both $p60^{src}$ and $p56^{lck}$ are myristylated at their amino-terminal glycine residues [11,41], and this myristylation is probably involved in the association of these proteins with cell membranes [42,43]. Since all *src*-like kinases share a common Met-Gly-Ser/Cys motif at their amino termini, it is likely that all are myristylated and that all will prove to be similarly membrane associated. In this sense, the variable amino-terminal structures of each *src*-family member may be viewed as ideally positioned to interact with cytoplasmic portions of transmembrane receptors, permitting the carboxy-terminal kinase domain to serve as the catalytic effector in a transmembrane signal transduction mechanism. For the *lck* gene, the putative receptor must be assumed to be present on lymphoid cells in general. Conservation of this amino-terminal region in both the human and murine *lck* sequences (Fig. 2) is consistent with this view. Thus it is provocative that treatment of resting human T lymphocytes with activating regimens of mitogen and phorbol ester induces rapid reductions in the level of *lck* transcripts in these cells as well as an altered pattern of expression of $p56^{lck}$ protein [9].

lck Expression as a Marker for the Lymphoid Lineage

By analyzing the pattern of appearance of *lck* transcripts in developing human fetal thymus, we demonstrated that *lck* transcripts are first detectable at the time when the

thymic rudiment becomes colonized with hematopoietic elements (Fig. 5). We cannot, however, determine whether lymphoid stem cells, either fetal or adult, already contain *lck* transcripts. In general, *lck* mRNA is barely detectable in murine bone marrow (data not shown). Since lymphoid stem cells capable of reconstituting irradiated hosts are present at frequencies of 10^{-3} or less in bone marrow populations [44], we cannot determine whether these precursors express *lck* transcripts before arrival in the thymus. Nevertheless, the production of *lck* mRNA appears to occur uniquely in lymphocytes, among normal cells, and may well be a marker for commitment within the lymphoid lineage.

Complexity of Protein Tyrosine Kinase-Mediated Regulation

In addition to *lck* transcripts, lymphocytes frequently contain mRNA encoding other related *src*-like protein tyrosine kinases, for example c-*fgr* [45] and *fyn* [36,37]. While this array of potential growth-regulating elements may be viewed as complementary to the large repertoire of signals to which lymphocytes respond, it is clear that considerable effort will be required to identify specific phosphorylation pathways within this complex web of kinases and their substrates. Under these circumstances, the unique lineage specificity of the *lck* gene in man and mouse is especially interesting since it suggests that the molecules with which the *lck* gene product interacts may be similarly specialized.

ACKNOWLEDGMENTS

We thank Patrick Chou and Yim-Foon Lee for preparing oligonucleotides, Pat Concannon, Lee Hood, and Dan Littman for providing human lymphocyte cDNA libraries, Ed Baetge for the SKN neuroblastoma cell line, Betsy Mellins for melanoma and EBV-transformed lymphoid cell lines, and our colleagues for helpful discussions. This work was supported in part by grants from the American Cancer Society and the National Institutes of Health.

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