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Inhibition of T-cell receptor β-chain gene rearrangement by overexpression of the non-receptor protein tyrosine kinase p56^{lck}

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The variable region genes of the T cell receptor (TCR) α and β chains are assembled by somatic recombination of separate germline elements. During thymocyte development, gene rearrangements display both an ordered progression, with β chain formation preceding α chain, and allelic exclusion, with each cell containing a single functional β chain rearrangement. Although considerable evidence supports the view that the individual loci are regulated independently, signaling molecules that may participate in controlling TCR gene recombination remain unidentified. Here we report that the lymphocyte-specific protein tyrosine kinase p56^{lck}, when overexpressed in developing thymocytes, provokes a reduction in Vβ−Dβ rearrangement while permitting normal juxtaposition of other TCR gene segments. Our data support a model in which p56^{lck} activity impinges upon a signaling process that ordinarily permits allelic exclusion at the β-chain locus.

Key words: germline transcription/*lck* transgenic mice/Vβ rearrangement

Introduction

Functional T cells derive from bone marrow stem cells that differentiate in the thymus in response to a complex series of developmental cues. Thymocyte cell populations representing distinct developmental stages can be distinguished by several cell surface markers, among which the T cell receptor−CD3 complex and the coreceptors CD4 and CD8 are especially well characterized (reviewed in von Boehmer, 1988). An apparently linear progression from $CD4^-8^-3^-$ precursors through $CD4^+8^+3^{lo}$ intermediates to mature $CD4^+8^-3^{hi}$ or $CD4^-8^+3^{hi}$ cells has been postulated for the majority of αβ cells. Antigen receptor-bearing $CD4^+8^+$ cells undergo negative and positive selection to eliminate self-reactive cells and to establish a repertoire wherein antigen recognition requires self-MHC restriction. Since the end result of the maturation sequence is a T cell repertoire selected on the basis of receptor specificity, the assembly of antigen receptor genes by somatic recombination is an absolute requirement in T-cell differentiation. Moreover, thymocyte development does not proceed beyond the most immature stages when somatic gene recombination is defective, e.g. in mice deficient for genes that activate recombination, RAG-1 and RAG-2 (Schatz *et al.*, 1989; Oettinger *et al.*, 1990; Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992) or in mice carrying the severe combined immunodeficiency (*scid*) mutation (Bosma and Carroll, 1991).

The potential for severe chromosomal damage resulting from inappropriate somatic recombination mandates strict control of this activity. Analysis of rearrangement events during T cell development suggests that both the expression of recombinase activity and the accessibility of the DNA templates are potential targets for regulation. For instance, the RAG-1 and RAG-2 genes are expressed in immature, but not mature, thymocytes (Boehm *et al.*, 1991; Turka *et al.*, 1991). In addition, complete T cell receptor (TCR) genes are assembled exclusively in developing T cells, the individual elements remaining largely in germline configuration even in B lymphocytes. This specificity implies cell type targeting of the appropriate loci, since the same recombinase components and recognition sequences function in both B and T cell lineages (Yancopoulos *et al.*, 1986; Hesse *et al.*, 1989). The recombinational competence of germline TCR elements has previously been correlated with tissue-specific transcriptional activity, suggesting that transcription may impose (or at least reflect) the unique chromatin state capable of undergoing recombination (Alt *et al.*, 1986; Ferrier *et al.*, 1990).

Analysis of receptor expression in fetal and adult thymocytes suggests that rearrangement of receptor genes begins in $CD4^-8^-$ thymocytes and progresses in an ordered fashion (Snodgrass *et al.*, 1985a,b; Raulet *et al.*, 1985). The first detected events are the joining of Dβ to Jβ elements and the resulting expression of a 1.0 kb (Dβ−Jβ−Cβ) RNA transcript. Vβ elements are subsequently joined to the Dβ−Jβ locus, initiating synthesis of a full-length 1.3 kb (Vβ−Dβ−Jβ−Cβ) transcript. Finally, Vα to Jα recombination occurs and is detected by expression of a 1.6 kb (Vα−Jα−Cα) transcript. Successful rearrangement of both α and β chains leads to surface expression of the heterodimeric antigen receptor in association with non-polymorphic CD3 proteins. Since the genes for the dimeric receptor are assembled sequentially, β chain elements are likely to be selectively inaccessible to recombinase during assembly of the α chain, thus preventing alteration of a functional β gene and excluding potentially productive rearrangement of two alleles. This inactivation of the β locus has been proposed as the molecular basis of allelic exclusion. The protein product of a functional β gene may itself provide such 'feedback inhibition', since thymocytes which contain a functional β chain transgene fail

to rearrange endogenous Vβ elements (Uematsu *et al.*, 1988).

Although TCR gene rearrangements are developmentally ordered, the biochemical mechanisms responsible for regulating this process remain enigmatic. In particular, the signaling system that attenuates β chain gene rearrangement while permitting Vα−Jα joining is undefined. Among those molecules that may potentially participate in this feedback regulation process, protein tyrosine kinases exhibit attractive characteristics. Previous studies demonstrate that the non-receptor protein tyrosine kinases $p59^{fyn}$ and $p56^{lck}$ both participate in T cell signaling, in the former case almost certainly as a result of direct interaction with the CD3 complex itself (Samelson *et al.*, 1990), and in the latter case by virtue of a specific association with the CD4 and CD8 coreceptor molecules (Veillette *et al.*, 1988; Shaw *et al.*, 1989; Turner *et al.*, 1990).

To investigate the functions of $p59^{fyn}$ and $p56^{lck}$ in T cell development, we generated transgenic mice in which these kinases are specifically overexpressed in thymocytes under the control of the thymocyte-specific proximal *lck* promoter (Abraham *et al.*, 1991a; Cooke *et al.*, 1991). Thymocytes from animals overexpressing $p59^{fyn}$ retained normal developmental characteristics, but proved especially responsive to TCR-mediated stimulation (Cooke *et al.*, 1991). These observations are consistent with the reported association of $p59^{fyn}$ with CD3 components. Moreover, $p59^{fyn}$ is not ordinarily expressed until relatively late in thymocyte development (Cooke *et al.*, 1991; Sancho *et al.*, 1992), an ideal characteristic for a signal transducing molecule principally engaged in mediating the responses of functional T cells to antigenic challenge.

In contrast, $p56^{lck}$ is present in immature T lineage cells (Perlmutter *et al.*, 1988; Reynolds *et al.*, 1990; Wildin *et al.*, 1991) and thymocyte maturation is substantially disrupted in mice which either lack or overexpress $p56^{lck}$ (Abraham *et al.*, 1991a; Molina *et al.*, 1992). The unique phenotype of $p56^{lck}$ overexpression is characterized by a transgene-dependent dose-related decrease in the number of thymocytes bearing functional antigen receptors. In addition, transgenic lines expressing the highest levels of $p56^{lck}$ activity reproducibly develop thymic tumors of immature ($CD4^-8^-3^-$) surface phenotype (Abraham *et al.*, 1991b).

We have previously shown that a lack of detectable RNA transcripts corresponding to functionally rearranged TCR β chains accompanies the absence of surface CD3 expression in mice expressing high levels of $p56^{lck}$ (Abraham *et al.*, 1991a). We report here, however, that thymocytes from

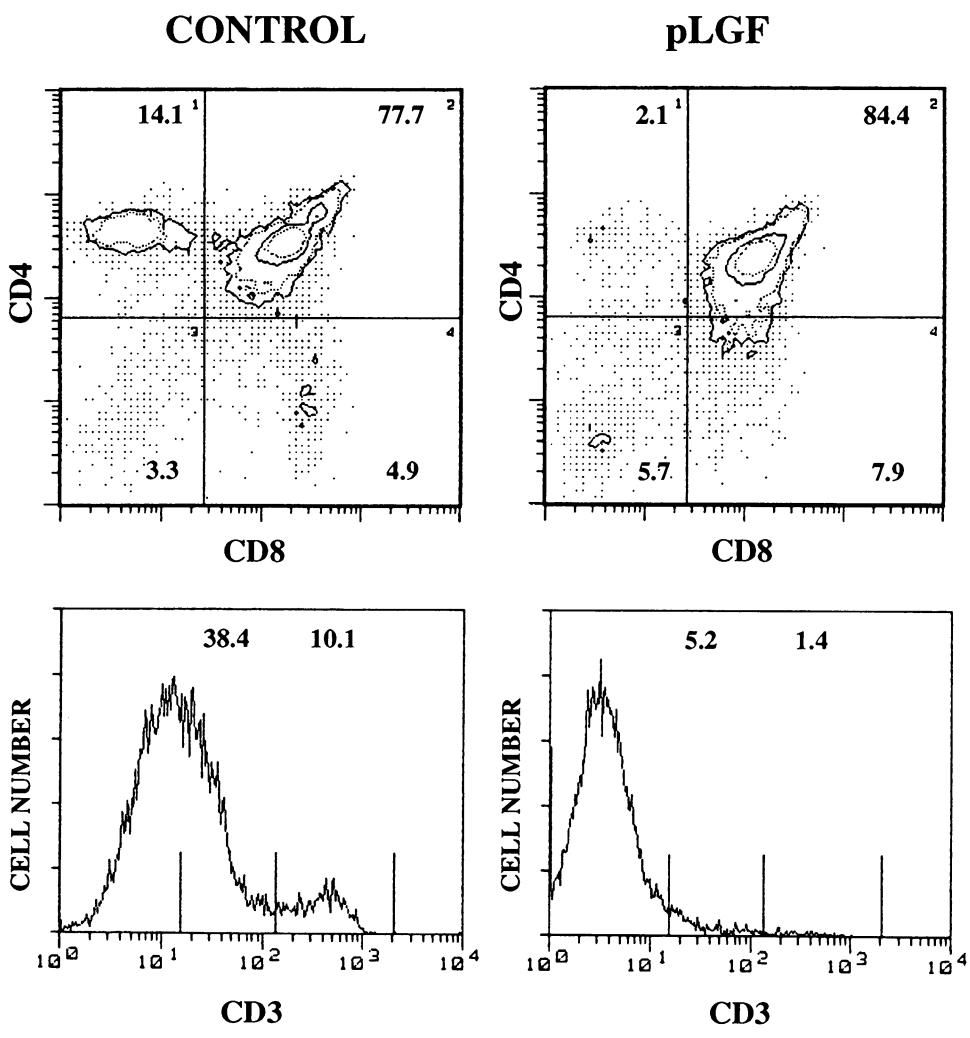

Fig. 1. Flow cytometric analysis of thymocytes from control and pLGF mice. Fluorescence staining profiles of surface CD4 and CD8 expression (top row) or CD3 expression (bottom row) on thymocytes obtained from control or pLGF 3073 transgenic littermates. Percentage of cells in each population are indicated.

these transgenic animals display normal levels of α chain transcripts, α chain protein monomers and CD3 components. Thymocytes from these mice display a specific reduction both in Vβ to Dβ−Jβ rearrangements and in germline Vβ element transcripts of accumulation. Moreover, antigen−receptor expression is restored in these thymocytes by the introduction of a functionally rearranged β chain transgene. These data argue that $p56^{lck}$ activity can impinge directly on a developmental pathway that ordinarily regulates Vβ−Dβ joining, a process that may be involved in allelic exclusion at the β chain locus.

Results

In a previous study, we observed that overexpression of the *lck* gene, under the control of its own thymocyte-specific proximal promoter, dramatically disturbed T cell development in transgenic mice (Abraham *et al.*, 1991a). This effect was especially profound in pLGF animals that express a mutant activated form of $p56^{lck}$ containing a phenylalanine for tyrosine substitution at position 505. The characteristics of thymocytes overexpressing activated $p56^{lckF505}$ are revealed by comparing the flow cytometric profiles of normal and pLGF mice. These phenotypic changes include: (i) a decrease in the proportion of mature $CD4^+8^-$ or $CD4^-8^+$ cells and an increase in the proportion of $CD4^-8^{lo}$ cells (Figure 1, top) and (ii) a dramatic decrease in cells expressing either low or high levels of surface CD3 (Figure 1, bottom).

Selective loss of TCR β chains in lck transgenic mice

To investigate the mechanism whereby $p56^{lck}$ overexpression results in loss of $CD3^+$ thymocytes, we examined the expression of various TCR components in normal and transgenic mice. As previously reported, RNA blot analysis reveals that pLGF thymocytes contain markedly decreased levels of full-length (1.3 kb) β locus transcripts, while transcripts corresponding to Dβ−Jβ−Cβ (1.0 kb) are present at high levels (Figure 2A). This pattern of expression is characteristic of immature $CD4^-CD8^-$ thymocytes prior to Vβ and Vα rearrangement (Snodgrass *et al.*, 1985a) and is not expected for a thymus consisting primarily of $CD4^+8^+$ cells. Surprisingly, the level of mature (1.6 kb) α chain transcripts in the pLGF thymocytes appears close to normal (Figure 2A), suggesting that the majority of pLGF thymocytes express TCRα chain in the absence of TCRβ. This expression pattern is unlike that seen in normal thymocyte populations.

To determine if the α chain transcripts in pLGF thymocytes result from functional gene rearrangements, $CD4^+8^+$ thymocytes from normal and transgenic mice were analyzed for the presence of intracellular α protein. NP-40 lysates of control and pLGF thymocytes were subjected to non-reducing SDS−PAGE and immunoblotting

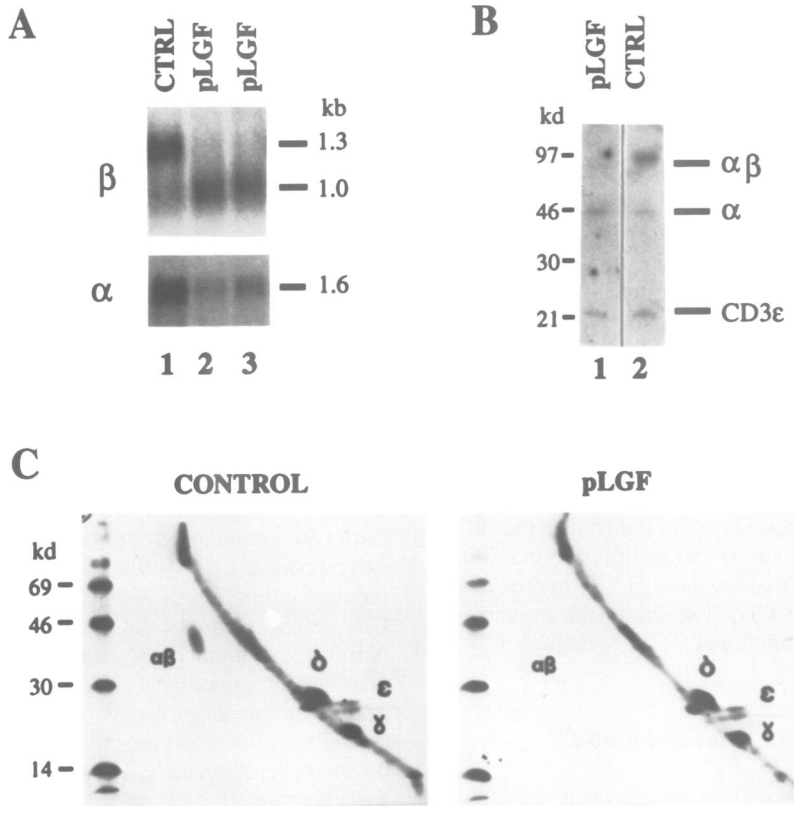

Fig. 2. Detection of TCR components. **A.** RNA blot probed with Cβ- (top) or Cα-specific (bottom) probes. Each lane represents 10 μg total thymocyte RNA from control (lane 1), pLGF3073 (lane 2) or pLGF2954 (lane 3) transgenic mice. Transcript sizes in kilobase pairs (kb) are indicated. **B.** NP-40 lysates from pLGF3082 (lane 1) or littermate control (lane 2) $CD4^+8^+$ thymocytes immunoblotted with a mixture of anti-TCRα (mAB H28-710) and anti-CD3ε (mAB HMT3-1) antibodies. Positions of αβ heterodimer, α monomer and CD3ε are indicated. Size markers are in kilodaltons (kd). **C.** Two-dimensional non-reducing/reducing SDS−PAGE of TCR−CD3 complexes immunoprecipitated with anti-CD3ε antibodies (mAB 145-2C11) from metabolically labeled littermate control (left) or pLFG3082 (right) $CD4^+8^+$ thymocytes. Positions of αβ and CD3 γ, δ and ε chains are indicated.

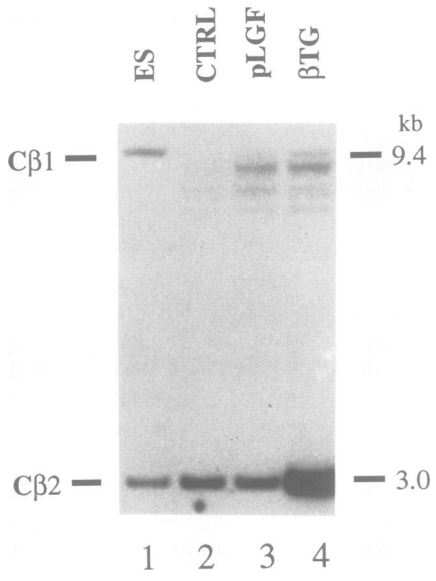

Fig. 3. Analysis of β locus rearrangement in control and pLGF thymocytes. Genomic DNA blot probed with Cβ-specific probe. Each lane represents 10 μg *Hin*dIII-digested DNA derived from the embryonic stem-cell line D3J8 (ES), thymocytes from control, pLGF3073 line or β chain transgenic mice as indicated. Sizes of germline Cβ1 and Cβ2 bands are indicated in kilobase pairs (kb).

using mAb H28-710 (anti-TCRα). Figure 2B reveals that α monomers are present in pLGF $CD4^+8^+$ thymocytes, indicating that the α chain transcripts direct the synthesis of intact α proteins. Consistent with the lack of surface TCR, however, αβ heterodimers were not detected in pLGF thymocytes. Immunoblotting at the same time with mAb HMT3-1 (anti-CD3ε) shows that CD3ε was present at normal levels in the pLGF thymocytes.

To examine the synthesis and assembly of TCR complexes in $CD4^+8^+$ thymocytes from pLGF and control mice, metabolically labeled cells were lysed in digitonin, intact TCR−CD3 complexes were immunoprecipitated using an anti-CD3ε mAb (145-2C11) and the resultant immune complexes were subjected to two-dimensional non-reducing/reducing SDS−PAGE. Figure 2C reveals that pLGF cells, despite the pronounced absence of αβ heterodimers, contain levels of newly synthesized CD3 γ, δ and ε complexes equivalent to those present in control $CD4^+8^+$ cells. Immunoprecipitation of the digitonin lysates with anti-TCR β mAB (H57-597) failed to detect TCR β chains, either as αβ heterodimers or β monomers, in the transgenic cells (data not shown). In as much as intracellular α and CD3 proteins are detected at normal levels in pLGF thymocytes, we conclude that surface CD3 expression is prevented primarily by the lack of functional TCR β chains.

Reduced Vβ rearrangement induced by p56^{lck} overexpression

Failure to synthesize mature β chain transcripts in the pLGF thymocytes suggests an absence of Vβ to Dβ rearrangement. To quantitate the relative abundance of Vβ rearrangements, we analyzed genomic DNA from normal and pLGF thymocytes by two means. First, *Hin*dIII digested DNA was subjected to genomic blot analysis with a Cβ-specific probe (Figure 3). Germline DNA, as represented by the embryonic

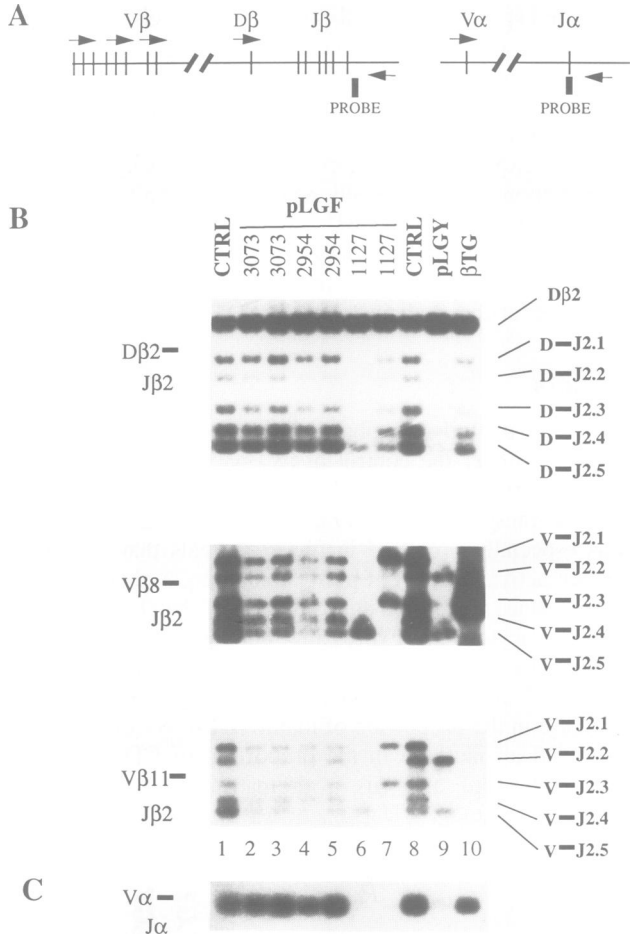

Fig. 4. Quantification of relative levels of Vβ and Vα rearrangements in control and pLGF thymocytes. **A.** Schematic representation of DNA PCR strategy. The positions of oligonucleotide primers used in amplification of β (left) and α (right) rearrangements are shown by arrowheads and the positions of the hybridization probes are indicated by solid bars. **B.** Blot hybridizations of gels containing products derived from PCR using the Jβ2 3′ primer with Dβ2- (top), Vβ8- (middle) and Vβ11-specific (bottom) 5′ primers. Filters were probed with the Jβ2-specific probe depicted in A. Sources of thymocyte DNA are indicated above each lane, and the positions of germline Dβ2 and specific Dβ−Jβ and Vβ−Jβ rearrangements are marked by solid lines. The pLGF1127 (lanes 6 and 7) and pLGY (lane 9) samples are derived from animals bearing *lck*-induced thymomas. **C.** PCR products derived from reactions using VαF3 and Jα pHDS58 specific primers probed with Jα pHDS58-specific oligonucleotide probe. Lanes are the same as in B.

stem cell line in lane 1, contains two hybridizing fragments corresponding to Cβ1 (9.4 kb) and Cβ2 (3.0 kb). Rearrangement of either Dβ or Vβ elements will alter or delete the Cβ1 band, whereas the Cβ2 band remains unchanged. Normal adult thymocytes (lane 2) are characterized by nearly complete elimination of the germline Cβ1 band and a lack of distinct rearranged bands. A different pattern is generated by cells which undergo only partial (Dβ−Jβ) rearrangements, as represented by DNA derived from mice transgenic for a functionally rearranged TCR β chain. This transgene, identifiable in the blot by the intensity of the Cβ2 band, has been shown to yield thymocytes in which endogenous Dβ, but not Vβ, elements are rearranged (Uematsu *et al.*, 1988). As seen in lane 4, the germline Cβ1 band persists in this sample and the limited Dβ−Jβ rearrangements can be visualized as discrete bands. The rearrangement pattern of

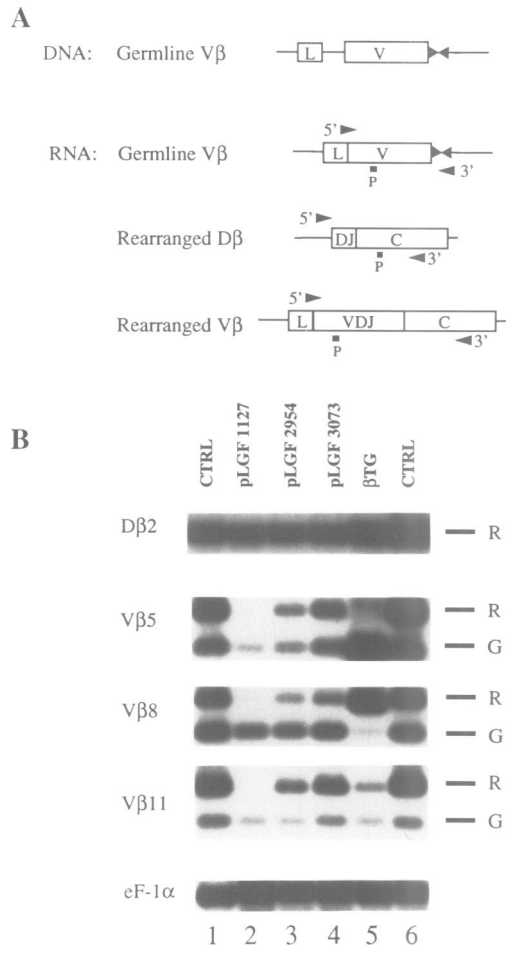

Fig. 5. Quantitation of rearranged and germline Vβ element transcription. **A**. Schematic representation of RNA PCR strategy. Recombination recognition sequences (7−9mer) are indicated by triangles 3′ to the V exon. The positions of 5′ and 3′ primers are indicated by arrowheads. The 5′ Vβ primer is complementary to sequences formed by RNA splicing of the leader (L) and variable (V) exons and does not amplify genomic DNA. Positions of hybridization probes (P) are indicated. **B**. Blot hybridization analysis of gels containing PCR products from the indicated Dβ or Vβ elements. R, rearranged transcript; G, germline transcript; eF-1α, elongation factor control. The source of thymocyte RNA is indicated above each lane. cDNA samples for lanes 1−4 were generated in simultaneous reactions, whereas those for lanes 5−6 were generated separately.

DNA from pLGF thymocytes (lane 3) is similar to that of the TCRβ transgenics, displaying germline Cβ1 persistence and limited bands corresponding to β locus rearrangements. This pattern is also characteristic of early fetal thymocytes in which only Dβ rearrangements have occurred (Snodgrass *et al.*, 1985b), suggesting that the majority of pLGF thymocytes contain β locus alleles with partial (Dβ−Jβ) rearrangements.

Further insight into the efficiency of Vβ gene rearrangement in *lck* transgenic mice was obtained using a recently described PCR assay (van Meerwijk *et al.*, 1990) shown schematically in Figure 4A. Primers complementary to Vβ or Dβ elements were used in combination with a primer positioned immediately 3′ to the Jβ2 cluster, allowing amplification of rearranged, but not germline, Vβ gene segments. The products of these reactions were visualized after electrophoresis, blot transfer and hybridization with a Jβ2-specific probe. We first confirmed the observation of previous reports (Schlissel and Baltimore, 1989) that this type of PCR assay, when restricted by limited template and amplification cycles, produces amplified products that are proportional to the amount of input DNA (see Materials and methods). The results presented in Figure 4B, corresponding to rearrangements of Dβ2 (top), Vβ8 (middle) and Vβ11 (bottom) to the Jβ2.1−Jβ2.5 elements, were generated using 0.5 μg thymocyte DNA, with each lane representing an individual animal. The top panel shows that levels of Dβ2 to Jβ2 rearrangements are equally high in normal, β chain transgenic and pLGF thymocytes. This finding is consistent with the abundance of 1.0 kb Dβ-initiated transcripts in both normal and transgenic cells. In contrast to the abundant Dβ rearrangements, products generated from rearranged Vβ elements were detected at lower levels in pLGF samples when compared with controls. Quantification of the PCR products shown in Figure 4 indicates that thymic DNA from animals of the pLGF 3073 and 2954 lines contains only 8−18% the amount of specific Vβ8 or Vβ11 rearrangements found in control samples. The assay also confirms the almost complete lack (4% of control) of endogenous Vβ rearrangement in β chain transgenic thymocytes, where only the transgene-specific Vβ8 rearrangement is abundant. Vβ4, Vβ5, Vβ8, Vβ11, Vβ12 and Vβ16 gene segments (Figure 4B and data not shown) all show reduced rearrangements in pLGF cells, consistent with the extremely low levels of 1.3 kb Vβ-containing transcripts in these thymocytes. Thus, both the genomic blot and PCR quantification data indicate that the lack of functional TCR β chain expression in pLGF mice is correlated with a dramatic reduction in Vβ gene rearrangement.

Also shown in Figure 4B are the rearrangement patterns observed in samples from three animals bearing *lck*-induced thymomas (pLGF1127, lanes 6 and 7; pLGY4220, lane 9). These samples display patterns of Vβ rearrangements that suggest that the tumors are comprised primarily, although not entirely, of cells that are clonal at the level of Dβ−Jβ joining. Specifically, we detect rearrangement of multiple Vβ elements to the same one or two Jβ elements unique to each sample (Vβ8 and Vβ11, Figure 4B; Vβ4 and Vβ16, data not shown). This pattern suggests that the immortalization event mediated by $p56^{lck}$ overexpression follows Dβ rearrangement and that discrete Vβ rearrangements occur subsequently within individual cells.

In as much as thymocytes from *lck* transgenic mice contain near normal levels of α chain transcripts, we utilized the PCR strategy to examine the status of the α locus in these mice. In contrast to the reduction in Vβ recombination, specific Vα to Jα rearrangements in pLGF3073 and 2954 thymocytes were present at levels comparable to those observed in controls. Figure 4C shows the results of a PCR assay utilizing primers specific for the VαF3 family (Chou *et al.*, 1986) and JαpHDS58 (Winoto *et al.*, 1985). Equivalent results were obtained with all other Vα and Jα combinations tested (data not shown), suggesting that overall Vα rearrangement occurs normally in these cells despite the suppression of the β locus. Only tumors derived from the pLGF 1127 (lanes 6 and 7) and pLGY 4220 (lane 9) lines display greatly reduced levels of specific Vα rearrangements, a likely consequence of the pauciclonal nature of these tumors.

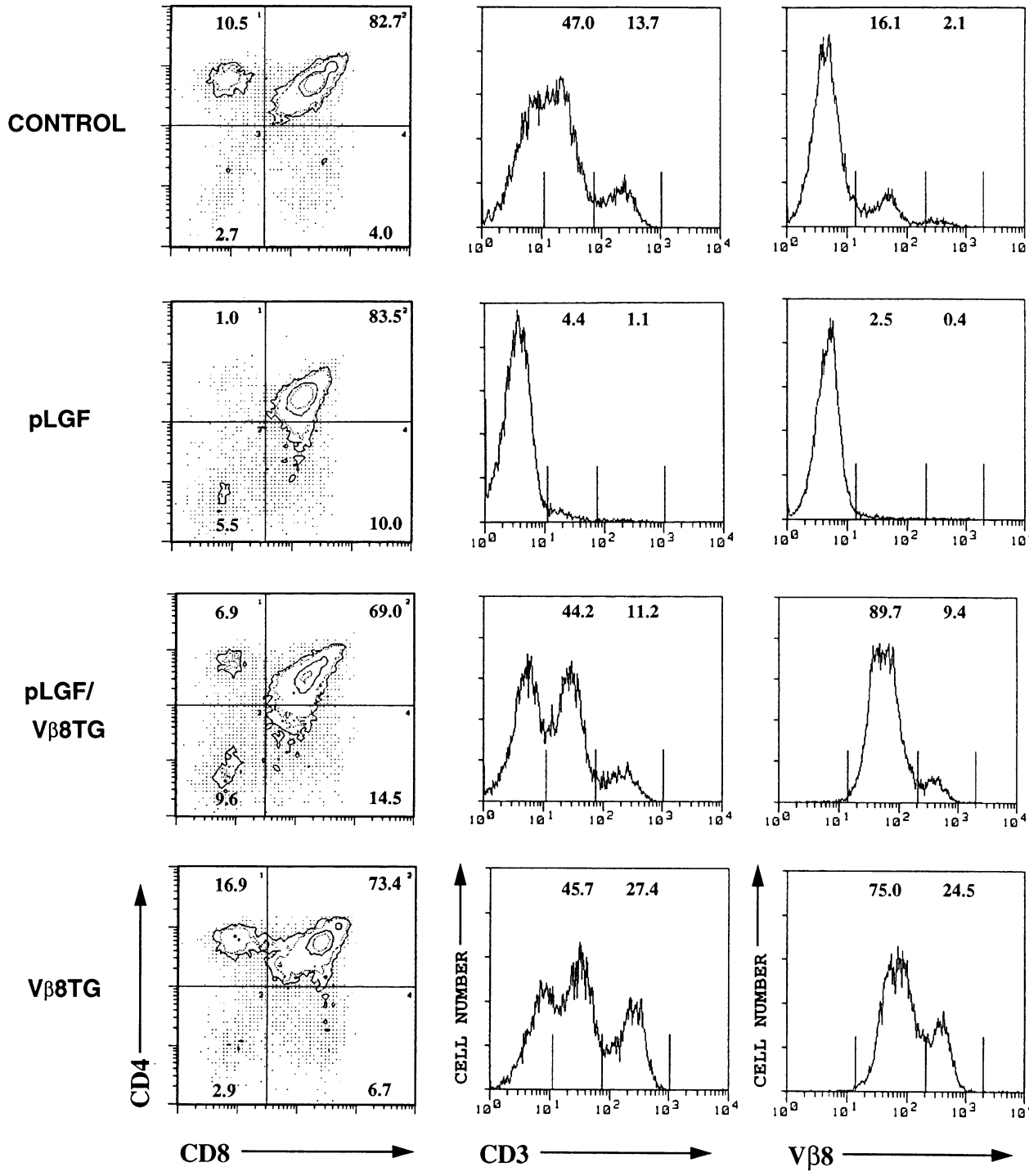

Fig. 6. Flow cytometric analysis of thymocytes from four offspring of pLGF × Vβ8 matings. Fluorescence staining profiles of surface CD4 and CD8 (column 1), CD3 (column 2) and Vβ8 (column 3). Thymocytes were isolated from four littermates that had been identified as non-transgenic control, pLGF2954 transgenic, Vβ8 transgenic or pLGF2954 plus Vβ8 double transgenic. Percentage of cells in each population is indicated.

Efficiency of Vβ rearrangement correlates with levels of germline Vβ transcripts

Although many mechanisms can be postulated to explain the specific interdiction of Vβ to Dβ joining in *lck* transgenic mice, transcription of germline V elements is a potential regulatory step that has previously been correlated with recombination potential for TCR β chain genes (Ferrier *et al.*, 1990). We therefore employed a sensitive PCR assay to measure the relative levels of germline Vβ transcription in normal and pLGF thymocytes. Random-primed cDNA generated from pools (2–4 animals) of total thymocyte RNA was amplified by PCR using primers that distinguish germline from rearranged transcripts. The 5′ primers in each case uniquely specify individual Vβ genes and are designed to bridge the splice junction between the first and second exons. This effectively prevents amplification of genomic DNA present in some RNA samples. Detection of germline transcription utilizes 3′ primers complementary to a region

within or 3′ to the recombination recognition sequences, a region that is deleted upon recombination. Transcripts derrived from rearranged elements were detected by using a primer complementary to Cβ sequences. Both types of products, which differ in size, can be amplified in a single reaction and visualized after gel electrophoresis, blot transfer and hybridization with Vβ-specific oligonucleotide probes. This PCR strategy is presented in Figure 5A, and examples of assays for transcripts containing Dβ2, Vβ5, Vβ8 and Vβ11 gene segments are shown in Figure 5B. The equivalence of cDNA in each sample was demonstrated by amplification of elongation factor-1α transcripts (Figure 5B, bottom panel). The PCR assay, although much more sensitive, yielded results that paralled those of RNA blot analysis of Figure 2, namely that Dβ-containing transcripts were present at high levels in both control and pLGF samples, while the levels of rearranged Vβ-containing transcripts in pLGF thymocytes were consistently lower. In addition, the PCR assay demonstrated that germline Vβ5, Vβ8 and Vβ11 gene segment transcripts were decreased in pLGF cells when compared with control thymocytes. The relative levels of both rearranged and germline Vβ-containing transcripts in pLGF thymocytes appear to follow the transgene dose-dependent pattern (Control > 3073 > 2954 > 1127) previously reported for surface TCR expression (Abraham *et al.*, 1991a).

β chain transgenic animals, which display markedly reduced rearrangement of endogenous Vβ elements, were also analyzed and compared with the pLGF mice. Transcripts derived from the rearranged Vβ8.2 and germline Vβ5 elements included in the transgene were present at higher than control levels. The high level of Vβ5 expression probably reflects both the presence of multiple transgene copies and the unusual transcription pattern of the transgene, where the rearranged Vβ8.2 gene segment utilizes the germline Vβ5.1 promoter (Chou *et al.*, 1987; Uematsu *et al.*, 1988). Nevertheless, transcripts representing both rearranged and germline endogenous Vβ genes were present at levels lower than control (Figure 5B and data not shown). These data indicate that in both *lck* and TCRβ transgenic mice, reduced Vβ rearrangement can be correlated with a decreased level of germline Vβ transcription.

A functional β chain transgene rescues thymocyte development in pLGF mice

The conclusion that the lack of $CD3^+$ thymocytes in pLGF animals results directly from a specific reduction in Vβ rearrangement suggested that this developmental block could be bypassed if a functionally rearranged β chain were present in every cell. This genetic 'complementation' was accomplished by mating pLGF animals with mice carrying a Vβ8.2−Dβ2−Jβ2.3−Cβ2 transgene (Uematsu *et al.*, 1988). Non-transgenic, single transgenic and double transgenic offspring were generated from these crosses and flow cytometric profiles of four littermates (one of seven experiments) are presented in Figure 6. Comparison of the double transgenic to the pLGF2954 littermate reveals several features indicative of improved thymic development. The proportion of CD4 single positive thymocytes has increased from 1−7%, indicating the presence of mature, post-selection cells. This is confirmed in the CD3 profile, which shows that the double transgenic thymocytes contain 44% $CD3^{lo}$ and 11% $CD3^{hi}$ cells. These proportions are similar to those observed in the control animal (47% $CD3^{lo}$, 14% $CD3^{hi}$) and very much greater than those seen in pLGF animals (4% $CD3^{lo}$ and 1% $CD3^{hi}$). The V8TG animal and the double transgenic both display surface expression of the β chain on >98% of thymocytes, although the proportion of $CD3^{hi}$ cells differs by ~2.5-fold. Proliferation assays reveal that the double transgenic thymocytes respond normally to stimulation by Concanavalin A, anti-TCR (anti-CD3ε mAb 145-2C11) and the Vβ8-reactive superantigen staphylococcal enterotoxin B (data not shown). Interestingly, two features of the pLGF phenotype were preserved in the double transgenic animals and are evident from the flow cytometric profiles: an overabundance of $CD8^{lo}$ cells and a decreased mean fluorescence of CD4 and CD8 in the double positive population. Hence some effects of the *lck* transgene are independent of β chain expression. Nevertheless, we conclude that the introduction of a single TCR component, a rearranged β chain, allows mice overexpressing $p56^{lck}$ to develop mature, functional T cells.

Discussion

Assembly of functional antigen receptor repertoires in both T and B cell populations requires developmentally regulated site-specific gene rearrangement events that juxtapose multiple independent gene segments, positioned discontinuously in germline DNA, which when joined together form a functional variable region-encoding exon (Tonegawa, 1983). This rearrangement process yields heterogeneous populations of cells, where each individual cell expresses only a single type of receptor specificity. Moreover, functional gene rearrangements are ordinarily completed on only a single chromosomal homolog, a phenomenon known generally as allelic exclusion.

Recent studies have permitted the identification of some of the molecules that participate in the control of antigen receptor gene rearrangement. In particular, the RAG-1 and RAG-2 gene products are required for antigen receptor recombinase activity and may themselves encode components of the recombinase complex (Schatz and Baltimore, 1988; Schatz *et al.*, 1989; Oettinger *et al.*, 1990). Analysis of RAG-1 and RAG-2 expression reveals that both transcripts disappear rapidly following TCR-mediated stimulation of developing thymocytes (Turka *et al.*, 1991). This result suggests a mechanism whereby assembly of genes encoding functional TCR α and β chains might halt TCR gene rearrangement: the generation of a functional TCR would permit effective signaling, thereby provoking rapid elimination of the RAG-1 and RAG-2-encoded proteins required for recombinase activity. However, allelic exclusion at the β chain locus in T cells, similar to that observed at the H chain locus in B cells, is unlikely to result from alterations in RAG-1 and RAG-2 expression, since both are required for subsequent rearrangement of the α chain (or in B cells the L chain) genes. Instead, a more selective mechanism must act to specifically compromise rearrangement of one type of TCR gene component while permitting other TCR gene rearrangements to take place. Our studies make plain that $p56^{lck}$, when overexpressed, can activate a process that behaves in just this fashion. Indeed, thymocytes from pLGF transgenic mice exhibit a single prominent defect: the failure of Vβ gene segments to serve as satisfactory targets for recombinase activity.

Feedback regulation of β chain gene rearrangement

In general, antigen receptor gene rearrangement events are developmentally ordered in both the T and B cell lineages. Thus, although a single recombinase acting on a common set of recognition signals seems always to mediate antigen receptor gene rearrangement (Yancopoulos *et al.*, 1986; Hesse *et al.*, 1989), during thymocyte development the β chain gene segments undergo rearrangement before those encoding the α chain (Raulet *et al.*, 1985; Snodgrass *et al.*, 1985b). The mechanism whereby nearly identical recombinase templates become selectively activated or repressed remains enigmatic; however, considerable evidence supports the view that the synthesis of a complete β chain protein in T cells, or of H chain in B cells, blocks subsequent rearrangements at these loci. In mice bearing a complete H chain-encoding transgene (Storb *et al.*, 1986; Nussenzweig *et al.*, 1987), functional joining of V_H gene segments to D_H-J_H units appears to block subsequent heavy chain rearrangements. Similarly, thymocytes from mice bearing a functionally rearranged TCR β chain transgene manifest a profound disturbance in the rearrangement of endogenous Vβ gene segments (Figures 3 and 4; Uematsu *et al.*, 1988) associated with nearly uniform surface expression of the Vβ transgene. This phenomenon has been widely interpreted to provide evidence supporting the regulation of allelic exclusion via a feedback mechanism. According to this model, functional TCR β chains must interact with some other cellular components to provide a signal that halts subsequent Vβ gene rearrangements. Simple selection of cells bearing a transgene-encoded TCR β chain cannot explain the failure to observe endogenous Vβ gene rearrangements in β chain transgenic animals, since a truncated β transgene encoding a protein that lacks Vβ sequences also blocks endogenous β locus rearrangements effectively (Krimpenfort *et al.*, 1989). Both full-length and truncated β chain transgene products permit normal Dβ and α chain gene rearrangements. Hence any satisfactory model for the control of TCR gene rearrangement must accommodate two observations: Vβ rearrangement can be regulated selectively, independent of the efficiency with which Dβ−Jβ joining occurs, and the β chain protein, specifically the constant region of the β chain, provides the necessary regulatory information to suppress subsequent Vβ recombination. Participation of the β chain in such feedback inhibition implies that this protein is capable of forming a signaling complex, which thereafter alters the efficiency of recombinase interaction with specific targets in the nucleus.

p56^{lck} can impinge upon mechanisms regulating TCR rearrangement

The pLGF and β chain transgenic mice share several characteristics with respect to endogenous TCR gene rearrangements. First, Dβ rearrangements occur in both systems at normal levels, yielding the 1.0 kb transcript characteristic of such events. This suggests that p56^{lck} overexpression, similar to the TCR β product, alters thymocyte development at a point prior to Vβ rearrangement but subsequent to Dβ−Jβ joining. The apparent timing of p56^{lck}-induced transformation in thymocytes from mouse strains expressing very high levels of the kinase supports this conclusion. Secondly, Vα rearrangements proceed normally in both pLGF and β chain transgenic mice, in the former case generating intracellular monomers and in the latter case giving rise to cell surface αβ heterodimers. Finally, the lack of Vβ rearrangement is accompanied by a reduction in the appearance of transcripts derived from unrearranged Vβ gene segments. Together these observations suggest a mechanism whereby p56^{lck} may selectively regulate the rearrangement of Vβ gene segments by reducing the activity of nuclear factors that regulate Vβ transcription (Anderson *et al.*, 1989). If transcription of unrearranged Vβ gene segments is required to permit recombinase access, the observed selective decrease in Vβ−Dβ joining is understandable.

Although reduced levels of germline Vβ transcripts appear in both pLGF and Vβ transgenic thymocytes, there is no obvious defect in expression of a functionally rearranged β chain-encoding transgene in either case. Presumably this result reflects the overwhelming influence of enhancer elements located 3′ to Cβ that act to improve initiation at the Vβ promoter following rearrangement (Krimpenfort *et al.*, 1988; Gottschalk and Leiden, 1990; Takeda *et al.*, 1990). Viewed in this way, p56^{lck} may exert its regulatory effect on the β locus by reducing the activity of Vβ-specific transcriptional control elements, presumably through one or more intermediary molecules that convey information from the cytoplasm to the nucleus. Alternatively, the reduction in germline Vβ transcript abundance observed in thymocytes from *lck* transgenic mice may reflect a process that acts independently, perhaps at locus control sequences, to block β locus gene rearrangement.

Does p56^{lck} ordinarily regulate β chain rearrangements?

Our data support the view that when p56^{lck} is overexpressed it can activate regulatory machinery that specifically suppresses Vβ, but not Dβ−Jβ, gene segment recombination. These results provide a satisfying explanation for the block in thymocyte development observed in *lck* transgenic animals. Moreover, phenotypic rescue of the *lck*-induced developmental defect by a functionally rearranged β chain gene provides precise information regarding the requirement for TCR gene expression in T cell development. Nevertheless, our data leave the question of whether p56^{lck} ordinarily participates in the control of TCR gene rearrangements unaddressed. Previous studies demonstrate that *lck* expression commences at or before the time when hematopoietic progenitors first colonize the thymic anlage and *lck* transcripts appear in all thymocyte subsets (Perlmutter *et al.*, 1988; Reynolds *et al.*, 1990; Wildin *et al.*, 1991). Moreover, very subtle variations in p56^{lck} abundance substantially compromise thymocyte development, as even a 2-fold increase in the level of *lck* transcripts provokes dramatic suppression of Vβ gene segment recombination. This effect is independent of the well-known interaction of p56^{lck} with the CD4 and CD8 coreceptors, since mutant *lck* transgenes, encoding a variant protein that cannot bind to CD4 or CD8, are also capable of blocking Vβ rearrangements (S.D.Levin, K.M.Abraham, K.A.Forbush and R.M.Perlmutter, manuscript in preparation). The extraordinary sensitivity of Vβ recombination to p56^{lck} abundance strongly suggests that this protein participates in a signaling pathway that acts to promote thymocyte maturation by sensing synthesis of a β chain protein. The formation and detection of a functional β chain may be a first critical step in T cell development, since thymocytes

in mice defective in somatic gene recombination fail to develop beyond the $CD4^-8^-$ stage (Bosma and Carroll, 1991; Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992). Indeed, this block is partially relieved in SCID mice by the presence of a transgenic TCR β chain (Kishi *et al.*, 1991). Interestingly, analysis of mice deficient in $p56^{lck}$ points to a critical role for this signaling molecule in the development of $CD4^+8^+$ thymocytes (Molina *et al.*, 1992). The demonstration that transgenic β chains, in conjunction with CD3 components, can appear on the cell surface of immature $CD4^-8^-$ thymocytes (Kishi *et al.*, 1991; Groettrup *et al.*, 1992) provides a framework for envisioning a mechanism by which the presence of a functional β chain can be coupled to nuclear changes: the β chains could participate in complexes that utilize $p56^{lck}$ to initiate a cascade of intracellular signals which both promotes thymocyte development and affects the recombinational competence of the TCR β locus. We cannot, however, exclude the possibility that transgene-encoded $p56^{lck}$, though active in selectively blocking Vβ gene segment rearrangement, simply mimics the effects of a distinct regulatory apparatus. In either case, the data reported here provide compelling independent support for the view that allelic exclusion at the β locus is an active process, controlled by an *lck*-sensitive pathway that acts specifically at the Vβ−Dβ recombination step. Elucidation of the mechanism that permits $p56^{lck}$ to block Vβ rearrangement should illuminate underlying features of the signaling processes that order TCR gene recombination and control thymocyte development.

Materials and methods

Transgenic mouse lines

The construction of the $p56^{lck}$ transgenes and the generation of transgenic mouse lines (pLGF 3082, 3073, 2954 and 1127; pLGY 4220) have been described previously by Abraham *et al.* (1991a,b). Expression of wild type $p56^{lckY505}$ (pLGY) or mutant $p56^{lckF505}$ (pLGF) is directed by the thymus-specific *lck* proximal promoter. Mice transgenic for a functionally rearranged Vβ8.2 gene have been described previously by Uematsu *et al.* (1988) and breeding pairs were kindly provided by Dr Michael Bevan. All transgenic lines were maintained by breeding transgene-positive males to C57BL/6 female mice.

Flow cytometric analysis

Single-cell suspensions were obtained by teasing from freshly removed thymuses and were stained with antibodies as previously described by Garvin *et al.* (1990). Cells were stained for surface expression of CD4, CD8, CD3 and Vβ8 using phycoerythin-conjugated GK1.5 (Becton-Dickinson, Mountain View, CA) fluorescein-conjugated 53.6.71 (Becton-Dickinson), biotinylated 500A2 (Havran *et al.*, 1987) and unconjugated F23.1 (Staerz *et al.*, 1985), respectively. Detection of biotinylated and unconjugated antibodies was facilitated using phycoerythrin-conjugated streptavidin (Caltag Labs, San Francisco, CA) and fluorescein-conjugated goat anti-mouse Ig (Becton-Dickinson), respectively. Each analysis included 10 000 events collected in list mode on a FACSCAN flow cytometer (Becton-Dickinson) and analyzed using FACSTAR Consort 30 software.

RNA and DNA blot analysis

For Northern blot analysis of TCR expression, total RNA was isolated from mouse thymuses as described by Cathala *et al.* (1983); 10 μg was resolved on a 1.5% agarose−formaldehyde gel, transferred, hybridized and washed according to standard procedures (Sambrook *et al.*, 1989). For Southern blot analysis of β chain rearrangement, high molecular weight thymocyte DNA (10 μg) was digested to completion with *Hin*dIII, separated on a 0.8% agarose gel, transferred, hybridized and washed according to standard procedures (Sambrook *et al.*, 1989). Plasmid inserts specific for Cβ (Hedrick *et al.*, 1984) or Cα (Fink *et al.*, 1986), gifts of Dr Pamela Fink, were labeled to high specific activity by random priming (Feinberg and Vogelstein, 1983).

Immunoblotting

NP-40 lysates of purified $CD4^+8^+$ thymocytes from control or pLGF mice were applied to a 12.5% SDS−polyacrylamide gel under non-reducing conditions and then subjected to electrotransfer, immunoblotting with anti-TCRα (mAB H28-710) (Becker *et al.*, 1989) and anti-CD3ε (mAB HMT3-1) (Born *et al.*, 1987), and visualization with the use of ^{125}I-labeled protein A as described by Nakayama *et al.* (1990).

Metabolic labeling, immunoprecipitation and gel electrophoresis

Purified $CD4^+8^+$ thymocytes from control or pLGF mice were preincubated in methionine-free RPMI with 10% fetal calf serum at 37°C for 15 min and the cells labeled for 60 min at 37°C at 2×10^7/ml with 0.5 mCi/ml [^{35}S]methionine (Tran^{35}S-label, ICN Radiochemical, Irvine, CA). After washing, the labeled cells were lysed with 1.0% digitonin (Nakayama *et al.*, 1990) and intact TCR−CD3 complexes were immunoprecipitated with anti-CD3ε (mAb 145-2C11) (Leo *et al.*, 1987) that had been adsorbed to protein A−Sepharose (Pharmacia LKB, Uppsala, Sweden). The precipitates were analyzed by two-dimensional non-reducing/reducing SDS−PAGE.

Detection of rearrangement by PCR

We performed PCR-based analysis of β chain rearrangement using an assay modified from that described by van Meerwijk *et al.* (1990). High molecular weight thymocyte DNA was extracted (1× phenol, 2× phenol:chloroform, 1:1), ethanol-precipitated and resuspended in TE [10 mM Tris−HCl (pH 7.5), 1 mM EDTA] prior to quantification and use. PCR amplifications were performed in 50 μl reaction buffer [50 mM KCl, 20 mM Tris−HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml BSA] containing 0.5 μg template DNA, 1 mM 5′ and 3′ primers, 0.2 mM each dNTP (Pharmacia) and 1 U AmpliTaq DNA polymerase (Perkin−Elmer Cetus). The amplification cycle (1 min at 95°C, 2 min at 63°C and 10 min at 72°C) was repeated 24 times. 12.5 μl of the PCR reaction was fractionated on a 1.8% agarose gel, blot transferred and the filters hybridized with a Jβ2 specific probe (a 263 bp fragment originating from PCR amplification of the region immediately 5′ of the *Cla*I site which is 3′ of Jβ2.6), which is completely upstream of the 3′ amplification primer.

Proportionality of the assay was determined by analysing the products generated by amplification of Vβ8 or Vβ11 rearrangements from serial dilutions of control thymocyte DNA. The total amount of DNA in each reaction was kept constant by the addition of embryonic stem cell DNA. A portion of each reaction was dotted onto a nitrocellulose filter, hybridized with a Jβ2-specific oligonucleotide probe and quantified by either scintillation counting or analysis with a Molecular Dynamics PhosphorImager. The PCR generated products which varied in a linear fashion as template DNA was varied from 1.0−0.025 μg, thereby allowing quantitative comparison of rearrangement products over a >10-fold range.

Vα to Jα rearrangements were detected using the same PCR strategy, utilizing 5′ primers specific for Vα elements and 3′ primers specific for individual Jα segments. The amplification cycle (1.5 min at 95°C, 2 min at 63°C and 3.5 min at 72°C) was repeated 24 times and products were detected with Jα-specific oligonucleotide probes located upstream of the 3′ PCR primer.

Analysis of rearranged and germline β chain transcripts by PCR

An RNA PCR assay was modified from the procedures described by Schlissel and Baltimore (1989). cDNA was generated from 5 μg total thymocyte RNA in a 20 μl reverse transcription reaction containing 50 mM Tris−HCl (pH 8.3), 5 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 1 mM each dNTP (Pharmacia), 1.0 μl RNasin (Boehringer Mannheim), 5 mM random hexanucleotide primers (Boehringer Mannheim) and 20 U AMV reverse transcriptase (Life Sciences). The reaction was incubated at 41°C for 2 h, diluted to 100 μl with DEPC-treated water and stored at −20°C. Aliquots of this cDNA were further diluted 1:20 and 5 μl was used in a 50 μl PCR reaction containing 1× reaction buffer (see above), 0.5 mM 5′ and 3′ primers, 0.2 mM each dNTP (Pharmacia) and 1 U AmpliTaq DNA polymerase (Perkin−Elmer Cetus). The amplification cycle (1 min at 95°C, 2 min at 58°C and 3 min at 72°C) was repeated 28 times. 12.5 μl of the PCR reaction was fractionated on a 1.8% agarose gel, blot transferred and the filters were hybridized at 60°C with ^{32}P end-labeled oligonucleotide probes. eF-1α PCR products were detected using a random-primed plasmid insert.

Oligonucleotide primers

The following oligonucleotides were used for PCR and hybridization: 5′ PCR primers and probes:

Dβ2 5′:	5′ GTAGGCACCTGTGGGGAAGAAACT 3′
Vβ5 LV:	5′ CTCCTGGGAACAAGTTCAGCAA 3′
Vβ5 V:	5′ CCCAGCAGATTCTCAGTCCAACAG 3′
Vβ8 LV:	5′ CTCCTGIGIRCAAAACACATG 3′
Vβ8 V:	5′ GCATGGGCTGAGGCTGATCCATTA 3′
Vβ11 LV:	5′ GCTTCTTGAGAGCAGAACCAACA 3′
Vβ11 V:	5′ TGCTGGTGTCATCCAAACACCTAG 3′
VαF3 V:	5′ ACCCAGACAGAAGGCCTGGTCACT 3′
eF-1α A:	5′ CTGCTGAGATGGGAAAGGGCT

3′ PCR primers and probes:

Vβ5 3′:	5′ GATTAAGTTACAGAAAGCCAGTAGC 3′
Vβ8 3′:	5′ GTTCTCCTTATTTCCTTTCTGTGCAGAG 3′
Vβ11 3′:	5′ GGAAGCGTATGGTTTCTACCTCAG 3′
Jβ2 3′:	5′ TGAGAGCTGTCTCCTACTATCGATT 3′
Cβ2 A:	5′ ATTCACCCACCAGCTCAGCTCCACGTG 3′
Cβ2 B:	5′ GCTATAATTGCTCTCCTTGATGGCCTG 3′
Jα pHDS58 A:	5′ GTGCCAGATCCAAATGTCAGCGCA 3′
Jα pHDS58 B:	5′ GAAGACACACTTACATGGTAGACATGG 3′
eF-1α B:	5′ TTCAGGATAATCACCTGAGCA 3′

Vβ V oligonucleotides were used as 5′ primers in DNA PCR and were also end-labeled for use as hybridization probes to detect RNA PCR products. These primers are complementary to sequences 3′ to the Vβ LV splice primers and are therefore specific for correct PCR products. Cβ2 B and Jα pHDS58 B were used for RNA and DNA PCR, respectively, while the more 5′ Cβ2 A and Jα pHDS58 A were used only as probes. Inosines (I) and a mixture of A + G (R) were incorporated in the Vβ8 LV splice oligomer to maximize homology to both Vβ5 and Vβ8 leader exons because of the unusual splicing pattern of Vβ8.2 (Chou *et al.*, 1987) and to allow detection of all Vβ8 genes.

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