Reconstitution of the Subclass-specific Expression of CD4 in Thymocytes and Peripheral T Cells of Transgenic Mice: Identification of a Human CD4 Enhancer

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Summary

During thymic maturation, CD4$^-$CD8$^-$TCR$^-$ immature thymocytes differentiate through a CD4$^+$CD8$^+$TCR^{lo} intermediate into two functionally distinct mature T cell subsets: helper T cells expressing CD4 and a major histocompatibility complex (MHC) class II–restricted T cell receptor (TCR), and cytotoxic T cells expressing CD8 and an MHC class I–restricted TCR. The mutually exclusive expression of CD4 and CD8 is maintained in the periphery during expansion of these mature T cell subsets. To elucidate the mechanisms controlling CD4 and CD8 expression on differentiating thymocytes and mature peripheral T cells, we have examined the expression of human CD4 gene constructs in the lymphoid tissues of transgenic mice. Our analyses demonstrate that sequences contained within or closely linked to the human CD4 gene are sufficient to reconstitute the appropriate regulation of human CD4 expression on all thymocyte and mature peripheral T cell subsets. Specifically, appropriate developmental regulation was dependent on two sets of sequences, one contained within a 1.3-kb restriction fragment located 6.5 kb upstream of the human CD4 gene, and the other present within or immediately flanking the gene. Nucleotide sequence analysis identified the 1.3-kb restriction fragment as the likely human homologue of an enhancer found 13 kb upstream of the mouse CD4 transcription initiation site. The human CD4 transgenic mice provide a useful system for the identification and characterization of additional sequence elements that participate in human CD4 gene regulation and for the elucidation of regulatory mechanisms governing the developmental program mediating the maturation of the CD4$^+$ and CD8$^+$ peripheral T cell subsets.

Mature CD4$^+$CD8$^-$ class II–restricted and CD4$^-$CD8$^+$ class I–restricted lymphocytes differentiate intrathymically from CD4$^-$CD8$^-$TCR$^-$ cells that originate from fetal liver or bone marrow precursors (1). The major intermediate in the T cell differentiation pathway is the double-positive thymocyte, which expresses both CD4 and CD8 and low levels of the α/β TCR. Antibody blocking experiments (2, 3), as well as more recent studies in gene knockout mice (4, 5), have demonstrated that CD4 and CD8 are essential for the differentiation of double-positive thymocytes into mature class II– and class I–restricted T cells, respectively. On mature T cells, CD4 and CD8 participate in Ag recognition and T cell activation by binding to monomorphic determinants on MHC class II and class I proteins, respectively (6–12). Coengagement of an Ag–MHC complex by an α/β TCR and either CD4 or CD8 is believed to be a critical component of the T cell activation pathway (13–17). The lymphocyte-specific tyrosine kinase, p56^{lck}, is physically associated with the cytoplasmic domains of both CD4 and CD8 and can be activated by antibody crosslinking of either of these glycoproteins (18–21). Thus, coengagement of Ag–MHC by the TCR and CD4 or CD8 may trigger T cell activation by transducing transmembrane signals via p56^{lck} (22, 23). By binding MHC, CD4 and CD8 may also contribute to T cell activation as adhesion molecules that increase the avidity of T cell/target cell interactions (24–26). On maturing thymocytes, as on peripheral T cells, the functions of CD4 and CD8 are mediated through their binding to MHC proteins, either as coreceptors and/or as adhesion molecules (27–29).

The pattern of CD4 and CD8 expression during thymocyte development is quite complex. In the mouse, CD4 is expressed at low levels on multipotential hemopoietic stem cells in bone marrow (30) and on the earliest detectable thymic precursor cells (31), which subsequently differentiate into

double-negative CD4⁻CD8⁻ thymic precursor T cells. CD4 surface expression is later regained at the double-positive stage, when CD4 is coexpressed with CD8. Included within this double-positive subset are thymocytes that have productively rearranged and assembled α/β TCRs on the cell surface. Upon positive selection, CD4⁺CD8⁺ cells differentiate into CD4⁺ CD8⁻ and CD4⁻CD8⁺ single-positive thymocytes that express, respectively, class II- and class I–restricted TCRs (reviewed in reference 32). Thus, the maturation of a CD4⁺ CD8⁺ double-positive thymocyte into a class II–restricted T cell involves the loss of CD8 cell surface expression, whereas the maturation of a class I–restricted T cell involves the loss of CD4 cell surface expression. The loss of CD4 or CD8 expression during T cell selection is accompanied by the differentiation of double-positive cells into mature thymocytes, which exhibit increased surface levels of TCR and cease TCR α chain gene rearrangement (33, 34). The mutually exclusive loss of CD4 or CD8 coreceptor expression is also associated with the commitment of thymic precursors to either the mature cytotoxic or helper T cell phenotype. Presumably, the developmental decision to mature and to commit to a functional T cell phenotype involves changes in gene expression. Therefore, the study of CD4 and CD8 gene regulation during thymocyte development should enhance our general understanding of the mechanisms governing the apparent reprogramming of gene activity during the differentiation of the mature T cell subclasses.

The strict correlation between TCR specificity and CD4/ CD8 coreceptor expression indicates that the regulation of CD4 and CD8 expression must be inextricably tied to the T cell selection process. Although it is known from studies on TCR transgenic mice that the specificity of an α/β TCR for MHC class I or class II molecules determines the CD4⁻ CD8⁺/CD4⁺CD8⁻ phenotype of a mature T cell (35–38), the mechanism by which TCR specificity dictates the CD4 or CD8 phenotype has yet to be elucidated. Two models have been formulated to describe the regulation of CD4 and CD8 expression during the selection of mature thymocytes: instruction and stochastic/selection (39–41). In the most widely discussed form of the instruction model the CD4 and CD8 genes are continuously transcribed in the CD4⁺CD8⁺ cell, and coengagement of MHC by the TCR and CD4 or CD8 sends a signal to turn off either CD8 or CD4 gene transcription, respectively. In the stochastic/selection model, on the other hand, the CD4 or CD8 genes are transcriptionally silenced by a mechanism that acts independently of the MHC class specificity of the TCR, and the process of positive selection chooses those cells with the appropriate combination of CD4/CD8 and class I/II–restricted TCR for further differentiation. Studies on CD8 transgenic mice are consistent with the predictions of the instruction model (42–44). However, on the basis of analyses of neonatal thymocytes in class I–restricted TCR transgenic mice (45) and of T cell subsets in class II knockout mice (46, 47), other investigators have argued for the existence of a stochastic component to the appearance of the single-positive subsets. Northern analyses of CD4⁺CD8⁻ and CD4⁻CD8⁺ PBL indicate that the expression of the CD4 and CD8 genes is transcriptionally

controlled in mature T cells (48, 49), and both the instruction and stochastic/selection models make the assumption that the loss of CD4 or CD8 expression during thymocyte maturation is also mediated at the transcriptional level. However, little data exist on the transcriptional states of the CD4 and CD8 genes during T cell development in the thymus.

As a first step toward elucidating the mechanisms controlling CD4 and CD8 expression during the selection of mature thymocytes, we have examined the expression of three different human CD4 gene constructs in the lymphoid tissues of transgenic mice. One construct expresses the human CD4 transgene preferentially on mouse CD4⁺ T cells, including the CD4⁺CD8⁺ and CD4⁺CD8⁻ thymic and the CD4⁺ CD8⁻ splenic T cell subsets. Comparison of the three transgenes shows that the developmentally appropriate expression of a human CD4 transgene is dependent on a T cell–specific enhancer located 6.5 kb upstream of the CD4 gene and on sequences within or immediately flanking the gene. These findings demonstrate that the developmental reprogramming of human CD4 expression during thymocyte selection is mediated by sequences closely linked to the CD4 gene. Thus, the human CD4 transgenic mouse system described in these studies can be used to further dissect the regulatory mechanisms governing CD4 gene expression during thymocyte maturation.

Materials and Methods

Human CD4 Transgene Constructs. Two human CD4 genomic clones, λhg1 and λhg6, were kindly provided by D. R. Littman (University of California, San Francisco) for the construction of CD4-1. The insert of λhg1 begins at a HindIII site 3.5 kb upstream of the transcriptional initiation site and ends within the first 1 kb of the third intron, and the insert in λhg6 begins within the third intron, ~2 kb upstream of exon 4, and ends 9.5 kb downstream of exon 10. The inserts from these λ clones were recloned into pBluescript I (Stratagene, La Jolla, CA) to generate the plasmids phg1KS5 and phg6SK9. The phg1KS5 plasmid was partially digested with EcoRI, which cuts at either end of the insert. Linear 20-kb molecules (17-kb insert plus 3-kb vector) were then separated from form II DNA, and the 17-kb insert was separated by pulsed field gel electrophoresis (PFGE). The linear fragment was isolated by electroelution and then dephosphorylated by calf intestinal phosphatase (CIP). The EcoRI insert of phg6SK9 (18 kb) was purified from the pBluescript vector by gel electrophoresis and electroelution and then ligated to linearized phg1KS5 at 10 μg/ml. All transformants were screened by colony hybridization (50) to identify those colonies containing hg6 inserts. Miniprep DNA was prepared from those colonies hybridizing to an hg6-specific probe and analyzed by digestion with HindIII and BamHI to identify clones in the correct orientation. This yielded CD4-1 (51).

The 1.3-kb SphI-ClaI restriction fragment was obtained from a cosmid clone isolated from a pWE15 human placental DNA cosmid library kindly provided by G. A. Evans (Salk Institute). Three overlapping human CD4 cosmid clones were identified after a screen of 4.5 × 10⁵ clones with a radiolabeled 0.5-kb EcoRI-SacI fragment of the human CD4 promoter (51). The 1.3-kb SphI-ClaI fragment was isolated from one of these clones, gel purified, ligated to SalI linkers (New England Biolabs, Inc., Beverly, MA), and then subcloned into the SalI site of pBluescript II (Stratagene), creating pBluescript 1.3 SalI.

The 1.3 CD4-1 transgene construct was generated by ligation of the gel-purified 1.3-kb SalI-SalI restriction fragment from pBluescript 1.3 SalI to the SalI-linearized pBluescript CD4-1 construct.

The 1.3 mouse CD3–human CD4 cDNA/genomic (1.3 mCD3-hCD4c/g) transgene construct was prepared by ligating the 1.3-kb SphI-ClaI restriction fragment to a human CD4 minigene. This minigene was obtained from pNEZT4 (described in D. Grass, A. Garvin, D. Littman, N. Lee, and E. Lacy, manuscript in preparation) and consists of an EcoRI-SacI cDNA fragment encoding exons 2–4 and part of exon 5 fused to a genomic SacI-BamHI fragment encoding the remainder of exons 5, 6–9, and part of 10 (49). The transcriptional initiation and termination signals of the pNEZT4-encoded minigene are provided, respectively, by a 0.5-kb BclI-BclI fragment from the mouse CD3-δ promoter (52) and a 0.24-kb BclI-BamHI fragment containing the SV40 poly(A) site. To prepare 1.3 mCD3-hCD4c/g, pNEZT4 was digested with XhoI and then incubated with Klenow fragment of DNA polymerase I to generate blunt ends. This linear plasmid was then digested with NotI to release a fragment containing the mCD3-hCD4-SV40 sequences. A second 1.3 subclone (pBluescript 1.3 RV) was prepared that contains the Klenow fragment–treated SphI-ClaI fragment inserted into the EcoRV site of pBluescript II. The pBluescript 1.3 RV plasmid was digested with EcoRI, blunted by treatment with Klenow fragment, and then digested with NotI. The final 1.3 mCD3-hCD4c/g construct was prepared by ligating the XhoI-NotI fragment from pNEZT4 to the EcoRI-NotI fragment from pBluescript 1.3 RV.

Preparation of DNA Fragments for Microinjection. Large-scale plasmid preparations of transgene constructs were performed according to Sambrook et al. (53). All three constructs were digested with XhoI and NotI to release the inserts from vector sequences. Digests were fractionated by electrophoresis through 0.7% (CD4-1 and 1.3 CD4-1) or 1.0% (1.3 mCD3-hCD4c/g) agarose gels. The inserts were isolated by one of three methods: (*a*) electrophoresis onto DEAE membranes (Schleicher & Schuell, Inc., Keene, NH) and purification according to the instructions provided by the manufacturer, followed by CsCl density gradient centrifugation (54) and ethanol precipitation; (*b*) by excision of a gel slice containing the insert and electrophoresis of the DNA fragment into 0.5× Tris-borate-EDTA buffer (53) contained within dialysis tubing, followed by CsCl density gradient centrifugation and ethanol precipitation; or (*c*) by PFGE, excision of the gel slice, and electroelution of the insert, followed by phenol extraction and two rounds of ethanol precipitation (51). DNA pellets were washed in 70% ETOH and then resuspended in deionized H_2O and diluted to a concentration of 2–8 μg/ml with 1× injection buffer (54).

Transgenic Mice. The human CD4 transgene constructs were microinjected into fertilized eggs from (C57Bl/6 × CBA/CA)F$_1$ matings and the injected eggs were transferred to pseudopregnant female CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA) for development to term. Transgenic founders were identified by Southern blot hybridization of HindIII-digested tail DNAs to a radiolabeled human CD4 cDNA.

Northern Analysis. Total RNAs from tissues and purified cells of CD4-1 and 1.3 CD4-1 transgenic mice were isolated by the acid-guanidinium thiocyanate-phenol-chloroform extraction method (55). Thymocyte and splenocyte suspensions were prepared by grinding whole thymus and spleen between two frosted glass slides in cold PBS, 1% BSA, and 0.1% sodium azide. Suspensions were filtered through Nitex (Tetko, Inc., Elmsford, NY). RBC were lysed in 0.15% NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA. The cells were washed twice in PBS, 1% BSA, and 0.1% sodium azide,

then resuspended in acid-guanidinium thiocyanate solution, and disrupted by expulsion through an 18-gauge needle. B cells were depleted from splenocyte suspensions using nylon wool columns (Polysciences Inc., Warrington, PA; 56). The efficiency of the depletions was determined by FACS® analysis (Becton Dickinson & Co.) of aliquots of B cell–depleted splenocytes (see below). Depletions removed 93–97% of all B220$^+$ splenocytes. 15 μg of total RNA was subjected to electrophoresis through 1% agarose-formaldehyde gels and blotted onto GeneScreen nylon membranes (New England Nuclear, Boston, MA). Blots were hybridized to either a radiolabeled human CD4 cDNA lacking sequences encoding the highly conserved cytoplasmic domain, or a radiolabeled 143-bp PstI-PstI restriction fragment derived from the 3' untranslated portion of the mouse CD4 cDNA (57). Prehybridizations (0.5–1 h) and hybridizations (16–24 h) were performed at 55°C in 0.5× nylon wash, 30% formamide, and 1% BSA. (1× nylon wash consists of 14% SDS, 80 mM Na$_2$HPO$_4$·7H$_2$O, and 14 mM Na$_4$EDTA·2H$_2$O, pH 7.2; 58). Washes were performed twice, for 20 min each, at 65°C in 0.4× nylon wash, 0.2× nylon wash, and 0.1× nylon wash. Normalization of RNA amounts in each lane was achieved by hybridizing Northern blots with an oligonucleotide specific for 28S rRNA (59).

FACS® Analysis. Thymocyte and splenocyte suspensions were prepared as described above. Aliquots of 10^6 cells were stained for 25 min at 25°C with combinations of the following mAbs: PE-anti–mouse B220 (Pharmingen, San Diego, CA), FITC-anti–mouse CD8, PE-anti–mouse CD4, FITC-anti–human CD4, and biotin-anti–human CD4 (Becton Dickinson & Co.). In cases where biotin-conjugated antibodies were used, stained cells were washed twice in PBS, 1% BSA, 0.1% sodium azide and then stained with Streptavidin-RED613 (Gibco-BRL, Gaithersburg, MD). After incubation with antibodies and Streptavidin reagents, cells were then washed once in PBS, 0.1% sodium azide and fixed in PBS, 0.1% sodium azide, and 1% formaldehyde. Fluorescence intensity was measured on a FACScan® flow cytometer using FACScan® research software (Becton Dickinson & Co.) and plotted on a logarithmic scale. Between 15,000 and 50,000 cells were analyzed per plot. Dead cells were eliminated from the analysis on the basis of forward and sideways light scatter.

Nucleotide Sequence Analysis. The sequence of the 1.3-kb SphI-ClaI restriction fragment was determined by dideoxy sequence analysis using reagents provided in the sequence kit (U.S. Biochemical Corp., Cleveland, OH) according to instructions provided by the manufacturer. SK and KS, primers that anneal to the polylinker of pBluescript II plasmid vectors, were purchased from Stratagene. Additional internal primers were prepared by the Memorial Sloan-Kettering Cancer Center Microchemistry Core Facility. Sequence comparisons were performed using Microgenie Sequence Software (Beckman Instruments, Inc., Palo Alto, CA; 60).

Results

Design of Human CD4 Transgene Constructs. The developmental cues regulating CD4 gene expression are likely to occur exclusively in thymocytes maturing in the presence of thymic stromal cells. Therefore, elucidation of the mechanisms required for the establishment of the complex pattern of CD4 expression during thymocyte development may necessitate the use of an in vivo system that supports all stages of T cell differentiation. Consequently, we chose to define the *cis*-acting DNA sequence elements regulating the developmental

stage and cell type–specific expression of CD4 in transgenic mice. We have used the human, rather than the mouse, CD4 gene for these studies, because the human gene and its mRNA and protein products can be readily distinguished from those of the mouse.

Three human CD4 transgene constructs were prepared and tested for expression in transgenic mice. Restriction maps for two of these transgenes, CD4-1 and 1.3 CD4-1, are shown in Fig. 1 *A*. CD4-1 was constructed by ligating together the inserts from two genomic λ clones. The 5′ λ clone begins at a HindIII site 3.5 kb upstream of the transcriptional initiation site and ends within the first 1 kb of the third intron. The 3′ λ clone begins within the third intron, ∼2 kb upstream of exon 4, and ends 9.5 kb downstream of exon 10. This ligation deleted 18 kb of sequence from the third intron, but left the other 8 introns and 10 exons intact. Thus all human CD4 protein coding sequences are present within CD4-1 and the total length of CD4-1 is 35 kb. The second transgene construct, 1.3 CD4-1, includes, in addition to the

CD4-1 sequences, a 1.3-kb SphI-ClaI restriction fragment normally located 6.5 kb upstream of the transcriptional initiation site of the human CD4 gene. This fragment contains a T cell–specific DNase I hypersensitive site, designated HS4 (51). DNase I hypersensitive site mapping experiments revealed that HS4 was present in chromatin from a number of human CD4+ T cell lines, including Jurkat and CEM, as well as from human PBL depleted of CD8+ cells. HS4 was absent in chromatin from a CD4− human B cell line, Raji, and a CD4+ human macrophage cell line, U937 (51; data not shown).

The third transgene construct, 1.3 mCD3-hCD4c/g, is diagrammed in Fig. 1 *B*. 1.3 mCD3-hCD4c/g contains the 1.3 SphI-ClaI fragment joined to a human CD4 minigene. The minigene was prepared by fusing an EcoRI-SacI cDNA fragment encoding exons 2–4 and part of exon 5 (49) to a genomic SacI-BamHI fragment encoding the remainder of exons 5, 6–9, and part of 10 (D. Grass, A. Garvin, D. Littman, N. Lee, and E. Lacy, manuscript in preparation). The minigene
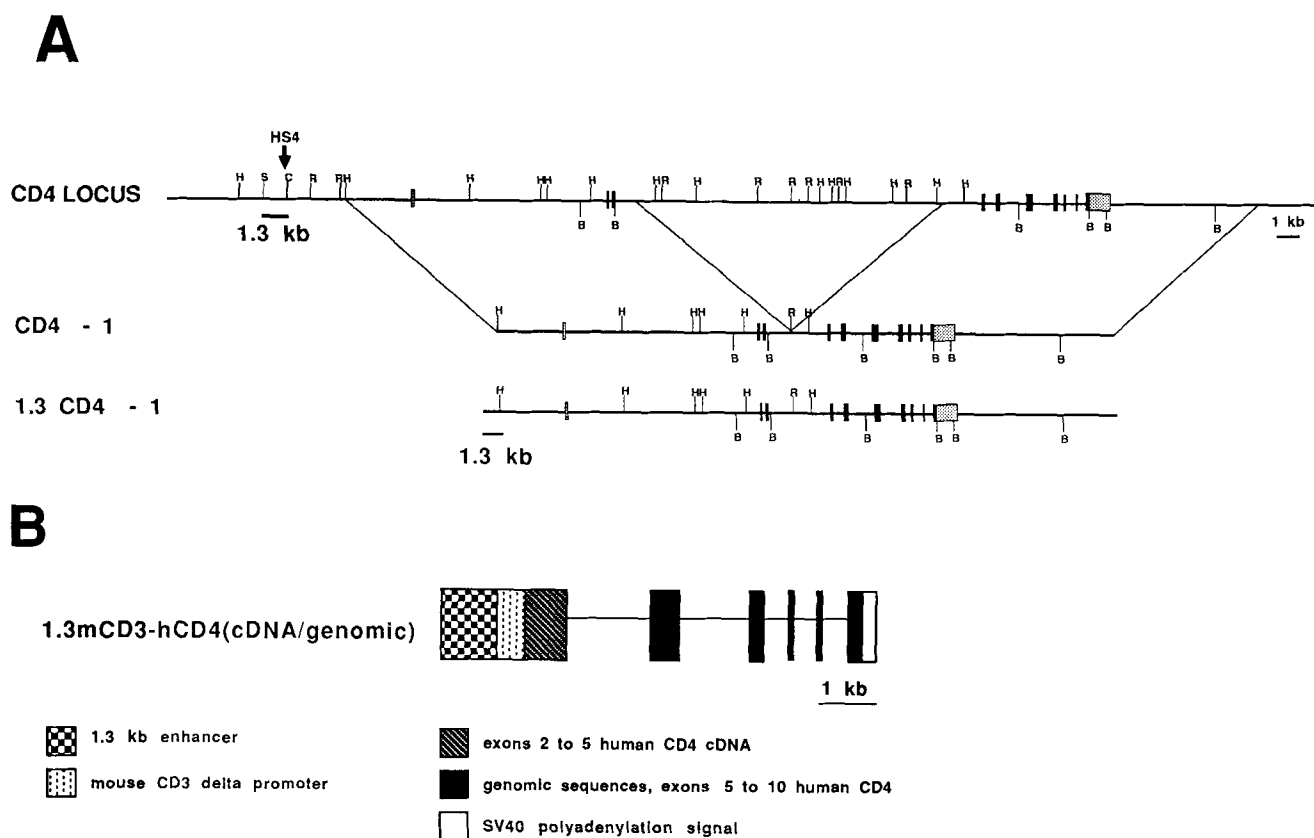

Figure 1. Map of the human CD4 locus and human CD4 transgene constructs. (*A*) The top line shows a restriction map of the human CD4 genomic locus with restriction sites designated as follows: HindIII (*H*), BamHI (*B*), EcoRI (*R*), SphI (*S*), and ClaI (*C*). The SphI and ClaI sites have not been completely mapped. Boxes indicate exons 1–10, with stippled boxes representing untranslated regions and filled boxes representing translated regions. The bold vertical arrow (*HS4*) denotes the location of a T cell–specific DNase I hypersensitive site identified in the chromatin of two human CD4+ T cell lines and in the chromatin of human PBL depleted of CD8+ T cells. The underlined region shows the 1.3-kb SphI-ClaI restriction fragment encompassing HS4 that was used in the generation of two transgene constructs. The middle line shows a restriction map of the CD4-1 transgene construct. Diagonal lines between the CD4 locus and the CD4-1 transgene denote the limits of the inserts from two genomic λ clones that were fused to construct the CD4-1 transgene. The third line shows a restriction map of the 1.3 CD4-1 transgene construct. (*B*) Map of the 1.3 mCD3-hCD4c/g transgene construct. The diagram indicates the five sequence elements comprising the 1.3 mCD3-hCD4c/g transgene construct. Each of the sequence elements is denoted by a differently patterned block, as shown beneath the diagram.

includes all of the human CD4 protein coding sequence. Transcriptional initiation and termination signals were provided, respectively, by a 0.5-kb BclI-BclI fragment from the mouse CD3-δ promoter (52) and a 0.24-kb BclI-BamHI fragment containing the SV40 poly(A) site.

Each of the three transgene constructs was microinjected into fertilized eggs derived from matings between (C57Bl/6J × CBA/CA)F$_1$ mice. Founder mice were identified by hybridizing Southern blots of HindIII-digested tail DNA to a radiolabeled human CD4 cDNA probe. Five founder mice were produced with the CD4-1 transgene and transgenic lines were obtained from three of these. Two founders that carry the 1.3 CD4-1 transgene were generated and bred. Injection with the 1.3 mCD3-hCD4c/g transgene yielded 12 lines. Human CD4 transgene expression was examined in all lines, and all lines carrying a particular transgene exhibited qualitatively similar patterns of human CD4 expression. Below we present data from three lines: CD4-1, no. 74; 1.3 CD4-1, no. 1509; and 1.3 mCD3-hCD4c/g, no. 25. These lines were chosen because they contain similar numbers of human CD4 transgenes ($\sim$50 copies).

Human CD4 Expression in the Spleens of CD4-1 and 1.3 CD4-1 Transgenic Mice: B Cell Expression. To examine the tissue specificity of CD4-1 and 1.3 CD4-1 transgene transcription, we performed Northern analyses on total RNA isolated from kidney, lung, skeletal muscle, brain, liver, testis, ovary, and spleen of adult mice. These experiments showed that both the CD4-1 and 1.3 CD4-1 transgenes were expressed preferentially in spleen (data not shown). 1.3 CD4-1–encoded transcripts were detected only in spleen, indicating that 1.3 CD4-1 transgene expression is highly specific for lymphoid tissue. In contrast, CD4-1–encoded transcripts were found in brain and lung, as well as in spleen. Subsequent analyses of the CD4-1 transgenic mice demonstrated that the presence of human CD4 transcripts in brain and lung most likely resulted from expression of the transgene in resident macrophages (51; G. Wong, D. Grass, D. Littman, and E. Lacy, manuscript in preparation).

Fig. 2 compares the expression of the CD4-1 and 1.3 CD4-1 transgenes in whole spleen and B cell–depleted splenocytes. Lane *1* contains total spleen RNA purified from a (C57BL/6 × CBA/CA)F$_1$ nontransgenic mouse; the absence of a hybridization signal indicates that the human CD4 cDNA probe, which lacks the highly conserved cytoplasmic region, does not crosshybridize to mouse CD4 transcripts. Both the CD4-1 and 1.3 CD4-1 transgenic mice were found to express full-length 3-kb human CD4 transcripts in whole spleen (Fig. 2, *top*, lanes *2* and *4*). However, CD4-1 spleens expressed markedly more of the transgene than did 1.3 CD4-1 spleens. Interestingly, no human CD4 transcripts were detected in the B cell–depleted splenocytes prepared from CD4-1 mice (Fig. 2, *top*, lane *3*). In contrast, hybridization of the human CD4 cDNA probe to RNA isolated from whole spleen or from B cell–depleted splenocytes of 1.3 CD4-1 mice (Fig. 2, *top*, lanes *4* and *5*) produced bands of approximately equal intensity. Reprobing the Northern blots in Fig. 2 with an oligonucleotide specific for 28S ribosomal RNA showed that the whole spleen lanes contained about twofold more RNA
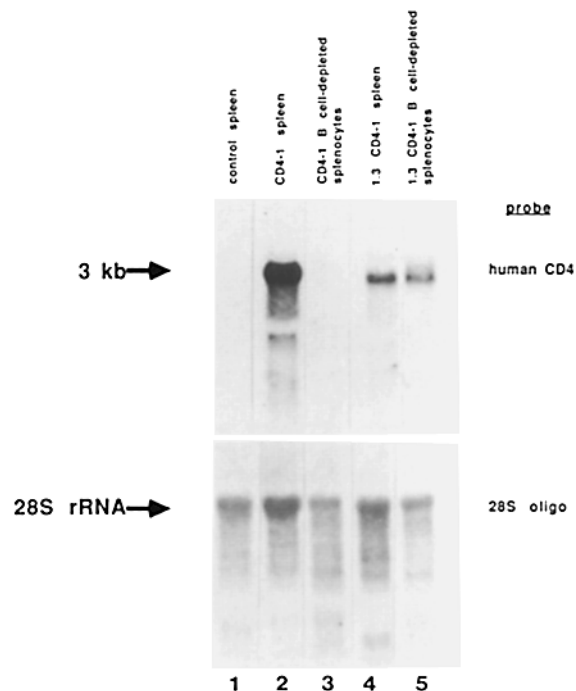

Figure 2. (*Top*) Northern blot analysis of human CD4 expression in whole spleen from a (C57Bl/6 × CBA/CA)F$_1$ control mouse (lane *1*), a CD4-1 transgenic mouse (lane *2*), and a 1.3 CD4-1 transgenic mouse (lane *4*), and in B cell–depleted splenocytes from a CD4-1 transgenic mouse (lane *3*) and a 1.3 CD4-1 transgenic mouse (lane *5*). The probe used for hybridization is a human CD4 cDNA that lacks the sequences encoding the highly conserved cytoplasmic region. (*Bottom*) Autoradiogram of the blot shown (*top*) after hybridization to a radiolabeled oligonucleotide probe specific for the 28S rRNA. All lanes derive from the same gel and autoradiogram and were repositioned for the figure.

than the B cell–depleted spleen lanes (Fig. 2, *bottom*). Thus, the human CD4 transcripts were, in fact, slightly enriched in the B cell–depleted splenocytes isolated from the 1.3 CD4-1 mouse. These results suggest that the CD4-1 transgene is transcribed primarily in splenic B cells, whereas the 1.3 CD4-1 transgene is transcribed predominantly in a non-B cell population, most likely splenic T cells.

To confirm our conclusions from the Northern assays, we examined the cell surface expression of human CD4 by FACS® analyses of purified splenocytes. Fig. 3 shows two-color FACS® profiles of individual CD4-1 and 1.3 CD4-1 mice, after staining with anti–human CD4 and anti–mouse B220 antibodies. The B220 antigen is expressed on both pre-B and B lymphocytes (61), and therefore served as a B cell marker in this experiment. Both animals contained similar numbers of human CD4–expressing splenocytes: 18% for the CD4-1 mouse and 15% for the 1.3 CD4-1 mouse. As indicated in the upper right quadrant, 13% of the CD4-1 splenocytes, but only 2% of the 1.3 CD4-1 splenocytes, costained with the anti–human CD4 and anti–mouse B220 antibodies. Thus, 73% of the human CD4$^+$ splenocytes stained as B cells in the CD4-1 mouse, whereas only 12% of the human CD4$^+$ splenocytes stained as B cells in the 1.3 CD4-1 mouse. Table 1 documents human CD4 and B220 staining in four animals from
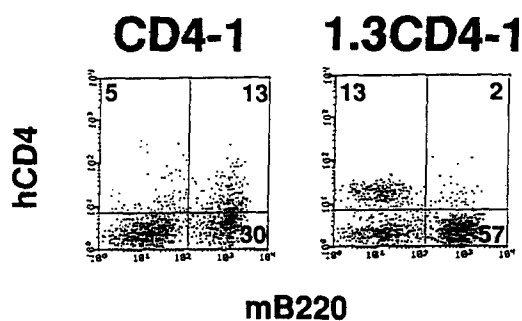
CD4-1   1.3CD4-1

Figure 3. Human CD4 and mouse B220 cell surface expression on splenocytes isolated from a CD4-1 and a 1.3 CD4-1 transgenic mouse. For each sample, a total of 50,000 cells was analyzed by FACS®. The number in each quadrant is the percentage, rounded to the nearest whole number, of gated cells falling in that quadrant. Values for the human CD4+ quadrants were corrected for background staining by subtracting values obtained in those quadrants in a nontransgenic control mouse. Fluorescence intensity is indicated on a logarithmic scale.

each of the CD4-1 and 1.3 CD4-1 lines. These data substantiate the findings from the individual animals shown in Fig. 3; on average, 78% of the human CD4+ splenocytes in CD4-1 mice and 14% of the human CD4+ cells in 1.3 CD4-1 mice were B cells. When the percentage of human CD4+ cells that express B220 is normalized to the percentage of B220+ cells in each mouse, there is a sixfold reduction of human CD4 expression on B cells in 1.3 CD4-1 mice compared with CD4-1 mice (0.26/1.6; see Table 1). These results suggest that the 1.3-kb SphI-ClaI fragment significantly inhibits the inappropriate expression of the CD4-1 transgene in splenic B cells.

Human CD4 Expression in the Spleens of CD4-1 and 1.3 CD4-1 Transgenic Mice: T Cell Expression. Human CD4 expression on the CD4+ and CD8+ peripheral T cell subsets was examined in both the CD4-1 and 1.3 CD4-1 transgenic lines by three-color FACS® analysis. Splenocyte suspensions were prepared from four 6–10-wk-old animals in each line; these cells were stained with anti–mouse CD4, anti–mouse CD8, and anti–human CD4 antibodies, and then subjected to flow cytometric analysis. Fig. 4 *A* shows the mouse CD8 vs. mouse CD4, mouse CD8 vs. human CD4, and mouse CD4 vs. human CD4 staining profiles from single, representative CD4-1 and 1.3 CD4-1 transgenic mice. The findings from these animals, and from three other mice in each line, are compiled in Table 2. As demonstrated by both the mouse CD4/CD8 plots (Fig. 4 *A*, *top*, and Table 2), neither the CD4-1 nor the 1.3 CD4-1 transgene altered the percentages or ratios of single-positive CD4+ and CD8+ splenocytes from those observed in control mice (22.1 ± 4% CD4+ and 10.1 ± 2.1% CD8+). The mouse CD8 vs. human CD4 staining profiles (Fig. 4 *A*, *middle*) showed virtually no detectable human CD4 staining on the CD8+ T cell subset in either the CD4-1 or the 1.3 CD4-1 transgenic mouse. In contrast, the mouse CD4 vs. human CD4 plots (Fig. 4 *A*, *bottom*) indicated that both the CD4-1 and 1.3 CD4-1 transgenes were expressed on the mouse CD4+ T cell subset. 4% of CD4-1 and 11% of 1.3 CD4-1 splenocytes costained for both mouse and human CD4. These data (Fig. 4 and Table 2) clearly demonstrate that both human CD4 transgenes are preferentially expressed in CD4+ cells relative to CD8+ cells.

In the experiment presented in Fig. 4 *A*, 13% of the splenocytes from the CD4-1 mouse and 11% from the 1.3 CD4-1

Table 1. *Summary of Human CD4 Expression on Splenic B Cells in CD4-1 and 1.3 CD4-1 Transgenic Mice*

	Sample no.	Percent human CD4+	Percent human CD4+ that are B220+	Percent B220+	Percent human CD4+ that are B220+/ percent B220+
CD4-1	2883	1.93	82.1	57.8	1.4
	3061	22.7	76.4	45.8	1.7
	3062	20.3	78.9	53.0	1.5
	2884	17.2	73.3	42.7	1.7
	Average	19.9 ± 2.3	77.7 ± 3.7	49.8 ± 6.9	1.6 ± 0.15
1.3 CD4-1	2896	16.6	12.2	52.5	0.23
	2900	13.5	19.2	56.8	0.34
	3066	15.0	12.2	58.5	0.21
	3067	15.7	13.8	52.6	0.26
	Average	15.2 ± 1.3	14.4 ± 3.3	55.1 ± 3.0	0.26 ± 0.06

Splenocytes from four CD4-1 and four 1.3 CD4-1 transgenic mice were analyzed by flow cytometry for cell surface expression of human CD4 and mouse B220. Averages for each column are provided along with SD. Four nontransgenic control mice gave values for percent B220+ cells of 64.6, 67.4, 53.9, and 43.5 (average, 57.4 ± 10.9%). The FACS® profiles for mice 2884 and 3066 are presented in Fig. 3. Discrepancies between the percentages shown in Fig. 3 and here result from rounding the percentages to the nearest whole number in Fig. 3 and to the first decimal place here.

Table 2. *Summary of Human CD4 Expression on Splenic T Cells from CD4-1 and 1.3 CD4-1 Transgenic Mice*

	Sample no.	Percent human CD4$^+$	Percent human CD4$^+$ that are mouse CD8$^+$	Percent mouse CD8$^+$	Percent human CD4$^+$ that are mouse CD4$^+$	Percent mouse CD4$^+$	Percent human CD4$^+$ that are mCD8$^+$/ percent mouse CD8$^+$	Percent human CD4$^+$ that are mCD4$^+$/ percent mouse CD4$^+$
CD4-1	2883	15.1	16.7	14.0	25.6	16.7	1.2	1.5
	3061	12.8	4.8	10.2	27.0	22.6	0.47	1.2
	3062	11.3	3.1	7.0	30.1	17.9	0.44	1.7
	2884	8.4	6.7	10.7	28.6	15.5	0.62	1.8
	Average	11.9 ± 2.8	7.8 ± 6.1	10.5 ± 2.9	27.8 ± 2.0	18.2 ± 3.1	0.6 ± 0.35	1.5 ± 0.26
1.3 CD4-1	2896	14.8	2.9	8.7	97.8	24.6	0.33	4.0
	2900	10.0	2.2	8.1	95.3	18.9	0.27	5.0
	3066	10.8	0.8	7.4	97.7	19.3	0.11	5.1
	3067	11.2	0.8	5.4	100	22.7	0.15	4.4
	Average	11.7 ± 2.1	1.7 ± 1.1	7.4 ± 1.4	97.7 ± 1.9	21.4 ± 2.7	0.22 ± 0.1	4.6 ± 0.52

Splenocytes from four CD4-1 and four 1.3 CD4-1 transgenic mice were analyzed by flow cytometry for cell surface expression of human CD4, mouse CD4, and mouse CD8. Averages for each column are provided along with SD. Four nontransgenic control mice gave values for percent mouse CD4$^+$ cells of 19.8, 23.1, 27.3, and 18.3 (average, 22.1 ± 4.0%) and for percent mouse CD8$^+$ cells of 7.0, 10.9, 11.3, and 11.3 (average, 10.1 ± 2.1%). The FACS® profiles for mice 3061 and 3066 are presented in Fig. 4 *A*, and those for mice 3062 and 2896 are in Fig. 4B. Discrepancies between the percentages shown in Fig. 4 and here result from rounding the percentages to the nearest whole number in Fig. 4 and to the first decimal place here.

mouse expressed human CD4 (*bottom*). Notably, all of the human CD4$^+$ cells in the 1.3 CD4-1 mouse also expressed mouse CD4. However, only 31% of the human CD4$^+$ cells in the CD4-1 mouse coexpressed mouse CD4, and 8% stained as mouse CD8$^+$; the remaining 61% stained as non-T cells and are presumably B cells. Thus, as previously indicated by Northern analyses (Fig. 3), expression of the 1.3 CD4-1 transgene is quite T cell restricted relative to the expression of the CD4-1 transgene. The CD4$^+$ subclass specificity of the CD4-1 and 1.3 CD4-1 transgenes, as well as the T cell specificity of the 1.3 CD4-1 transgene, are demonstrated more clearly when human CD4$^+$ splenocytes are assayed for the expression of CD4 and CD8 (Fig. 4 *B*). Both transgenes exhibited significant T cell expression exclusively in the CD4$^+$ subset. Furthermore, while the vast majority of human CD4$^+$ cells in the 1.3 CD4-1 mice stained as T cells (mouse CD4$^+$CD8$^-$), most of the human CD4$^+$ cells in the CD4-1 mouse stained as non-T cells (mouse CD4$^-$CD8$^-$).

These data argue that sequence elements within the CD4-1 construct are sufficient to direct transgene expression preferentially to the CD4$^+$ subset of peripheral T cells, albeit to only a small percentage of the CD4$^+$ cells. The data also reveal that the 1.3-kb SphI-ClaI fragment enhances the CD4$^+$ subset-specific expression of human CD4 in 1.3 CD4-1 transgenic splenocytes. This enhancement is manifested as an increase in the number of mouse CD4$^+$ cells that coexpress human CD4 in the 1.3 CD4-1 mice compared with that in the CD4-1 animals. However, since CD4-1-encoded transcripts

were not detected in B cell–depleted splenocytes (Fig. 2, *top*), it is likely that the 1.3-kb SphI-ClaI fragment is also enhancing the levels of human CD4 transcripts per cell. In addition, the Northern and FACS® analyses show that the 1.3-kb SphI-ClaI fragment can confer a T cell specificity on the relatively B cell–specific CD4-1 transgene.

Human CD4 Expression in the Thymuses of CD4-1 and 1.3 CD4-1 Transgenic Mice. To assess whether the CD4-1 and 1.3 CD4-1 transgenes can direct human CD4 expression to the thymus, we hybridized a radiolabeled human CD4 cDNA probe to Northern blots of total RNA, extracted from either whole thymus or isolated thymocytes. The results are shown in Fig. 5, *top*. Lane *1* contains total thymus RNA purified from a (C57BL/6 × CBA/CA)F$_1$ nontransgenic mouse; as in the Northern of the splenic RNAs, the absence of a hybridization signal indicates that the human CD4 cDNA probe did not crosshybridize to mouse CD4 transcripts. Full-length human CD4 transcripts were detected in whole thymus RNA prepared from either a CD4-1 (lane *2*) or a 1.3 CD4-1 mouse (lane *4*), demonstrating that the thymus is a major site of transcription for both transgenes. However, a hybridization signal was barely detectable in RNA isolated from purified CD4-1 thymocytes (lane *3*), whereas a significant enrichment of human CD4 transcripts was observed in purified 1.3 CD4-1 thymocytes (lane *5*). A duplicate of the Northern shown in Fig. 5, *top*, was hybridized to a mouse CD4 probe, and the resulting autoradiogram, displayed in Fig. 5, *bottom*, showed a similar enrichment of mouse CD4 transcripts in
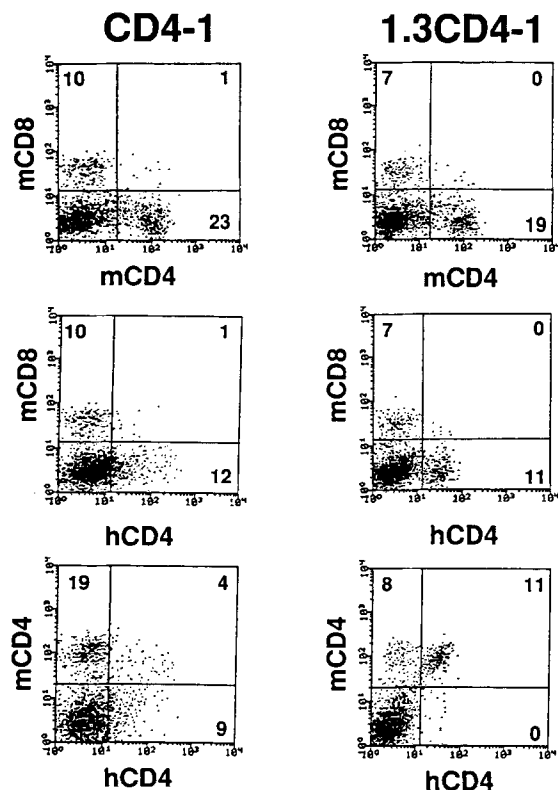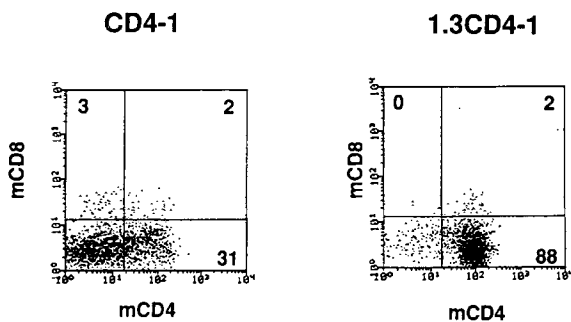
A

CD4-1

1.3CD4-1

Figure 5. Northern blot analysis of total RNA isolated from whole thymus of a (C57Bl/6 × CBA/CA)F₁ control mouse (lane 1), a CD4-1 transgenic mouse (lane 2), and a 1.3 CD4-1 transgenic mouse (lane 4), and from purified thymocytes of a CD4-1 transgenic mouse (lane 3) and a 1.3 CD4-1 transgenic mouse (lane 5). (*Top*) The hybridization probe was a human CD4 cDNA clone that lacks the highly conserved cytoplasmic region. (*Bottom*) The hybridization probe is a mouse CD4 cDNA clone derived from the 3' untranslated region, which is not well conserved between the mouse and human CD4 genes. Hybridization of the two blots to an oligonucleotide specific for 28S ribosomal RNA indicated that all lanes contained an equivalent amount of RNA (data not shown). All lanes derive from the same gel and autoradiogram and were repositioned for the figure.

B

CD4-1

1.3CD4-1

Figure 4. (*A*) Human CD4, mouse CD4, and mouse CD8 cell surface expression on splenocytes from a CD4-1 and a 1.3 CD4-1 transgenic mouse. For each sample, a total of 15,000 cells was analyzed by three-color FACS® analysis. The number in each quadrant is the percentage, rounded to the nearest whole number, of gated cells falling in that quadrant. Values for the human CD4⁺ quadrants were corrected for background staining by subtracting values obtained in those quadrants in a nontransgenic control mouse. Fluorescence intensity is indicated on a logarithmic scale. (*B*) Mouse CD4 and mouse CD8 cell surface expression on human CD4⁺ splenocytes from CD4-1 and 1.3 CD4-1 transgenic mice.

isolated thymocytes relative to whole thymus in both the CD4-1 and 1.3 CD4-1 mice. These findings suggest that the human CD4 expression detected in the 1.3 CD4-1 mouse resulted primarily from transgene transcription in thymic T
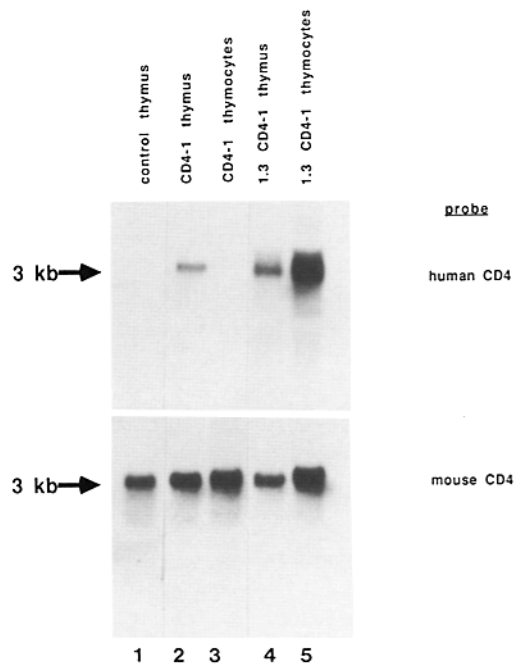
cells. In contrast, the human CD4 expression observed in the CD4-1 mouse appears to have derived from transcription in a non-T component of the thymus, possibly a bone marrow–derived component of the thymic stroma (51; G. Wong, D. Grass, D. R. Littman, and E. Lacy, manuscript in preparation).

We performed FACS® analyses on thymocytes isolated from CD4-1 and 1.3 CD4-1 transgenic mice to confirm the Northern data and to examine the expression of the human CD4 transgenes in the different thymocyte subsets. Suspensions of thymocytes from 6–10-wk-old mice were stained with anti–mouse CD4, anti–mouse CD8, and anti–human CD4 antibodies and subjected to flow cytometric analysis. Representative FACS® profiles from individual CD4-1 and 1.3 CD4-1 mice are shown in Fig. 6 *A*. Fig. 6, *top* and *middle*, shows the mouse CD4/CD8 staining profiles, which indicated that the CD4-1 and 1.3 CD4-1 transgenic mice had normal numbers and ratios of the CD4⁻CD8⁻ double-negative, CD4⁺CD8⁺ double-positive, and CD4⁺CD8⁻ and CD4⁻ CD8⁺ single-positive thymocytes. Control animals contained 4.2 ± 0.64% double-negative, 73.9 ± 5.3% double-positive, 17.3 ± 5.4% CD4 single-positive, and 4.7
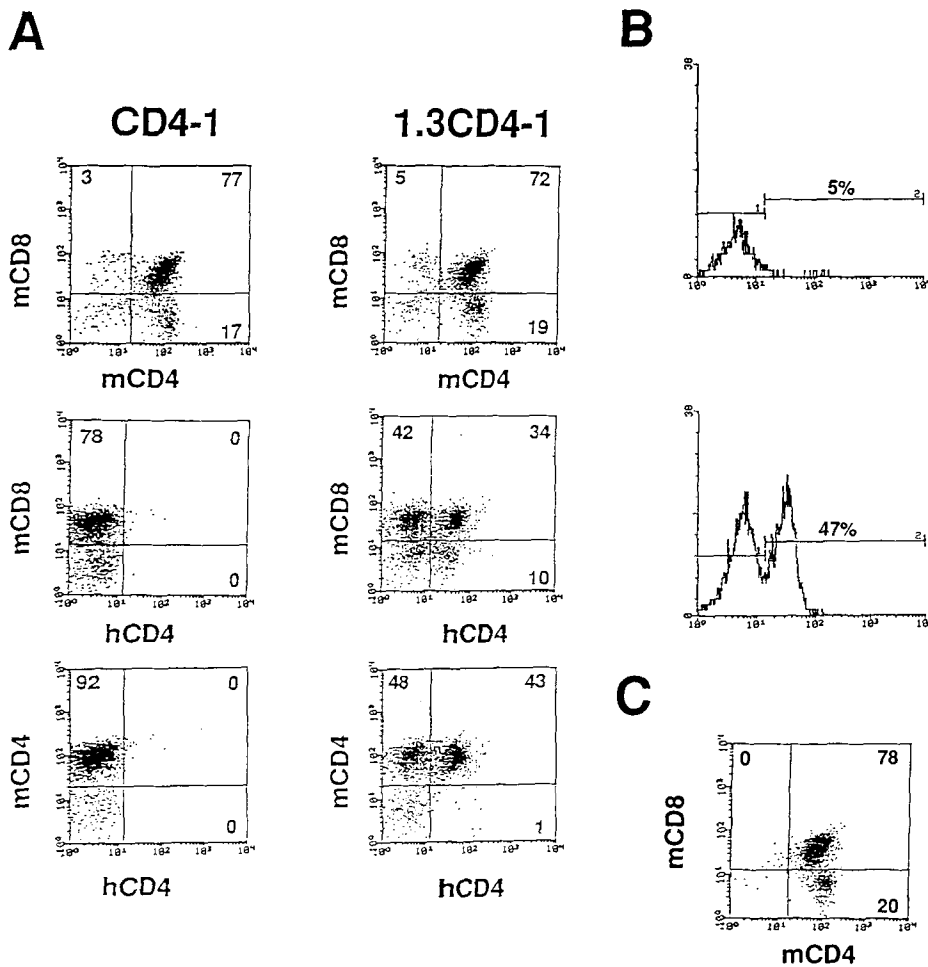
A

CD4-1

mCD8 | 3 | 77
mCD4 | | 17

mCD8 | 78 | 0
hCD4 | | 0

mCD4 | 92 | 0
hCD4 | | 0

1.3CD4-1

mCD8 | 5 | 72
mCD4 | | 19

mCD8 | 42 | 34
hCD4 | | 10

mCD4 | 48 | 43
hCD4 | | 1

B

5%

47%

C

mCD8 | 0 | 78
mCD4 | | 20

Figure 6. (*A*) Human CD4, mouse CD4, and mouse CD8 cell surface expression on thymocytes from a CD4-1 and a 1.3 CD4-1 transgenic mouse. A total of 15,000 cells was examined by three-color FACS® analysis for each sample. The number in each quadrant is the percentage, rounded to the nearest whole number, of gated cells falling in that quadrant. Values for human CD4+ quadrants were corrected for background staining by subtracting values obtained in those quadrants in a nontransgenic control mouse. Fluorescence intensity is denoted on a logarithmic scale. (*B*) Human CD4 expression on gated mouse CD8 single- positive thymocytes (*top*) and gated mouse CD4 single-positive thymocytes (*bottom*) from the 1.3 CD4-1 transgenic mouse shown in *A*. Horizontal bars delimit the human CD4-negative thymocytes (*1*) and the human CD4 positive thymocytes (*2*). The percentage of cells falling within the limits of *2* are indicated above the bar. Fluorescence intensity is denoted on a logarithmic scale. (*C*) Mouse CD8 and mouse CD4 cell surface expression on gated human CD4+ thymocytes from the 1.3 CD4-1 transgenic mouse shown in *A*.

± 2.5% CD8 single-positive thymocytes. Consistent with the Northern results in Fig. 5, no human CD4 expression was detected on either the mouse CD4+ or CD8+ thymocytes of the CD4-1 mouse (Fig. 6 *A*, *bottom* and *middle left*, respectively). The 1.3 CD4-1 transgenic mouse, on the other hand, exhibited human CD4 expression on 44% of all thymocytes. 77% of the human CD4+ thymocytes costained with mouse CD8; 98% costained with mouse CD4 (Fig. 6 *A*, *middle* and *bottom right*).

The FACS® profiles shown in Fig. 6 *A* indicate that the 1.3 CD4-1 transgene must be expressed on at least some of the double-positive thymocytes. To determine the distribution of human CD4 expression on the CD4 and CD8 single-positive thymocyte subsets in the 1.3 CD4-1 mice, we plotted histograms of human CD4 fluorescence for the CD4−CD8+ and CD4+CD8− cells. Fig. 6 *B*, *top*, shows the profile from one representative mouse and reveals that only 5% of mouse CD8 single-positive thymocytes express human CD4. In contrast, Fig. 6 *B*, *bottom*, shows that 47% of the mouse CD4 single-positive thymocytes stain for the human CD4 transgene. Thus, the subclass-specific expression of the 1.3 CD4-1 transgene observed on splenic T cells is also evident on mature thymocytes. Histograms of human CD4 fluorescence were

also plotted for the CD4−CD8+ and CD4+CD8− thymocytes of CD4-1 mice, and no mature single-positive thymocytes were found that expressed human CD4 (data not shown). Fig. 6 *C* presents the mouse CD4/CD8 staining profile of the human CD4+ thymocytes from the same 1.3 CD4-1 transgenic mouse shown in Fig. 6 *B*. 78% of the human CD4+ cells fall into the double-positive subset, and 20% into the CD4 single-positive subset. The double-negative and CD8 single-positive thymocytes largely failed to express human CD4. Thus, the pattern of expression of the human CD4 transgene is identical to that of mouse CD4. Similar findings were obtained from three other 1.3 CD4-1 transgenic mice, and these are summarized in Table 3. These data indicate that the CD4-1 transgene lacks the sequences required to drive CD4 expression in either immature or mature thymocytes and that the 1.3-kb SphI-ClaI fragment provides CD4-1 with the missing information needed to fully recapitulate the stage and subclass-specific expression of CD4 in the thymus.

Transgene Expression in 1.3 mCD3-hCD4c/g Transgenic Mice. To evaluate the contribution that the 1.3-kb SphI-ClaI fragment makes to the subclass-specific expression of the 1.3 CD4-1 transgene, we produced transgenic lines carrying the 1.3 mCD3-hCD4c/g construct (Fig. 1 *B*). In 1.3 mCD3-

Table 3. *Summary of Human CD4 Expression on Thymocytes of 1.3 CD4-1 Transgenic Mice*

Sample no.	Percent human CD4⁺	Percent human CD4⁺ that are double positive	Percent human CD4⁺ that are mCD8 single positive	Percent human CD4⁺ that are mCD4 single positive	Percent human CD4⁺ that are double negative
2896	51.1	85.1	0.53	13.5	0.86
2900	42.5	88.0	1.2	9.6	1.2
3066	43.4	78.2	0.93	20.1	0.79
3067	37.6	84.0	1.0	13.4	1.7
Average	43.7 ± 5.6	83.8 ± 4.1	0.91 ± 0.3	14.2 ± 4.4	1.2 ± 0.4

Thymocytes from four CD4-1 and four 1.3 CD4-1 transgenic mice were analyzed by flow cytometry for cell surface expression of human CD4, mouse CD4, and mouse CD8. Averages for each column are provided along with SD. Four nontransgenic control mice gave values for percent mouse CD4 single-positive cells of 16.8, 23.6, 18.3, and 10.6 (average, 17.3 ± 5.4%); for percent mouse CD8 single-positive cells of 1.6, 4.7, 4.5, and 7.8 (average, 4.7 ± 2.5%); for percent mouse CD4⁺CD8⁺ cells of 78.1, 67.0, 72.4, and 77.9 (average, 73.9 ± 5.3%); and for percent mouse CD4⁻CD8⁻ cells of 3.6, 4.7, 4.8, and 3.7 (average, 4.2 ± 0.64%). The FACS® profiles for mice 3061 and 2900 are presented in Fig. 6 *A*, and those for mouse 3066 in Fig. 6 *B*, and *C*. Discrepancies between the percentages shown in Fig. 6 and here result from rounding the percentages to the nearest whole number in Fig. 6 and to the first decimal place here.

hCD4c/g, the human CD4 promoter has been replaced by the mouse CD3-δ promoter and a number of other human CD4 genomic sequences have been deleted: the 5' and 3' flanking sequences present in CD4-1, exon 1, introns 1–4, and the untranslated portion of exon 10. In six transgenic lines that carry a control mCD3-hCD4c/g transgene, which lacks the 1.3-kb SphI-ClaI fragment, human CD4 was expressed on all subsets of T cells (M. Blum and E. Lacy, unpublished results). Thus, no inherent CD4⁺ cell subclass specificity is encoded by the mouse CD3-δ promoter and the CD4 sequences included in the cDNA/genomic hybrid gene.

Splenocytes from adult 1.3 mCD3-hCD4c/g transgenic mice were stained with anti–mouse CD4, anti–mouse CD8, and anti–human CD4 antibodies and examined by flow cytometric analysis. As shown in Fig. 7, both the mouse CD8⁺ and CD4⁺ splenocyte subsets expressed the human CD4 transgene. Similarly, both CD4 and CD8 single-positive thymocytes, as well as double-positive thymocytes, expressed human CD4 (data not shown). Thus, the 1.3-kb SphI-ClaI fragment appears incapable, on its own, of restricting transgene expression specifically to the CD4⁺ subsets of immature and mature T cells. Fig. 7, *middle* and *bottom*, reveals that >90% of the splenocytes in the 1.3 mCD3-hCD4c/g mouse were stained by the anti–human CD4 antibody, indicating that most B cells also express the human CD4 transgene. Expression of the transgene in B cells was confirmed by three-color FACS® analyses using anti-B220, anti-CD3, and anti–human CD4 antibodies (data not shown). These data argue that the 1.3-kb SphI-ClaI fragment alone cannot inhibit transgene expression in B cells. Therefore, the T cell–restricted expression of the 1.3 CD4-1 transgene may have been mediated by an interaction between the SphI-ClaI fragment and sequences within CD4-1 that are absent in 1.3 mCD3-hCD4c/g.

Nucleotide Sequence of the 1.3-kb SphI-ClaI Fragment Reveals Homology to a Mouse CD4 Enhancer. We determined the

nucleotide sequence of the 1.3-kb SphI-ClaI fragment (Fig. 8 *A*) and compared it with the 772-bp sequence reported by Sawada and Littman (62) for a mouse CD4 enhancer. The 772-bp mouse sequence is located ∼13 kb upstream from the transcriptional start site of the mouse CD4 gene; it was shown to provide T cell–specific enhancer activity by transient transfection assays using a chloramphenicol acetyl transferase (CAT)
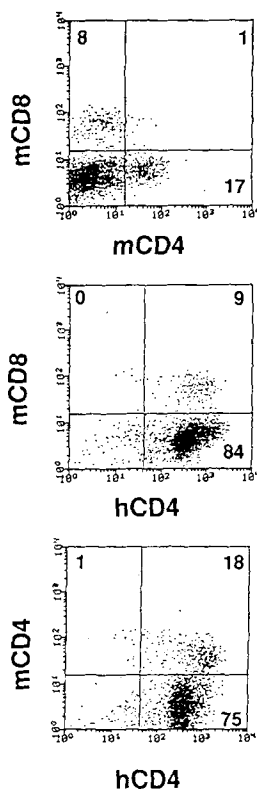

Figure 7. Human CD4 cell surface expression on splenocytes from a 1.3 mCD3-hCD4c/g transgenic mouse. A total of 15,000 cells was analyzed by flow cytometry. The number in each quadrant is the percentage, rounded to the nearest whole number, of gated cells falling in that quadrant. Values for human CD4⁺ quadrants were corrected for background staining by subtracting the values obtained in those quadrants of a nontransgenic control mouse. Fluorescence intensity is denoted on a logarithmic scale.

A

```
CACCTGTGGTCCTAGTTATTCAGGAGGCTGAGGGAGAAGAATCACTGGAG   50
GCTAGGATTTCCAGGCTGTAGTGAGCCATGATTGTGCTATTTCACTCCAG  100
CTTGGGTGACAGGGTGAGATCCTGTGTCTAAAAAGAATAATAACAATAAT  150
AATAAACATTATCTCACACAACGAGGAATCCAGGAGCAGTCTTGCTGAGT  200
AGTTCTGGCACAGGGTTGCTCATGAGGTTGCTGCTGAGGTGAGGGTCAGG  250
TCTGGAGCCATCTGAAGGCTGGGATGGGGCTGGAGATTCTGCCTCCAAGA  300
CAGCTCACTCGGGAGGCTGTCGGCCAGAGGCTTAACTCCTCCCTGTGTGG  350
GTGTCTTTATGGGGCCTCTCACAACACGACACAGCAACTGGCTTCCCCCA  400
GAGGACAGCGTGGAAAAGACAAAAGCTACCACATCTTTATTTCAAGATTA  450
GATTTGATCTTGAAAGCAACGTGCCATAATTTCTGCCCTATTCTATTGAT  500
CACAGAGACCAACCTGGTGCAAGCAGGAGGGGGCAATGCAAGGGTGTGAA  550
CTGAGGGAGACAGGAGTCACTGGGGGGCCATCTTGCAGCCTGGCTGTCAGA  600
GTGGGGAGCAGTGCCTGAAACAGAGATAGGCCAAGAGACGGGAAGGGACG  650
TCTCTGAGCATCTCCATCCCCAGCCCTCTTCTCTTGTCCACTCTCCAGAA  700
GTTCTGGAAGCCAAGTGAGGTCCAGGGCCTTGGCAAGTCGAGTGTGTGAG  750
GTAGGAGGCGATACTGTGCACATTCAGCCTTGGAGTCGGGTTTGCCAGCG  800
GTTCCCCTGACCCCTACGTGGTCTTCTCAGCTCCATAGTGCAGAGGAGGA  850
GCCTCTGCAGGCCCAGAAATTTCCCTGCCCACCTCCCCTACCAGGTAGAG  900
ATGTTAGGGTTCAGGCCTGAGCCCCTGCTGCTATCCCTCCTTCAAAGGAG  950
GAGATCAAGGAGCTTAGGGTCCCCCGCACAGGCCACCCCAGGGTGGGGTT 1000
CTTCCTTTGAAGGGAATTGCTTTGGGGTGGGGTCGGTTCTATCTGCTCAC 1050
TCTGTGGCTGACAGTTTCTCCAAGGGGCTGCAGGTGTCAGCTGTCTGAGC 1100
CCGGGCTGAGCTCTGAAACGTGCCTACTCAAACTTCCCGTGGGGTAGGGG 1150
AGGCCCAGAACCACCCTCTGAGAGTGGCAAAAAGTGGTCCTGGAGCCAGG 1200
GGAAATGTGGATGGGGTAGAGATGGCATC                       1229
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B

```
       132                      158
HUMAN  AAAGAATAATAACAATAATAATAAACA
       ****** ** ** ** ** ** ****          74%
MOUSE  AAAGAAAAAAAAAAAAAAAAAAGAACA
       42                        68

       325                          356
HUMAN  CAGAGGCTTAACTC CTCCCTGTGTGGGTGTCT
       *** *** *** *** ** *** ** *         61%
MOUSE  CAG CTTTGCTCTCTCTGTGGCTGGCAGTTT
       155                          184

       902          926
HUMAN  TGTT AGGG TTCAGGCCTGAGCCCCT
       **** **** **** * ** ** ***          69%
MOUSE  TGTTTACAGGGCTTCATGGCTCAGAACCT
       -71                       -43

       978                   1001
HUMAN  ACAGGCC ACC  CCAGGGTG GGG TTC
       *** ** *** *** **** *** ***         66%
MOUSE  ACATCCCCAACCAAGCCAAGGTGTTGGGGTTC
       -21                        11

       1021                 1049
HUMAN  TTTGGGGTGGGGTCGGTTCTATCTGCTCA
       ** *** *** * *** ** *** ***         69%
MOUSE  TTGGGG TGGAGGCGGATC  CTG TCA
       134                      157

       1043                             1077
HUMAN  CT  GCTCACTCTGTGGCTGACAGTTTCTCCAAGGGG
       ** **** ********** ************ ***  86%
MOUSE  CTTTGCTCTCTCTGTGGCTGGCAGTTTCTCCAAAGGG
       159                              195

       1059               1085
HUMAN  TGACAGTTTCTCCAAGGGGCTGC AGGT
       ** *** *** ***** ** ** ****          75%
MOUSE  TG CAGCTTC  CAAGGTGCCGCCAGGT
       348                          372

       1081                                     1127
HUMAN  CAGGTGTCAGCTGTCTGAGCCCGGGCTGAGCTCTGAAACGTGCCTAC
       ************* ******* ****** * **** ** ** ****  83%
MOUSE  CAGGTGTCAGCTGGCTGAGCCTAGGCTGAACCCTGAGACATG CTAC
       199                                      247

       1096             1118
HUMAN  TGAGCCCGGGCTG AGCTCTG AAA
       ******* **** *** ** ***              71%
MOUSE  TGAGCCCCAGCTGTTAGCCCTCTGCAAA
       17                        44

       1176                   1204
HUMAN  GGCAAAAGTGGTC CTGG  AGCCAGGG GAA
       *** ** ** *** **** **** ** ***       61%
MOUSE  GGGTAACAGGTGTCAGCTGGCTGAGCCTAGGCTGAA
       193                           228

       1201                1229
HUMAN  GGAAATGTGGATGGGGTAGAGATGGCATC
       ** * ** ** ****** *** ** ***         72%
MOUSE  GGGAGTGGGGTTGGGGTGGAGGCGG ATC
       124                         151
```

Figure 8. (*A*) Nucleotide sequence of the 1.3-kb SphI-ClaI human CD4 genomic fragment encompassing HS4. Numbers to the right of each line of sequence refer to the last nucleotide in each line. Regions of homology with the mouse CD4 enhancer (numbered according to Sawada and Littman [62]; Genbank accession number M75688) are indicated in boldface. Two consensus binding sites for basic helix-loop-helix proteins are underlined.

reporter gene driven by the mouse CD4 promoter (62). Interestingly, a 339-bp minimal enhancer fragment, defined by the transient transfection experiments, functioned equally well in CD4$^+$ and CD8$^+$ T cell lines (62). Sequence comparison identified 11 regions of the 1.3-kb SphI-ClaI fragment that share homology with the 772-bp sequence of the mouse CD4 enhancer; these are shown in boldface in Fig. 8 *A* and are aligned with the mouse sequence in Fig. 8 *B*. Each of the 11 regions spans between 27 and 47 bp and shares between 61 and 86% homology with the mouse sequence. As depicted in Fig. 8 *A*, most of the homology lies within the 3' terminal 200–300 bp of the 1.3-kb SphI-ClaI fragment.

DNase I footprint analyses and electrophoretic mobility shift assays (EMSA) identified three nuclear protein binding sites within the minimal mouse CD4 enhancer (CD4-1, CD4-2, and CD4-3; 62). Consensus binding sites for basic helix-loop-helix (bHLH) proteins, 5'-CANNTG-3', were found in CD4-1 and CD4-3; CD4-1 contains a single bHLH motif and CD4-3 contains two tandem motifs, arranged with incomplete dyad symmetry (62). Mutations in the CD4-3 site were shown to reduce enhancer activity by >90% (62). CD4-2 was found to contain a consensus binding site for TCF-1α (5'-CANAG-3'), a T cell–specific transcription factor known to bind enhancers of a number of T cell–specific genes (63). However, mutations in CD4-2 reduced the activity of the minimal mouse CD4 enhancer only moderately (62). The sequence of the CD4-3 site is completely conserved within the 1.3-kb SphI-ClaI human CD4 fragment and it lies within a region (1081–1127) that exhibits 83% homology with the mouse CD4 enhancer. Another region of the 1.3-kb SphI-ClaI fragment (1176–1204) shows homology (61%) with the segment of the mouse CD4 enhancer that includes the CD4-3 site; however, the CD4-3 site itself is not well conserved in the human sequence. A segment of the mouse CD4 enhancer that contains the CD4-1 site shares 71% homology with the 1.3-kb SphI-ClaI fragment (1096–1118), but the sequence of the CD4-1 site is not conserved. We also identified a number of TCF-1α consensus sites within the 1.3-kb SphI-ClaI fragment, but none of them was located within the areas of homology with the mouse CD4 enhancer. Of possible importance is our finding that the region of greatest homology (86%) does not contain any of the previously defined nuclear protein binding sites within the mouse CD4 enhancer. In

(*B*) Regions of homology between the human CD4 1.3-kb SphI-ClaI restriction fragment (*top line*) and the mouse CD4 enhancer (*bottom line*). Nucleotides in the top lines are numbered according to the human sequence in *A*. Nucleotides in the bottom lines are numbered according to the mouse sequence recorded under the Genbank accession number M75688. The 339-bp mouse CD4 minimal enhancer extends from nucleotide 80 to 418 of the Genbank sequence. Asterisks between the lines indicate identities. Interruptions were introduced to permit optimal alignment of homologous regions. Percentages to the right of each sequence comparison indicate the amount of sequence identity, as determined using Microgenie software (Beckman Instruments, Inc.; 60). Nucleotides within the mouse CD4 enhancer that comprise the CD4-3 DNase I footprint (62) are indicated in boldface. Underlined regions denote consensus binding sites for basic helix-loop-helix proteins. These sequence data are available from EMBL/Genbank/DDBJ under accession number X70672.

addition, we identified two regions of the 1.3-kb SphI-ClaI fragment (902–926 and 1059–1085) that share homology with sequences outside of the minimal mouse CD4 enhancer.

Discussion

In this study, we have reconstituted the stage and T cell subclass-specific expression of the CD4 gene in transgenic mice. Our findings demonstrate that the developmentally appropriate expression of a human CD4 transgene depends on at least two sets of sequences, one contained in the 1.3-kb SphI-ClaI upstream fragment and one present within the 35-kb CD4-1 construct. As shown by the Northern data in Fig. 5 and the FACS® analyses in Fig. 6, CD4-1 transgenes lacking the 1.3-kb SphI-ClaI fragment are not expressed in thymocytes. When this fragment is included in the transgene, human CD4 is expressed exclusively on double-positive and CD4 single-positive thymocytes. Thus, the 1.3-kb SphI-ClaI fragment appears to be functioning as an enhancer element, and we will, therefore, refer to this fragment as the human CD4 enhancer.

The differentiation of CD4$^-$CD8$^-$ thymocytes into CD4$^+$CD8$^+$ cells is a regulated transition that depends on the expression of the TCR β chain (64). CD4 transgene expression is not detected on the double-negative thymocyte subset in the 1.3 CD4-1 transgenic mice, and is first found at significant levels on the double-positive subset. Therefore, the 1.3 CD4-1 transgene is apparently capable of activating human CD4 expression at the correct stage of thymocyte development. Since the human CD4 protein can be expressed on the surface of CD4$^-$CD8$^-$ thymocytes in the 1.3 mCD3-hCD4c/g transgenic mice (M. Blum and E. Lacy, unpublished observations), the absence of the human CD4 protein on CD4$^-$CD8$^-$ thymocytes in the 1.3 CD4-1 mice suggests that the developmental stage–specific expression of CD4 is regulated at the transcriptional level. However, alternate explanations are possible. For example, the 1.3 mCD3-hCD4c/g-encoded transcript is not identical to the 1.3 CD4-1-encoded transcript. The former lacks untranslated sequences present in exons 1 and 10 of CD4, as well as introns 1–4. Thus, the 1.3 CD4-1 transgene may be transcribed in both double-negative and double-positive thymocytes and the stage-specific expression of human CD4 on the CD4$^+$CD8$^+$ cells achieved by a posttranscriptional mechanism. We have not yet examined human CD4 expression on multipotential hemopoietic bone marrow stem cells and early thymic precursor cells, and consequently, we do not know whether the 1.3 CD4-1 transgene is appropriately expressed on these progenitor cells.

The sequence elements that direct 1.3 CD4-1 transgene expression specifically to the CD4$^+$CD8$^-$ peripheral T cell subset appear to reside within the 35-kb CD4-1 construct. The FACS® analyses presented in Fig. 4 and summarized in Table 2 show that both the CD4-1 and the 1.3 CD4-1 transgenes were expressed preferentially on the mouse CD4 single-positive subset of splenic T cells. The 1.3-kb SphI-ClaI fragment enhanced the subclass-specific expression of the CD4-1-encoded transgene, as evidenced by the increase in the

number of CD4$^+$CD8$^-$ peripheral T cells that coexpressed human CD4 in the 1.3 CD4-1 mice compared with the CD4-1 mice. Possibly, the presence of the human CD4 enhancer shielded the CD4-1 construct from chromosomal position effects that are known to obstruct transgene expression (65–68). The 1.3 mCD3-hCD4c/g transgenic mice expressed the human CD4 transgene on all subclasses of T cells (Fig. 7); thus the human CD4 enhancer cannot, on its own, direct CD4 subclass-specific expression of a transgene. Consequently, the sequences that target human CD4 expression to CD4$^+$CD8$^-$ peripheral T cells may be contained entirely within the CD4-1 construct. Since the CD4-1 transgene was not expressed in the thymus, it is unclear whether the same CD4-1-encoded sequences that are sufficient for CD4 subclass-specific expression in peripheral T cells are also required for CD4 subclass-specific expression of the 1.3 CD4-1 construct in thymocytes.

A comparison of the 1.3 CD4-1- and 1.3 mCD3-hCD4c/g-encoded transgenes identifies a number of possible candidates for the sequences that mediate human CD4 subclass-specific expression on peripheral T cells. In the 1.3 mCD3-hCD4c/g construct, which is not expressed subclass specifically, the human CD4 promoter has been replaced by the mouse CD3-δ promoter. In addition, the 1.3 mCD3-hCD4c/g transgene lacks several other human genomic sequences that are present in 1.3 CD4-1; these include 5' and 3' flanking sequences, exon 1, introns 1–4, and an untranslated portion of exon 10. The experiments of Siu et al. (69) indicate that, in the mouse CD4 gene, the CD4 promoter itself may be a key component of the mechanism specifying subclass-specific expression. They found that a 172-bp fragment of the mouse CD4 promoter directed high levels of expression of a luciferase reporter in mature CD4$^+$CD8$^-$ T cell lines but not in mature CD4$^-$CD8$^+$ T cell lines. In addition, they reported that the mouse CD4 promoter did not function in cloned immature CD4$^+$CD8$^+$ thymomas, unless linked to the TCR β chain enhancer. This pattern of mouse CD4 promoter activity in cloned T cell lines recapitulates the pattern of CD4-1 transgene expression we observe in thymocytes and splenic T cells. Such a finding suggests that the human CD4 promoter may mediate the CD4 subclass-specific expression of the CD4-1 transgene. However, although the human CD4 promoter present in the CD4-1 transgene responds to the CD4 enhancer in mature CD4$^+$ cells, the activity of the mouse CD4 promoter was not increased by a T cell–specific enhancer in mature CD4$^+$CD8$^-$ lines. This difference between our results and the transient transcription studies of Siu et al. (69) may reflect an interaction between the CD4 enhancer and promoter that cannot be duplicated with the TCR β chain enhancer.

In contrast to the findings of Siu et al. (69), Sawada and Littman (62) reported that a CAT construct, driven by both the mouse CD4 promoter and enhancer, was active in CD4$^-$CD8$^+$, as well as CD4$^+$ cell lines. Thus, other sequences besides the CD4 promoter may be required in vivo to achieve CD4 subclass-specific expression. Interestingly, Sands and Nikolić-Žugić (70) have identified T cell–specific DNase I hypersensitive sites near the first exon and intron of the mouse

CD4 gene that vary significantly in intensity in different CD4 and CD8 subsets. They have proposed that proteins bound to DNA sequences at these sites may be involved in repressing CD4 transcription during thymocyte development (70). In preliminary mapping experiments, we too have located DNase I hypersensitive sites within the first intron of the human CD4 gene (51; G. Wong and E. Lacy, unpublished results). Consistent with the premise that sequences in addition to the promoter are involved in specifying CD4 subclass-specific expression, the DNA sequences encoding these hypersensitive sites are present in CD4-1 but not in 1.3 mCD3-hCD4c/g. Using specifically modified versions of the human CD4 transgenes described in this paper, it will now be possible to define the identity and locations of the cis-acting elements that direct CD4 gene expression to the mature CD4$^+$CD8$^-$ T cell subset.

The patterns of CD4-1 and 1.3 CD4-1 transgene expression in spleen indicate that the 1.3-kb SphI-ClaI fragment produces T cell-specific expression of the CD4-1 construct not only by acting as an enhancer but also by inhibiting expression in B cells (Figs. 2–4). Since the 1.3 mCD3-hCD4c/g construct was expressed in B cells (Fig. 7), the human enhancer is not sufficient to preclude expression in B cells. Presumably it must interact with sequences within CD4-1 to block B cell expression. It could accomplish this by permitting binding of a repressor and/or by preventing recognition and binding of B cell transcription factors. Interestingly, although CD4 expression is not found on B cells in humans, it has been detected on a number of human B cell lines in culture (71–73). This indicates that the human gene does have an inherent ability to interact functionally with transcription factors present in B cells. It also suggests that such cultured B cell lines lack the transcriptional controls responsible for maintaining the normal pattern of B cell-vs. T cell-specific gene expression. Possibly, in the human CD4 gene, these controls act through the 1.3-kb upstream enhancer identified in this study.

The nucleotide sequence of the 1.3-kb SphI-ClaI fragment argues that the HS4 DNase I hypersensitive site identifies the location of the human homologue of the mouse CD4 enhancer defined by Sawada and Littman (62). Of the three DNase I footprints defined in the minimal mouse CD4 enhancer, only the sequence of the CD4-3 site is conserved in the human CD4 enhancer (Fig. 8 B). This finding is consistent with the mutational analysis of the mouse CD4 enhancer, which showed that mutations in the CD4-3 site reduced enhancer activity >90%, while mutations in the CD4-1 and/or CD4-2 sites generated only a two- to fourfold loss of enhancer activity (62). 11 regions of the 1.3-kb SphI-ClaI fragment were found to share homology with a 772-bp sequence containing the mouse CD4 enhancer (Fig. 8 B). Two of these regions (902–926 with 69% homology and 1059–1085 with 75% homology) lie outside the minimal mouse CD4 enhancer; a third region, and the one with the greatest sequence homology (86%), does not contain any of the nuclear protein binding sites thus far defined within the minimal mouse CD4 enhancer. Thus, in vivo, the CD4 enhancer may function through the binding of nuclear factors that were either absent or undetectable in the nuclear extracts so far examined (62). With the identification of a construct that is expressed appropriately during thymic maturation in transgenic mice, it should now be possible to screen T cell lines for those that recapitulate the developmental regulation of the CD4 gene. Such lines may permit the identification and isolation of regulatory proteins that bind to the other regions of homology shared by the mouse and human CD4 enhancers.

Our analyses of the CD4-1 and 1.3 CD4-1 transgenes demonstrate that cis-acting sequences closely linked to the human CD4 gene direct CD4 expression to the CD4$^+$CD8$^-$ mature T cell subset and preclude CD4 expression in the CD4$^-$CD8$^+$ subset. However, these data do not establish whether the CD4 regulatory sequences respond to signals generated by coengagement of an MHC molecule by CD4/CD8 and a TCR, as predicted by the instruction model, or to signals produced by some other developmental event that commits a thymocyte to the CD4 lineage, as predicted by the stochastic/selection model.

Our observation that the CD4-1 transgene is expressed in splenic CD4$^+$CD8$^-$ T cells but not in thymocytes suggests that different mechanisms may operate to regulate the transcription of the CD4 gene in immature and mature T cells. For example, one set of transcription factors may be required to activate CD4 expression in CD4$^+$CD8$^+$ and CD4$^+$CD8$^-$ thymocytes, and a second, distinct set of factors may drive expression in mature CD4 single-positive cells. Our data, showing that the expression of the CD4-1 transgene in thymus is dependent on the 1.3-kb SphI-ClaI fragment, suggests that the activity of the first set of transcription factors would be mediated through the human enhancer. Possibly, the second set of factors would be brought into play only after positive selection of a class II–restricted TCR and/or export of a T cell to the periphery. Further studies in transgenic mice should allow us to distinguish between reactivation and repression as mechanisms for maintaining CD4 gene expression in the CD4$^+$CD8$^-$ T cell subset and silencing it in the CD4$^-$CD8$^+$ T cell subset. Deletion of sequence elements required for subclass-specific expression should result in CD4 expression in both the CD4 and CD8 subsets, if the loss of CD4 on CD4$^-$CD8$^+$ cells is mediated by repression. On the other hand, deletion of these regulatory sequences would result in the absence of transgene expression on both mature T cell subsets, if the subclass-specific expression of CD4 is generated by reactivation in class II–restricted cells.

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