

An Enhancer That Directs Lineage-Specific Expression of CD8 in Positively Selected Thymocytes and Mature T Cells

Wilfried Ellmeier,*† Mary Jean Sunshine,*†
Kasia Losos, Farah Hatam,*† and Dan R. Littman*††

*Division of Molecular Pathogenesis
Skirball Institute of Biomolecular Medicine

†Howard Hughes Medical Institute
New York University Medical Center
New York, New York 10016

Summary

Positive selection of CD4⁺CD8⁺ T cells to the CD4⁺CD8⁻ helper and CD4⁻CD8⁺ cytotoxic lineages is a multistep process that involves complex regulation of coreceptor gene expression. By analyzing expression of a reporter gene in transgenic mice, we have identified a DNA segment, located between the murine *CD8β* and *CD8α* genes, that has enhancer activity restricted to CD8 lineage cells. Remarkably, this enhancer functions in thymocytes undergoing positive selection to the CD4⁻CD8⁺ phenotype but not in immature double-positive thymocytes. The enhancer also functions in gut intraepithelial lymphocytes that express CD8α but not CD8β, suggesting that it is specific for CD8α expression. The tight correlation between activation of this enhancer and the final step in positive selection has important implications for understanding the mechanism of lineage commitment in thymocytes.

Introduction

The two major subsets of T lymphocytes in the peripheral immune system express either the CD4 or the CD8 coreceptor and generally have a helper or cytotoxic phenotype, respectively. These single-positive (SP) cells develop in the thymus from a common progenitor that expresses both CD4 and CD8 during a process known as positive selection. Double-positive (DP) thymocytes with a T cell receptor (TCR) specific for major histocompatibility complex (MHC) class II molecules develop toward the CD4 lineage, whereas DP cells with a TCR specific for MHC class I develop toward the CD8 lineage (Robey and Fowlkes, 1994; Fowlkes and Schweighoffer, 1995; Kisielow and von Boehmer, 1995; Guidos, 1996; Marrack and Kappler, 1997).

The molecular mechanism underlying the developmental choice toward either the helper or the cytotoxic phenotype is not known (Davis and Littman, 1994; von Boehmer, 1996). Since the expression of the CD4 or CD8 coreceptor proteins generally correlates with the phenotype of the differentiated T cell, factors that regulate transcription of these genes probably also participate in directing the development of the helper versus cytotoxic T cell lineages. An understanding of how the

expression of the CD4 and CD8 coreceptors is regulated therefore may also provide insight into molecular processes involved in lineage commitment.

The major regulatory elements governing the expression of murine CD4 have been shown to consist of a T cell-specific enhancer located approximately 13 kb upstream of the transcription start site (Sawada and Littman, 1991) and a lineage-specific silencing element residing within the first intron of *CD4* (Sawada et al., 1994; Siu et al., 1994). The elements regulating the expression of CD8 in vivo are not known. Thymus-derived T cells generally express CD8α and CD8β heterodimers on their surface, whereas extrathymically derived intraepithelial lymphocytes (IEL) and a subset of human natural killer (NK) cells express only CD8αα homodimers (Jarry et al., 1990; Lefrancois, 1991; Moebius et al., 1991). This indicates that the expression of the *CD8α* and *CD8β* genes, which are linked at a distance of about 36 kb on mouse chromosome 6 (Gorman et al., 1988), must be both coordinately and independently regulated. In addition, since CD4 is transcriptionally silenced in CD8 SP cells (Sawada et al., 1994; Siu et al., 1994), CD8 down-regulation in T cells may similarly involve transcriptional silencing. In fact, the expression of both CD4 and CD8 is strongly repressed in hybrids formed between CD4⁺CD8⁺ and CD4⁻CD8⁻ parental lymphoma clones, suggesting that negative factors (or putative silencing factors) in CD4⁻CD8⁻ cells regulate the expression of both CD4 and CD8 (Wilkinson et al., 1991). Taken together, these results point to positive and negative regulatory mechanisms for CD8 transcription.

Several candidate regulatory sequences for CD8 lineage specific expression have been previously reported. Multiple binding sites for the T cell-restricted transcription factor GATA-3 have been identified upstream of the mouse *CD8α* gene (Landry et al., 1993). These GATA-3 binding sites coincide with CD8 lineage-specific DNase I hypersensitivity (DH) sites, and it has been shown that GATA-3 is able to transactivate a CAT-reporter construct containing these sites. It is unlikely that GATA-3 is a major mediator for subset-specific expression of CD8, however, since it is also expressed in CD4 SP T cells (Zheng and Flavell, 1997). Another study implicated the region approximately 300 bp upstream of the GATA-3 binding sites in the transcriptional down-regulation of the *CD8α* gene upon fusion with the BW5147 thymoma (Lee et al., 1994). The role of these elements in vivo remains to be addressed. A T cell-specific enhancer has been mapped in the last intron of the human *CD8α* gene (Hambor et al., 1993). In transgenic mice, this enhancer directed expression of the human *CD8α* reporter gene in NK cells but not in T cells (Kieffer et al., 1996). Since a subset of human NK cells expresses CD8α homodimers (Moebius et al., 1991), the intronic enhancer might be involved in the regulation of CD8 expression in this cell type.

We and others have previously failed to achieve high-level, subset-specific expression of transgene genomic fragments containing the human *CD8α* gene, suggesting that multiple elements, spread over the whole *CD8*

† To whom correspondence should be addressed (e-mail: littman@saturn.med.nyu.edu).

locus, control the expression of the *CD8 α* and *CD8 β* genes (Kieffer et al., 1996; N. Lonberg et al., unpublished data). Recently, Hostert et al. (1997) performed a comprehensive DH study of the 80 kb murine *CD8* locus and identified four clusters of DH sites, three of which are specific for thymocytes. By generating transgenic mice with a P1 clone containing the whole *CD8* locus, they were able to achieve developmentally regulated, tissue-specific and CD8 subset-specific expression of the CD8 transgene. However, this study did not determine whether individual DH clusters or DH sites within these clusters are able to mediate expression.

We identified two DH sites that are specific for CD8-expressing cells and correspond to cluster III of Hostert et al. (1997). A genomic fragment containing the CD8-specific DH sites was tested for its ability to regulate expression of a human CD2 (hCD2) reporter gene in transgenic mice. In these mice, hCD2 was expressed specifically in CD8 SP thymocytes and peripheral CD8 lineage T cells, but not in DP thymocytes. In addition, hCD2 expression was observed in IEL expressing CD8 α homodimers. These results indicate that we have identified an enhancer that directs transcription of the CD8 α gene only in mature thymocytes and T cells. Furthermore, we provide evidence that the activity of this enhancer coincides with positive selection of CD8 lineage thymocytes. These findings are interpreted in the context of recent models that suggest that a specific signal is required for DP thymocytes to commit to the CD8 cytotoxic lineage.

Results

Identification of DH Sites

To identify *cis*-acting elements that are involved in the regulation of *CD8* gene expression, we conducted a study of DH sites (Gross and Garrard, 1988) in regions adjacent to the murine *CD8 α* and *CD8 β* genes. In earlier studies, we identified DH sites flanking the two genes, but, unlike the CD4 enhancer (Sawada and Littman, 1991), these had no significant enhancer activity in transfected cells (K. Chu and D. R. L., unpublished data) and corresponded to regions that failed to direct CD8 expression in transgenic mice (N. Lonberg et al., unpublished data). Therefore we extended our search to a region between the *CD8 β* and *CD8 α* genes and probed for DH sites in CD4 $^-$ CD8 $^+$ (1200M), CD4 $^+$ CD8 $^+$ (AKR1), and CD4 $^+$ CD8 $^-$ (BOH4) T cell lines; in the B cell line M12; and in NIH3T3 fibroblast cells. Two DH sites (designated HS-1 and HS-2) were identified in an approximately 9.0 kb genomic BamHI fragment only in cells that expressed CD8 (e.g., 1200M and AKR1) (Figure 1B). The probe used for Southern hybridization detected a BamHI restriction fragment length polymorphism (RFLP) of either a 9.0 kb fragment in 1200M and AKR1.G.1 cells or of a 7.6 kb fragment in BO4H and M12 cells. NIH3T3 cells appeared to be heterozygous for the BamHI RFLP, explaining the presence of both the 9.0 kb and the 7.6 kb fragments. HS-1 and HS-2, which map approximately 19 and 15 kb upstream of the *CD8 α* gene, respectively, overlap with two DH sites in a region designated cluster III by Hostert et al. (1997) (Figure 1A).

CD8 Subset-Specific Expression of an hCD2 Reporter Gene in Peripheral Lymphocytes

The correlation between CD8 expression and the presence of the DH sites prompted us to test the role of this intergenic segment in the regulation of CD8 expression *in vivo*. We therefore developed a transgenic reporter construct (designated TG-a) in which the 391 bp minimal murine CD8 α promoter (Nakauchi et al., 1987) was cloned upstream of an expression cassette containing a murine CD4 splicing module (composite of the untranslated exon I, part of intron I, and part of exon II) linked to the hCD2 cDNA and the SV40 polyadenylation signal (Sawada et al., 1994) (Figure 1C). Since most parts of intron I are deleted in the CD4 splicing module (including the CD4 silencer region), hCD2 can be expressed in both CD4 and CD8 SP T cells when appropriate regulatory elements are present. The fragment containing both DH sites in the *CD8* locus was isolated from a genomic 129 library homozygous for the 7.6 kb BamHI RFLP (data not shown). This genomic 7.6 kb BamHI fragment was then inserted upstream of the mCD8 α promoter, thereby generating TG-b. These constructs were injected into fertilized (B6/D2) F2-mouse eggs to generate transgenic animals. Transgenic founders identified by Southern blotting of tail DNA were either analyzed directly or backcrossed to C57BL/6 mice to generate transgenic lines that were then analyzed.

Expression of hCD2 was not observed in founders or lines that had integrated TG-a, indicating that the CD8 α promoter is not sufficient to direct expression of the reporter gene (Table 1). In contrast, four of five founders and their progeny prepared with TG-b displayed high-level expression of hCD2 in peripheral CD4 $^-$ CD8 $^+$ T cells but not in CD4 $^+$ CD8 $^-$ T cells (Table 1 and Figure 2A). Furthermore, no expression was observed in B cells (data not shown). This suggested that the 7.6 kb genomic fragment contained a CD8 lineage T cell specific enhancer; alternatively, as observed in the *CD4* gene, this fragment could contain an enhancer plus a silencer that shuts off expression in CD4 $^+$ CD8 $^-$ cells. To determine whether a silencer restricts expression of the reporter to the CD8 lineage, the 7.6 kb fragment was inserted upstream of the CD4 enhancer and promoter (construct c in Sawada et al., 1994). Transgenic mice prepared with this construct expressed the hCD2 reporter in both CD4 and CD8 lineage cells, suggesting that the 7.6 kb fragment contains only a lineage-specific enhancer (data not shown).

The percentage of CD8 SP cells expressing hCD2 varied between the TG-b transgenic lines and ranged from 0.3% to 100%. Some founders transmitted this variegated phenotype to F1 animals, whereas others were mosaic, and lines derived from them showed a higher percentage of hCD2-expressing CD4 $^-$ CD8 $^+$ T cells. In the transgenic line TG-b#4, which expressed hCD2 on all CD4 $^-$ CD8 $^+$ T cells, about 5%–10% of CD4 SP T cells also expressed hCD2, although at a 2.5-fold lower level compared to hCD2 in the CD8 SP population (Table 1 and Figure 2A). Similar observations were made in line TG-b#12, in which about 1.6% of CD4 SP cells expressed hCD2 at 6-fold lower levels compared to CD8 SP T cells. The low-level expression in some CD4 $^+$ CD8 $^-$ T cells most likely reflects positional effects due to the site of transgene integration.

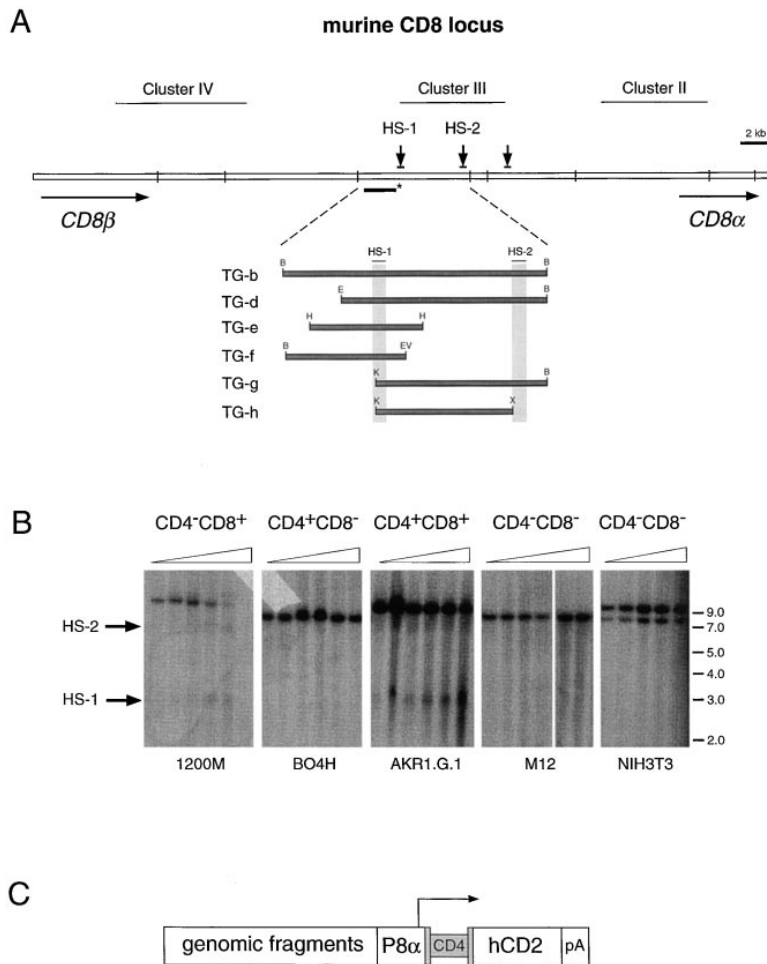


Figure 1. DH Sites in the Murine *CD8* Gene and Corresponding Segments Used for Preparing Transgenic Constructs

(A) (Top) Organization of the *CD8* locus (after Gorman et al., 1988, Hostert et al., 1997; our unpublished data). Vertical bars, BamHI sites; horizontal arrows, position and transcriptional orientation of the *CD8α* and *CD8β* genes. The arrangement of DH clusters II, III, and IV is according to Hostert et al. (1997). HS-1 and HS-2 represent the two CD8-specific DH sites identified in this study that are identical to two of three DH sites (vertical arrows) within cluster III. Bar with asterisk, 2.1 kb KpnI fragment used as a probe for hybridization of the genomic blot.

(Bottom) Map of the genomic fragments used for the generation of transgenic constructs TG-b, TG-d, TG-e, TG-f, TG-g, and TG-h. Light shaded areas, the approximate location of the DH sites HS-1 and HS-2. Restriction enzyme sites are BamHI (B), EcoRI (E), EcoRV (EV), HindIII (H), KpnI (K), and XbaI (X). Only sites relevant for the transgenic constructs are shown.

(B) Nuclei from the cell lines indicated were isolated and treated with increasing amounts of DNase I. Subsequently genomic DNA was isolated, BamHI digested, and transferred onto a gene screen membrane and hybridized with the probe shown in Figure 1A. Lanes 1–6 for 1200M, BO4H, AKR1.G.1, and M12 included 0, 2, 4, 6, 8, and 16 $\mu\text{g}/\mu\text{l}$ DNase I, respectively, whereas for NIH3T3 cells 0, 2, 4, 6, and 8 $\mu\text{g}/\mu\text{l}$ DNase I (lanes 1–5) were used. Arrows, DH sites. The presence of either a ~ 9.0 kb BamHI fragment (in 1200M and AKR1.G.1) or a ~ 7.6 kb BamHI fragment (in BO4H and M12) or both (NIH3T3) indicates a BamHI RFLP recognized by the probe used for Southern hybridization. Molecular size markers (kilobases) are shown at right.

(C) Schematic representation of the transgenic reporter construct, in which genomic fragments shown in Figure 1A were inserted upstream from the *CD8α* promoter.

To test whether the CD8-specific enhancer works exclusively in conjunction with the CD8 promoter or rather as an independent *cis*-regulatory element, construct TG-c was generated by linking the 7.6 kb genomic fragment with the murine CD4 promoter (Sawada and Littman, 1991). The enhancerless CD4 promoter construct does not mediate expression of hCD2 (Sawada et al., 1994). Analyses of peripheral T cells from TG-c founders and lines revealed that the 7.6 kb genomic element together with the CD4 promoter still functions as a CD8 SP subset-specific enhancer (Table 1 and Figure 2A). In addition, the enhancer functions in an orientation-independent manner, since the genomic fragment was inserted in the opposite orientation in TG-c compared to that in TG-b.

To narrow down the genomic region mediating the subset-specific expression of the hCD2 reporter gene, additional transgenic constructs containing overlapping segments of the 7.6 kb fragment were generated (Figure 1A). Founders or lines of transgenes TG-d (containing both DH sites on a 6 kb EcoRI–BamHI fragment) or TG-e

(containing HS-2 and at least part of HS-1 on a 5 kb KpnI–BamHI fragment; Figure 1A) expressed hCD2 at high levels in a CD8 subset-specific manner (Table 1 and Figure 2B). In these animals, no expression above background (defined by hCD2 antibody staining of non-transgenic littermates) was observed in $\text{CD4}^+\text{CD8}^-$ T cells. To test the role of the DH sites individually, constructs TG-e and TG-f, both containing only HS-1 (either in 3.3 kb HindIII or in 3.5 kb BamHI–EcoRV fragments, respectively) were generated. As shown in Table 1, transgenic constructs containing HS-1 failed to mediate expression of hCD2 in a high percentage of $\text{CD4}^-\text{CD8}^+$ T cells. In 4 of 21 founders (TG-e#3 and TG-e#6, and TG-f#9 and TG-f#36), however, a low but significant population (2%–4%) of CD8 SP T cells expressing hCD2 was observed (Table 1). Lines generated from some of these founders (e.g., TG-e#3) showed an increase (10%) in the $\text{CD4}^-\text{CD8}^+$ population that expressed hCD2, whereas in others the proportion remained low (e.g., TG-f#36). This result indicates that fragments containing HS-1 have a CD8 subset-specific activity, but that its

Table 1. hCD2 Expression on Peripheral CD4⁺CD8⁻ and CD4⁻CD8⁺ T Cells from Mice with Transgenes TG-a through TG-h

Construct	Founder		CD4 ⁺ T Cells		CD8 ⁺ T Cells	
		Copy Number	hCD2 ⁺ (%)	Mean Fluorescence	hCD2 ⁺ (%)	Mean Fluorescence
TG-a	1*	(10)	0	(—)	0	(—)
	7*	(8)	0	(—)	0	(—)
TG-b	2*	(30)	0.3	(—)	56.7	(135.6)
	3*	(6)	0.1	(—)	6.5	(127.9)
	4*	(4)	5.7	(48.8)	99.9	(116.9)
	7*	(4)	0.3	(—)	0.3	(—)
	12	(26)	1.4	(67.9)	27.7	(428.4)
TG-c	2*	(7)	1.6	(65.1)	30.3	(201.8)
	3	(2)	0.1	(—)	2.0	(22.5)
TG-d	3*	(10)	0.4	(62.3)	40.0	(317.2)
	9*	(7)	0.2	(—)	2.7	(211.8)
	11	(8)	0.2	(—)	3.0	(134.9)
	13	(6)	0.9	(27.5)	12.9	(153.0)
TG-e	14*	(2)	0.1	(—)	85.2	(56.5)
	3	(10)	0.2	(—)	1.9	(96.6)
	6	(6)	0.1	(—)	3.8	(34.9)
	8	(4)	0	(—)	0	(—)
	16	(4)	0.3	(—)	0.9	(49.6)
	25	(7)	0.2	(—)	0.5	(61.0)
	114	(5)	1.3	(19.3)	0	(—)
	125	(10)	0	(—)	0.1	(—)
	132	(14)	0.1	(—)	0.9	(133.2)
	134	(2)	0.4	(24.7)	0	(—)
	140	(10)	0.1	(—)	0.4	(137.8)
	150	(3)	0.1	(—)	0.3	(—)
	156	(8)	0.3	(—)	1.6	(29.5)
	157	(2)	0.1	(—)	0.1	(—)
TG-f	2	(1)	0	(—)	0.5	(154.8)
	9	(5)	0.2	(—)	3.1	(51.3)
	14	(1)	0.1	(—)	0	(—)
	24	(2)	0	(—)	0	(—)
	33	(5)	0.1	(—)	0.6	(110.5)
	36	(7)	0.1	(—)	1.9	(114.0)
	46	(4)	0	(—)	0.6	(110.1)
	52	(2)	0.4	(13.1)	0.2	(—)
	9	(6)	0.6	(26.2)	61.5	(69.3)
	13	(5)	0.1	(—)	27.9	(44.7)
TG-g	17	(6)	0.6	(124.2)	12.3	(167.6)
	20	(8)	0.2	(—)	46.6	(72.0)
	25	(14)	0.2	(—)	8.4	(157.6)
	10	(4)	0	(—)	1.6	(26.5)
	15	(20)	0	(—)	0.2	(—)
TG-h	16	(4)	0	(—)	0	(—)
	25	(4)	0	(—)	0.1	(—)
	27	(2)	0	(—)	0	(—)
	28	(3)	0	(59.9)	0.4	(50.8)
	30	(16)	0	(—)	1.2	(59.6)
	35	(2)	0.1	(39.6)	0	(—)
	37	(2)	0	(—)	0	(—)
	38	(5)	0	(—)	1.0	(36.4)
	40	(8)	0.1	(19.4)	0.4	(68.8)

Peripheral lymphocytes (from either lymph nodes or blood) were isolated and analyzed by three-color flow cytometry. The percentage of hCD2-expressing cells (together with the mean fluorescence) in either the CD4 SP or the CD8 SP T cell compartment is shown. The copy number of transgenes in each founder is indicated.

* A progeny was used for analysis.

activity is weak and highly susceptible to position effects.

To determine whether *cis*-acting sequences containing HS-2 are required for enhancer function, we deleted a 1kb XbaI-BamHI fragment containing this DH site from the insert of TG-g, thereby generating construct TG-h. Among the 11 TG-h founder animals analyzed, in only some there was a very low percentage of CD4⁻CD8⁺ T cells expressing hCD2, in the same range as observed for TG-e and TG-f (Table 1 and Figure 2).

This indicates that a major *cis*-regulatory element is located within the deleted 1 kb XbaI-BamHI fragment. It remains to be determined whether HS-2 alone or in combination with other sequences (including HS-1) within the 4 kb KpnI-XbaI fragment is required for optimal enhancer function.

hCD2 Expression on IEL

On thymus-derived peripheral T cells, the CD8 molecule exists predominantly as a heterodimer formed by the

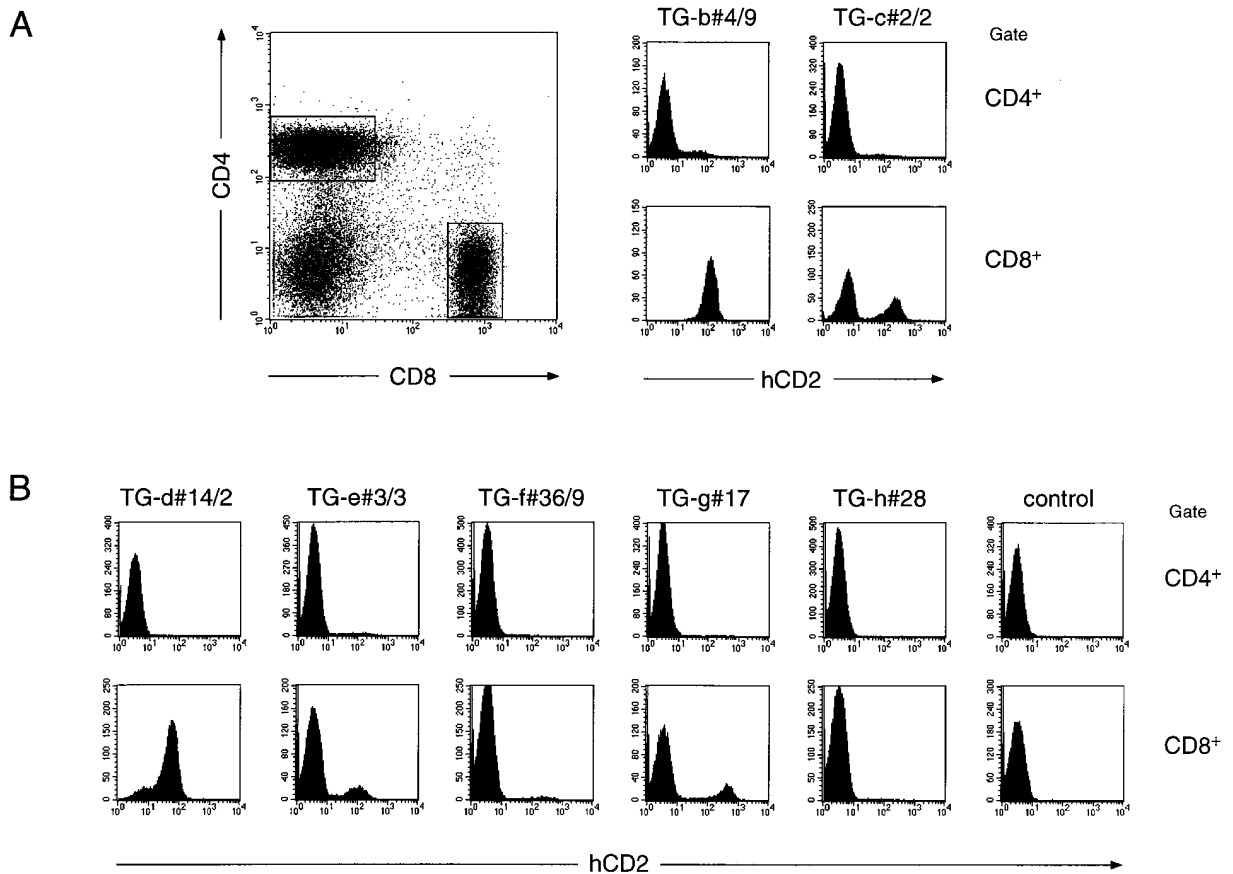


Figure 2. CD8 Subset-Specific Expression of an hCD2 Reporter in Peripheral Lymphocytes

Three-color flow cytometry analysis was performed on lymph node cells isolated from the transgenic lines indicated. Histograms show hCD2 expression on T cells gated either on the CD4⁺ or CD8⁺ population. Shown are representative gates for the CD4⁺ and CD8⁺ T cell population. (A) Expression of hCD2 from constructs containing the 7.6 kb genomic fragment together with the mCD8 α (TG-b) or the mCD4 promoter (TG-c).

(B) Expression of hCD2 from constructs containing the mCD8 α promoter together with subregions from the 7.6 kb genomic fragment. For founders TG-g#17 and TG-h#28 and for the transgenic lines TG-b#4/9 (9 indicates progeny #9), TG-c#2/2, and TG-d#14/2, Table 1 provides the percentage of hCD2-expressing cells within the CD4⁺ or CD8⁺ population. TG-e#3/3 and TG-f#36/9 represent progeny from founders (TG-e#3 and TG-f#36, respectively) included in Table 1.

CD8 α and CD8 β chains, whereas extrathymically derived CD4⁻CD8⁺ T cells, such as the majority of intraepithelial TCR $\gamma\delta$ and some TCR $\alpha\beta$ T lymphocytes (IEL) of the gut, express only CD8 $\alpha\alpha$ homodimers (Lefrancois, 1991; Moebius et al., 1991). Since CD8 α and CD8 β are linked on mouse chromosome 6 (Gorman et al., 1988) (Figure 1A), it is possible that the identified enhancer is specific for directing expression of CD8 α , CD8 β , or both genes. By analyzing CD8 SP T cells, one cannot distinguish among these possibilities.

However, if the CD8 enhancer is specific for CD8 α , one would expect expression of the transgenic hCD2 reporter on extrathymically derived IEL, unless the regulatory elements responsible for CD8 α expression are different in thymus-derived T cells and extrathymically derived IEL. To test this, IEL from the gut were isolated and analyzed for the expression of hCD2. CD8 $\alpha\alpha$ ⁺ IEL of TCR $\gamma\delta$ (Figure 3A) and TCR $\alpha\beta$ (data not shown) lineages expressed hCD2 in transgenic lines obtained with TG-b, TG-c, TG-d and TG-g. hCD2 reporter expression was also observed in CD8 $\alpha\beta$ -expressing IEL (Figure 3B), which are almost exclusively of the TCR $\alpha\beta$ lineage (Lefrancois, 1991). Thus, expression of the hCD2 transgenic

reporter correlates with expression of CD8 α in mature peripheral T lymphocytes, including IEL, suggesting that the enhancer is specific for the CD8 α gene. This result does not rule out the possibility that this enhancer is also required for CD8 β expression. However, additional positive or negative elements would then be required to account for the differential expression of CD8 β .

Expression Analysis during T Cell Development

To examine whether the enhancer is also involved in the regulation of CD8 gene expression during T cell development, we analyzed hCD2 expression in thymocytes from transgenic animals that had subset-specific expression in the periphery (e.g., TG-b, TG-d, and TG-g). In contrast to similar CD4 enhancer and silencer reporters (Sawada et al., 1994), hCD2 was expressed exclusively in CD8 SP thymocytes, and not in the DP compartment (Figure 4). In addition, the proportion of CD8 SP thymocytes expressing hCD2 was consistently lower than the proportion in the mature peripheral CD8 lineage cells. For example, in line TG-b#4, hCD2 was expressed in 100% of peripheral CD4⁻CD8⁺ T cells but in only 80%–90% of CD8 SP thymocytes.

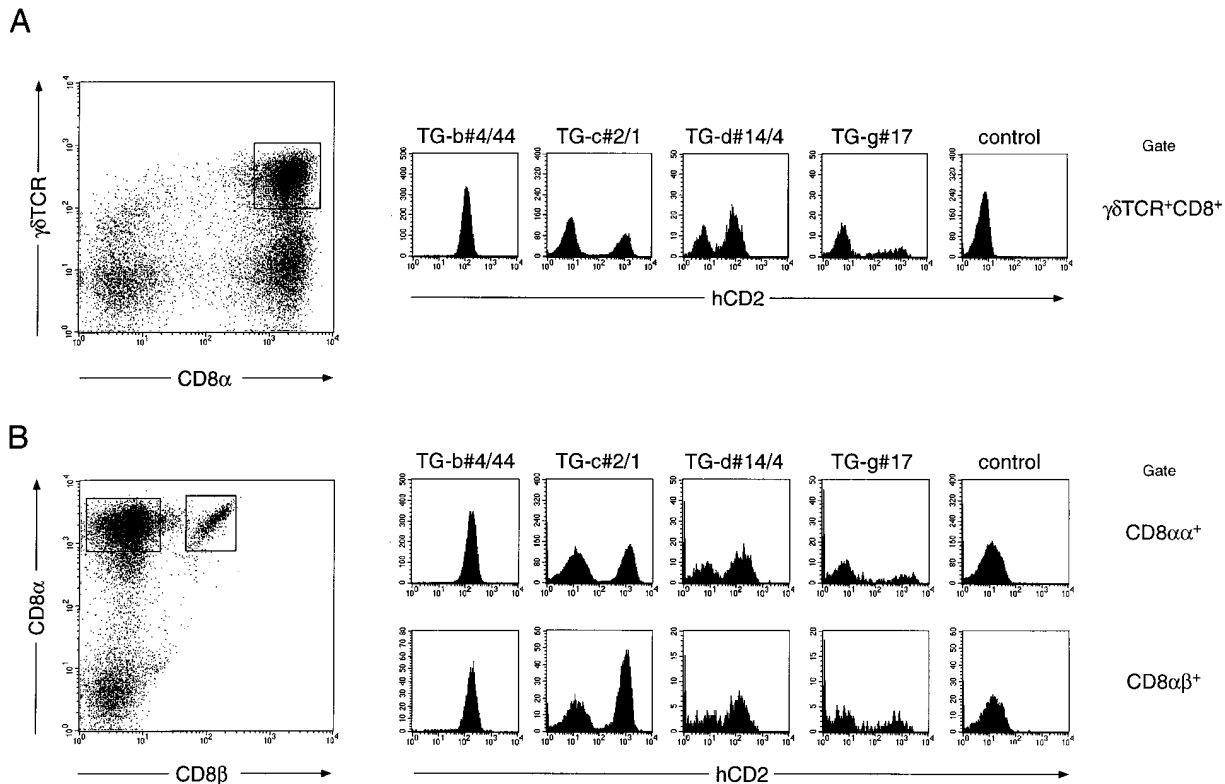


Figure 3. hCD2 Expression on IEL

Intestinal IEL from the transgenic lines indicated were isolated and analyzed for hCD2 expression by three-color flow cytometry. (A) Histograms show expression of hCD2 on $\text{TCR}\gamma\delta^+\text{CD8}\alpha\alpha^+$ IEL. A representative gate is shown at left. (B) Expression of hCD2 on $\text{CD8}\alpha\alpha^+$ and $\text{CD8}\alpha\beta^+$ subpopulations of IEL. Representative gates for the $\text{CD8}\alpha\alpha^+$ and $\text{CD8}\alpha\beta^+$ population are shown at left.

The population of thymocytes that express CD8 can be subdivided into cells at multiple stages. DP cells that undergo positive selection partially down-regulate surface CD4 and CD8 and then progress through $\text{CD4}^{\text{lo}}\text{CD8}^+$ to $\text{CD4}^-\text{CD8}^+$ stages in the presence of MHC class I ligand. During final maturation, the thymocytes up-regulate the level of TCR and down-regulate HSA expression (van Meerwijk and Germain, 1993). We next analyzed hCD2 expression in these CD8 lineage subsets to determine whether reporter gene transcription correlates with the developmental stage. Analysis of $\text{CD4}^+\text{CD8}^+$, $\text{CD4}^{\text{lo}}\text{CD8}^+$, and $\text{CD4}^-\text{CD8}^+$ thymocytes showed a progressive increase in the percentage of cells expressing the reporter gene, indicating a link between enhancer activity and maturation of thymocytes (Figure 5A).

To probe this further, we determined the developmental status of hCD2^+ versus hCD2^- cells within the $\text{CD4}^{\text{lo}}\text{CD8}^+$ compartment by analyzing the expression of $\text{TCR}\alpha\beta$, HSA, and CD69 on these cells. The $\text{CD4}^{\text{lo}}\text{CD8}^+$ population has been shown to contain thymocytes committed to the CD8 lineage (Lundberg et al., 1995; Suzuki et al., 1995; Lucas and Germain, 1996). As shown in Figure 5B, hCD2^- cells were of the immature phenotype ($\text{CD4}^{\text{lo}}\text{CD8}^+\text{HSA}^{\text{hi}}\text{TCR}\alpha\beta^{\text{int/hi}}$), and a proportion of these cells expressed CD69, indicating that they were in the process of positive selection (Bendelac et al., 1992; Swat et al., 1993). In contrast, the surface phenotype of hCD2^+

cells, $\text{CD4}^{\text{lo}}\text{CD8}^+\text{HSA}^{\text{lo}}\text{TCR}\alpha\beta^{\text{hi}}$, indicates that these cells were mature and had completed positive selection. Consistent with the mature status of these cells, they also up-regulated H-2K^b (Kisielow et al., 1984; Scollay and Shortman, 1985) and CD5 (Takahama and Singer, 1992) (data not shown). Similar surface phenotypes of hCD2^- and hCD2^+ cells were observed within the $\text{CD4}^-\text{CD8}^+$ compartment (data not shown). These results demonstrate that the CD8 enhancer described here is activated only in the latest stage of CD8 SP differentiation.

Discussion

In this study we describe the identification of an enhancer that directs expression of CD8 in vivo exclusively in mature thymocytes and T cells. This enhancer was found to colocalize with two CD8 lineage-specific DH sites in the region between the genes encoding the two CD8 subunits. Genomic fragments containing both DH sites directed expression of a reporter gene in CD8 SP thymocytes and T cells as well as in IEL, but not in DP thymocytes. The 7.6 kb BamHI fragment functioned in an orientation-independent manner and with either the murine CD8 α or the heterologous murine CD4 promoter, indicating that it contains independent *cis*-acting regulatory elements that mediate expression of CD8 selectively in mature T cells. In addition, up-regulation of reporter gene expression correlated with the transition

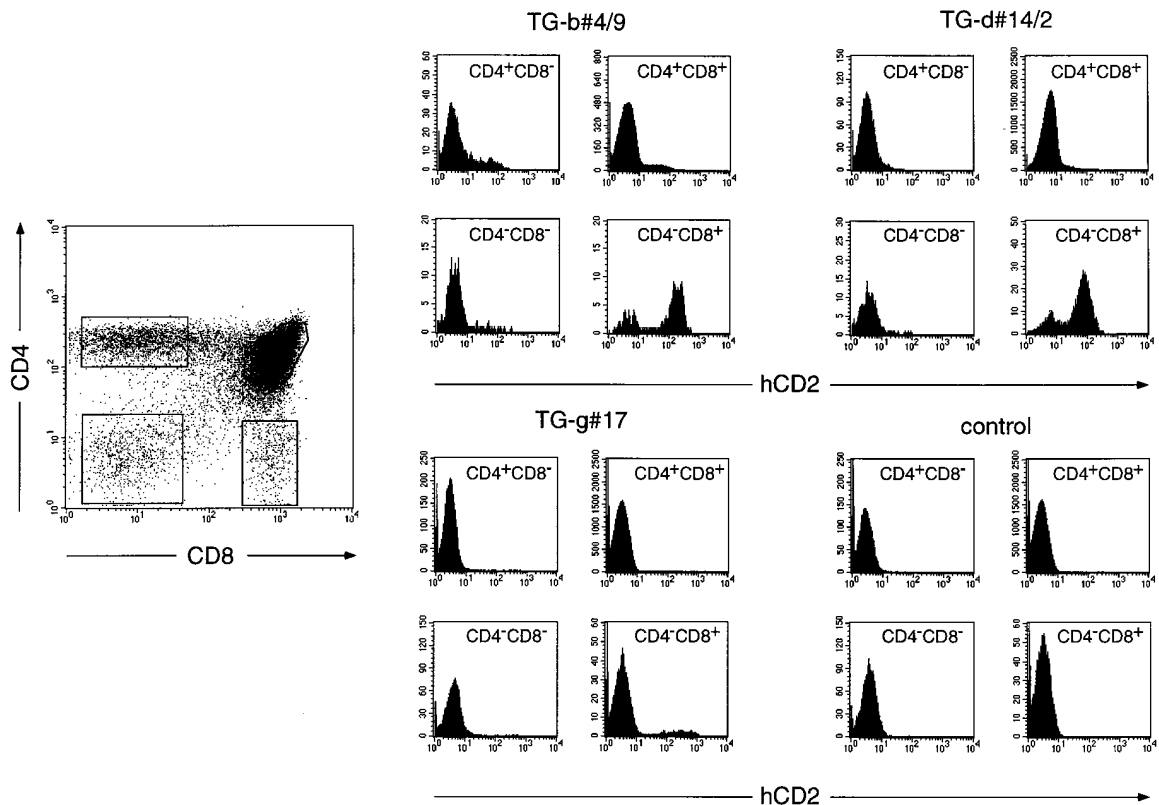


Figure 4. hCD2 Expression during Thymocyte Development

Thymocytes from the transgenic animals indicated were analyzed by three-color flow cytometry. Histograms show expression of hCD2 in the thymocyte population. Representative gates for either the $CD4^+CD8^-$, $CD4^+CD8^+$, $CD4^+CD8^-$, or $CD4^-CD8^+$ population are shown at left.

of committed CD8 lineage cells from the $HSA^{hi}TCR^{int/hi}$ to the $HSA^{lo}TCR^{hi}$ phenotype that marks completion of positive selection.

Recently, four clusters of DH sites within 80 kb encompassing the murine *CD8* locus were described. Transgenic mice prepared with a large genomic fragment that included all four DH clusters (three of which are specific for thymocytes) exhibited appropriate expression of the transgene in developing thymocytes and in peripheral T cells (Hostert et al., 1997). Expression of this large transgene was found to be mosaic, most likely due to position-effect variegation (Festenstein et al., 1996), prompting the authors to suggest that endogenous expression of the *CD8* genes might not be dependent on the presence of a locus control region (Grosveld et al., 1987; Greaves et al., 1989; Martin et al., 1996). By generating transgenic mice with HS-1 and HS-2, which overlapped two of the three thymocyte-specific DH sites within cluster III described by Hostert et al. (1997), we also observed mosaic patterns of transgene expression. This was not surprising, considering that position-effect variegation was observed even with the large genomic *CD8* locus (Hostert et al., 1997). However, we cannot exclude the possibility that the transgenes would be less susceptible to position effects if additional *cis* elements from the *CD8* locus were included.

The majority of thymus-dependent, MHC class I-restricted $CD4^-CD8^+$ T cells express $CD8\alpha\beta$ heterodimers on their surface, whereas a large population of

IEL from the gut express $CD8\alpha\alpha$ homodimers instead (Jarry et al., 1990; Lefrancois, 1991). The expression of hCD2 on IEL of the $CD8\alpha\alpha^+TCR\gamma\delta$ and $TCR\alpha\beta$ lineages (either of the $CD8\alpha\alpha$ or $CD8\alpha\beta$ subtype) indicates that the enhancer is specific for the *CD8\alpha* gene. However, it remains possible that the enhancer region also has an effect on the *CD8\beta* gene located upstream (relative to its transcriptional orientation) of the *CD8* enhancer. This would imply that a silencer element may repress expression of $CD8\beta$ in IEL or that the *CD8* enhancer may function in conjunction with the *CD8\beta* promoter in a cell type-restricted manner. A recent study suggests that the *CD8\beta* gene may indeed be negatively regulated. Transgenic mice prepared with a 95 kb human genomic DNA fragment that contained the entire *CD8\beta* gene were shown to express hCD2 not only in CD8 SP cells and $CD8\alpha\beta^+$ IEL of the $TCR\alpha\beta$ lineage, but also in $CD8\alpha\alpha^+TCR\alpha\beta$ IEL (Kieffer et al., 1997). Whether expression in the $CD8\alpha\alpha^+TCR\alpha\beta$ population reflects the absence of a negatively acting element in the transgene or is due to cross-species differences remains to be determined.

Maturation of thymocytes of the $TCR\alpha\beta$ lineage involves intricate regulation of the levels of cell surface CD4 and CD8 at different stages of development (Kisielow and von Boehmer, 1995; Zuniga-Pflucker and Lenardo, 1996). Double-negative thymocytes that express a cell surface pre-TCR following productive rearrangement of the *TCR\beta* gene undergo selection that is marked

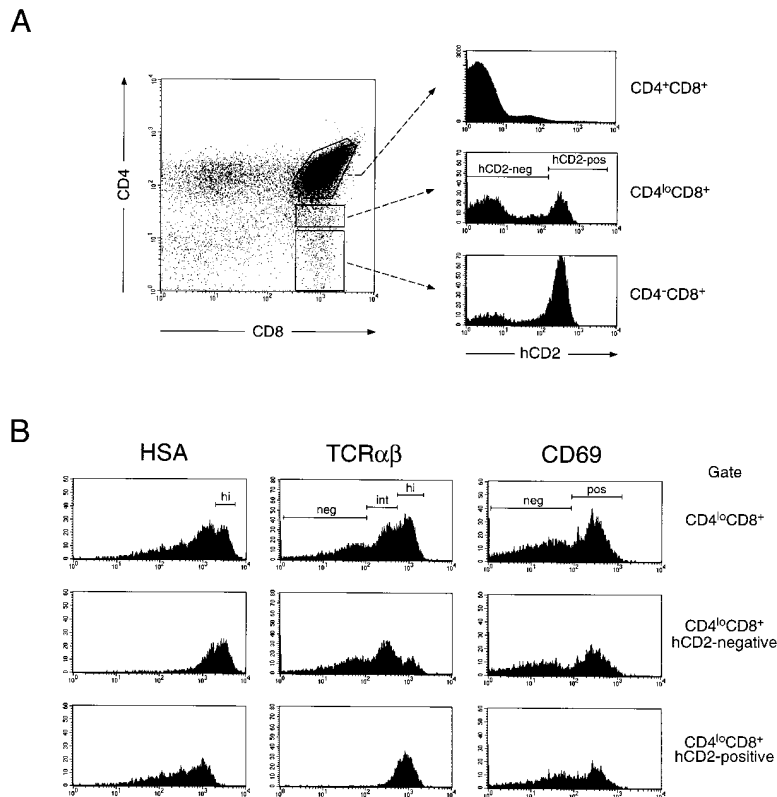


Figure 5. Onset of hCD2 Expression during CD8 SP Thymocyte Development

Thymocytes from the transgenic line TG-b#4 were analyzed by four-color flow cytometry for the expression of hCD2, HSA, TCRαβ, and CD69.

(A) Histograms showing hCD2 expression levels on different subpopulations of CD8⁺ thymocytes. Gating areas for the CD4⁺CD8⁺, CD4^{lo}CD8⁺, and CD4⁻CD8⁺ populations are shown at left. hCD2⁻ and hCD2⁺ regions in the CD4^{lo}CD8⁺ histogram indicate representative gates used for further analysis, presented in Figure 5B.

(B) Expression of HSA, TCRαβ, and CD69 on total CD4^{lo}CD8⁺ thymocytes and on subsets of this population that are either negative or positive for hCD2 expression, as defined in Figure 5A.

by initial expression of low levels of CD8 and a subsequent increase in both CD4 and CD8 levels to give rise to DP cells. During subsequent MHC ligand-dependent selection, DP cells commit to either the CD4⁺CD8⁻ T helper lineage or the CD4⁻CD8⁺ cytotoxic lineage. The mechanism by which DP thymocytes differentiate into mature SP cells remains controversial (von Boehmer, 1996). We and others have proposed that commitment to the CD4 or CD8 lineage is stochastic (Chan et al., 1993; Davis et al., 1993; van Meerwijk and Germain, 1993) and is followed by selection of thymocytes that have coreceptors with MHC specificity that matches that of the TCR. More recent studies, using in vitro and in vivo analyses of highly purified thymocyte subpopulations, favor a more complex mechanism involving an intermediate CD4⁺CD8^{lo} population that gives rise to both the CD4 and CD8 SP lineages (Lucas et al., 1995; Lundberg et al., 1995; Suzuki et al., 1995; Benveniste et al., 1996; Lucas and Germain, 1996). Differentiation of the intermediate cells appears to require initiation of positive selection by TCR signaling in response to either MHC class I or class II; however, subsequent differentiation toward the CD8 lineage, by way of CD4^{lo}CD8⁺ intermediate cells, occurs only if MHC class I is present (Suzuki et al., 1995; Benveniste et al., 1996).

This has led to the suggestion that in the absence of an instructional signal requiring TCR (and probably CD8) interaction with class I, thymocytes default to the CD4 lineage. It has been proposed that delivery of such a signal also involves activation of Notch, which has been shown to bias DP cells to commit to the CD8 lineage when it is expressed as a constitutively active protein in transgenic mice (Robey et al., 1996). Consistent with

this model, stimulation of DP thymocytes in vivo or in organ culture with anti-CD3 monoclonal antibodies results in the appearance of CD4 SP cells but not CD8 SP cells (Groves et al., 1995; Kearse et al., 1995; Cibotti et al., 1997). In addition, deficiency of ZAP-70 in humans results in complete loss of CD8 lineage cells, but only in a partial defect in maturation of CD4 lineage cells, suggesting that there may be more stringent signaling requirements for positive selection of CD8 lineage cells (Arpaia et al., 1994; A. C. Chan et al., 1994; Elder et al., 1994).

The correlation between the mature CD8 enhancer activity and positive selection can now be interpreted in the context of current models of thymocyte lineage commitment and may provide important insight into the underlying mechanisms. A major implication of our results is that there must exist distinct *cis*-acting elements that direct CD8 expression solely in DP cells. It is therefore likely that after DP thymocytes receive TCR-mediated signals to initiate positive selection, they down-regulate this early CD8 enhancer (E_{BDP}). An additional signal provided by interaction with MHC class I (possibly Notch-mediated) may then activate both the CD4 silencer (S₄) and the mature CD8 SP enhancer (E_{8SP}) described here, resulting in the transition from CD4⁺CD8^{lo} to CD4^{lo}CD8^{lo} to CD4^{lo}CD8⁺ to CD4⁻CD8⁺ cells (Figure 6). The gradual increase in the proportion of hCD2⁺ cells observed as thymocytes progress from DP to CD8 SP cells (Figure 5A) is consistent with this notion. In cells that fail to interact with class I, extinction of E_{BDP} function in the face of unopposed CD4 enhancer activity would mark commitment to the CD4 SP T helper lineage.

An obvious paradox is presented by the finding that

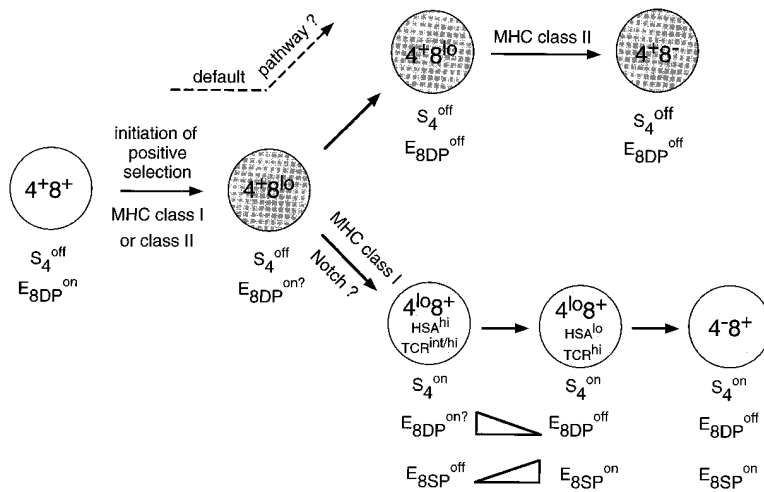


Figure 6. Model for CD8 Enhancer Function during Differentiation of DP to SP Thymocytes

Positive selection is postulated to initiate down-regulation of a putative CD8 enhancer specific for DP cells (E_{8DP}). In the absence of an additional signal, cells default to the CD4 lineage. If the thymocytes receive a CD8-specific instructional signal (possibly involving Notch), they activate the CD4 silencer (S_4) and the mature CD8 enhancer (E_{8SP}). The CD4 enhancer is postulated to be active throughout this sequence of differentiation.

E_{8SP} is activated only in mature ($TCR^{hi}HSA^{lo}$), and not in immature ($TCR^{int}HSA^{hi}$), $CD4^{lo}CD8^{+}$ thymocytes (Figure 5B). It therefore remains unclear how CD8 is initially down-regulated, upon receipt of the positive selection signal, and then reexpressed at high levels prior to activation of E_{8SP} (Figure 6). We favor a model in which down-regulation of CD8 (in $CD4^{+}CD8^{lo}$ cells) may be a transient posttranslational effect, due to endocytosis following MHC class I engagement by the TCR. In such a model, E_{8DP} in class I specific thymocytes would remain active longer than in the cells of the same surface phenotype destined to default to the CD4 lineage. Alternatively, this result may reflect a requirement for additional *cis*-acting sequences, absent in our transgenic constructs, to initiate E_{8SP} activity earlier in development. Resolution of this paradox will require the use of the putative E_{8DP} elements in experiments similar to those described here.

Our results suggest that CD4 silencer activity is initiated before that of E_{8SP} (Figure 6). This observation must be interpreted with caution, however, because recent studies suggest that there may also be distinct CD4 enhancer functions in DP versus CD4 SP thymocytes (Salmon et al., 1996; Adlam et al., 1997). Lucas and Germain (1996) have shown that suitable TCR engagement of DP thymocytes leads initially to down-regulation of both CD4 and CD8 ($CD4^{lo}CD8^{lo}TCR^{int}$), prior to reexpression of CD4 ($CD4^{+}CD8^{lo}TCR^{int/hi}$). This apparently transient decrease in CD4 expression may also be due to a switch in CD4 enhancer usage. Although it is clear that CD4 enhancer function is intact in CD8 SP cells (Sawada et al., 1994), further studies are required to determine whether there are additional elements involved in temporal regulation of CD4 expression during thymopoiesis.

As noted above, the element(s) responsible for expression of CD8 in the DP population remain to be identified. It will be important to determine whether DNA sequences within the peripheral enhancer, E_{8SP} , contribute to the function of E_{8DP} , or whether E_{8DP} is a distinct and independent enhancer. Future experiments, including deletion of E_{8SP} by homologous recombination, should help to answer this question.

The transcriptional regulation of the *CD4* and *CD8* genes is tightly linked to the functional program of the

developing T cell (S. H. Chan et al., 1994; Corbella et al., 1994; Robey et al., 1994). Thus, activation of *trans*-acting factors that regulate the CD4 silencer and the mature CD8 enhancer is likely to involve components that are common to a cytotoxic T cell differentiation program. To bridge our gap in understanding the nature of CD8 lineage-specific signaling and the commitment to the cytotoxic program, it will be necessary to identify the key nuclear factors. Elucidation of the mechanism of CD8 gene regulation will therefore provide important insights into how functionally distinct T lymphocytes arise from multipotential precursors.

Experimental Procedures

Cell Lines and Culture

The B cell line M12, the murine CD4 SP T cell hybridoma B04H, and the murine 1200M thymoma (CD8 SP) were gifts from R. Grosschedl (University of California, San Francisco), N. Shastri (University of California, Berkeley), and J. Allison (University of California, Berkeley), respectively. The murine thymoma AKR.1.G.1 ($CD4^{+}CD8^{+}$) and NIH3T3 fibroblasts were from the American Type Culture Collection. All cell lines were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum (FCS) and antibiotics.

DH Site Analysis

Nuclei were isolated and subjected to DNase I as described by Landry et al. (1993). In brief, 6×10^7 cells were washed once in phosphate-buffered saline and then lysed on ice for 5 min in 1 ml of reticulysate standard buffer (RSB) (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, and 3 mM MgCl₂) supplemented with 0.5% NP-40. The nuclei were pelleted and washed once with 1 ml of RSB and resuspended in 600 μ l of RSB. Nuclei (100 μ l) were added to Eppendorf tubes containing different amounts of DNase I (final concentrations 0, 2, 4, 6, 8, and 16 μ g/ μ l) and incubated at 37°C for 2 min. The DNase I reaction was stopped by adding 100 μ l of RSB containing 10 mM EDTA and 2% sodium dodecyl sulfate, and 40 μ g of proteinase K was added. After 2–5 hr of incubation at 37°C, the DNA was extracted with phenol and chloroform and precipitated with ethanol. Genomic DNA (20 μ g) was digested with BamHI and analyzed by Southern blotting (Sambrook et al., 1989).

Generation of the Transgenes

Transgene TG-a was generated from the mCD4 reporter construct (Sawada et al., 1994) by replacement of the XbaI-KpnI CD4 promoter fragment with a XbaI-KpnI polylinker and subsequent insertion of the PCR-amplified murine CD8 α promoter (nucleotides 1–391; Nakauchi et al., 1987). A BamHI fragment containing the two DH sites was isolated from a 129 genomic library (Stratagene) and subcloned

into pBS. The final transgenic constructs were derived either by direct cloning of genomic subfragment into TG-a or by means of pBS-CD8 α promoter-genomic fragment intermediates. Details of the protocol can be obtained upon request.

Generation of Transgenic Mice

F2 eggs of (B6/D2) mice were injected with the different transgenes according to standard procedures (Hogan et al., 1994). Founders were identified by Southern blotting with a probe from the CD4 splicing module (Sawada et al., 1994), and transgene copy number was determined by phosphorimager analysis. Transgenic founders were either analyzed directly or crossed to C57BL/6 mice to generate lines. All mice analyzed were aged 4–16 weeks.

Flow Cytometric Analysis and Antibodies

Thymocytes, spleen, and lymph nodes were removed from euthanized mice and placed into 60 mm tissue culture dishes containing phosphate-buffered saline supplemented with 2% FCS and 0.1% sodium azide (staining buffer). Cell suspensions were made by passing the tissue through a 70 μ m nylon cell strainer. The cell suspension was washed once in staining buffer, and flow cytometry was performed by staining $5\text{--}10 \times 10^5$ cells with the appropriate antibodies on ice for 30 min. Cells were washed and either analyzed or stained with secondary antibodies on ice for another 30 min. Cells were analyzed using a Becton Dickinson FACScan flow cytometer and CellQuest software. The following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-hCD2 (Leu 5b) from Becton Dickinson; FITC- or biotin (bio)-conjugated anti-hCD2 (clone G11), phycoerythrin (PE)-conjugated anti-mCD8 α (CT-CD8 α), FITC-anti-mCD8 β (CT-CD8 β), TC-anti-mCD4 (CT-CD4), PE- or bio-anti-mCD3 (Clone 500-A2), and TC-streptavidin from Caltag; and APC-anti-mCD8 (53-6.7), APC-anti-mCD4 (RM4-5), FITC- or bio-anti-HSA (M1/69), bio-anti-CD69 (H1.2F3), bio-anti-mTCR $\alpha\beta$ (H57-597), bio-anti-mTCR $\gamma\delta$ (GL3), bio-anti mCD3 ϵ (145-2C11), bio-anti-mCD5 (53-7.3), PE-anti-mH-2K b (AF6-88.5), and PE-streptavidin from Pharmingen.

Isolation of IEL from the Gut

To isolate IEL, the gut was removed from the peritonium of euthanized mice and the lumen was washed by flushing with RPMI supplemented with 2% FCS and antibiotics. The gut was turned inside-out over polyethylene tubing and incubated in 200 ml of RPMI (10% FCS, antibiotics, and 20 mM HEPES) at 37°C for 45 min in a shaker with low agitation to release the IEL from the intestinal epithelium into the medium. The lymphocytes were pelleted by centrifugation at 2000 rpm for 10 min at room temperature, resuspended in medium, and purified by 37% Percoll centrifugation (1750 rpm for 30 min at room temperature). After two washings with staining buffer, the IEL were first incubated for 5 min on ice with Fc-block (Pharmingen) and subsequently stained with the appropriate antibodies.

Acknowledgments

We thank D. Kioussis for communicating results prior to publication; J. Lafaille for help with isolation of IEL; A. Auerbach for help with the preparation of transgenic mice; and C. Davis, V. KewalRamani, and D. Unutmaz for comments on the manuscript. W. E. was supported by an Erwin-Schrödinger postdoctoral fellowship from the Fonds zur Förderung der wissenschaftlichen Forschung. D. R. L. is an investigator of the Howard Hughes Medical Institute.

Received August 12, 1997.

References

Adlam, M., Duncan, D.D., Ng, D.K., and Siu, G. (1997). Positive selection induces CD4 promoter and enhancer function. *Int. Immunol.* **9**, 877–887.
Arpaia, E., Shahar, M., Dadi, H., Cohen, A., and Roifman, C.M. (1994). Defective T cell receptor signaling and CD8 $^+$ thymic selection in humans lacking Zap-70 kinase. *Cell* **76**, 947–958.
Bendelac, A., Matzinger, P., Seder, R.A., Paul, W.E., and Schwartz,

R.H. (1992). Activation events during thymic selection. *J. Exp. Med.* **175**, 731–742.
Benveniste, P., Knowles, G., and Cohen, A. (1996). CD8/CD4 lineage commitment occurs by an instructional/default process followed by positive selection. *Eur. J. Immunol.* **26**, 461–471.
Chan, S.H., Cosgrove, D., Waltzinger, C., Benoist, C., and Mathis, D. (1993). Another view of the selective model of thymocyte selection. *Cell* **73**, 225–236.
Chan, A.C., Kadlecsek, T.A., Elder, M.E., Filipovich, A.H., Kuo, W.L., Iwashima, M., Parslow, T.G., and Weiss, A. (1994). ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science* **264**, 1599–1601.
Chan, S.H., Waltzinger, C., Baron, A., Benoist, C., and Mathis, D. (1994). Role of coreceptors in positive selection and lineage commitment. *EMBO J.* **13**, 4482–4489.
Cibotti, R., Punt, J.A., Dash, K.S., Sharrow, S.O., and Singer, A. (1997). Surface molecules that drive T cell development in vitro in the absence of thymic epithelium and in the absence of lineage-specific signals. *Immunity* **6**, 245–255.
Corbella, P., Moskophidis, D., Spanopoulou, E., Mamalaki, C., Tolaini, M., Itano, A., Lans, D., Baltimore, D., Robey, E., and Kioussis, D. (1994). Functional commitment to helper T cell lineage precedes positive selection and is independent of T cell receptor MHC specificity. *Immunity* **1**, 269–276.
Davis, C.B., Killeen, N., Crooks, M.E., Raulet, D., and Littman, D.R. (1993). Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell* **73**, 237–247.
Davis, C.B., and Littman, D.R. (1994). Thymocyte lineage commitment: is it instructed or stochastic? *Curr. Opin. Immunol.* **6**, 266–272.
Elder, M.E., Lin, D., Clever, J., Chan, A.C., Hope, T.J., Weiss, A., and Parslow, T.G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science* **264**, 1596–1599.
Festenstein, R., Tolaini, M., Corbella, P., Mamalaki, C., Parrington, J., Fox, M., Miliou, A., Jones, M., and Kioussis, D. (1996). Locus control region function and heterochromatin-induced position effect variegation. *Science* **271**, 1123–1125.
Fowlkes, B.J., and Schweighoffer, E. (1995). Positive selection of T cells. *Curr. Opin. Immunol.* **7**, 188–195.
Gorman, S.D., Sun, Y.H., Zamoyska, R., and Parnes, J.R. (1988). Molecular linkage of the Ly-3 and Ly-2 genes. Requirement of Ly-2 for Ly-3 surface expression. *J. Immunol.* **140**, 3646–3653.
Greaves, D.R., Wilson, F.D., Lang, G., and Kioussis, D. (1989). Human CD2 3'-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell* **56**, 979–986.
Gross, D.S., and Garrard, W.T. (1988). Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* **57**, 159–197.
Grosveld, F., van Assendelft, G.B., Greaves, D.R., and Kollias, G. (1987). Position-independent, high-level expression of the human β -globin gene in transgenic mice. *Cell* **51**, 975–985.
Groves, T., Katis, P., Madden, Z., Manickam, K., Ramsden, D., Wu, G., and Guidos, C.J. (1995). In vitro maturation of clonal CD4 $^+$ CD8 $^+$ cell lines in response to TCR engagement. *J. Immunol.* **154**, 5011–5022.
Guidos, C.J. (1996). Positive selection of CD4 $^+$ and CD8 $^+$ T cells. *Curr. Opin. Immunol.* **8**, 225–232.
Hambor, J.E., Mennone, J., Coon, M.E., Hanke, J.H., and Kavathas, P. (1993). Identification and characterization of an Alu-containing, T-cell-specific enhancer located in the last intron of the human CD8 α gene. *Mol. Cell. Biol.* **13**, 7056–7070.
Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). *Manipulating the Mouse Embryo* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
Hostert, A., Tolaini, M., Festenstein, R., McNeill, L., Malissen, B., Williams, O., Zamoyska, R., and Kioussis, D. (1997). A CD8 genomic fragment that directs subset-specific expression of CD8 in transgenic mice. *J. Immunol.* **158**, 4270–4281.
Jarry, A., Cerf-Bensussan, N., Brousse, N., Selz, F., and Guy-Grand,

- D. (1990). Subsets of CD3⁺ (T cell receptor $\alpha\beta$ or $\gamma\delta$) and CD3⁻ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. *Eur. J. Immunol.* **20**, 1097–1103.
- Kearse, K.P., Takahama, Y., Punt, J.A., Sharrow, S.O., and Singer, A. (1995). Early molecular events induced by T cell receptor (TCR) signaling in immature CD4⁺ CD8⁺ thymocytes: increased synthesis of TCR- α protein is an early response to TCR signaling that compensates for TCR- α instability, improves TCR assembly, and parallels other indicators of positive selection. *J. Exp. Med.* **181**, 193–202.
- Kieffer, L.J., Bennett, J.A., Cunningham, A.C., Gladue, R.P., McNeish, J., Kavathas, P.B., and Hanke, J.H. (1996). Human CD8 α expression in NK cells but not cytotoxic T cells of transgenic mice. *Int. Immunol.* **8**, 1617–1626.
- Kieffer, L.J., Yan, L., Hanke, J.H., and Kavathas, P.B. (1997). Appropriate developmental expression of human CD8 β in transgenic mice. *J. Immunol.*, in press.
- Kisielow, P., Leiserson, W., and von Boehmer, H. (1984). Differentiation of thymocytes in fetal organ culture: analysis of phenotypic changes accompanying the appearance of cytolytic and interleukin 2-producing cells. *J. Immunol.* **133**, 1117–1123.
- Kisielow, P., and von Boehmer, H. (1995). Development and selection of T cells: facts and puzzles. *Adv. Immunol.* **58**, 87–209.
- Landry, D.B., Engel, J.D., and Sen, R. (1993). Functional GATA-3 binding sites within murine CD8 alpha upstream regulatory sequences. *J. Exp. Med.* **178**, 941–949.
- Lee, W.H., Banan, M., Harriss, J.V., Hwang, I., Woodward, E., Youn, H.J., and Gottlieb, P.D. (1994). Cis-acting DNA elements and cell type-specific nuclear proteins which may play a role in regulation of mouse CD8 α (Lyt-2) gene transcription. *Int. Immunol.* **6**, 1307–1321.
- Lefrancois, L. (1991). Phenotypic complexity of intraepithelial lymphocytes of the small intestine. *J. Immunol.* **147**, 1746–1751.
- Lucas, B., and Germain, R.N. (1996). Unexpectedly complex regulation of CD4/CD8 coreceptor expression supports a revised model for CD4⁺ CD8⁺ thymocyte differentiation. *Immunity* **5**, 461–477.
- Lucas, B., Vasseur, F., and Penit, C. (1995). Stochastic coreceptor shut-off is restricted to the CD4 lineage maturation pathway. *J. Exp. Med.* **181**, 1623–1633.
- Lundberg, K., Heath, W., Kontgen, F., Carbone, F.R., and Shortman, K. (1995). Intermediate steps in positive selection: differentiation of CD4⁺ 8^{int} TCR^{int} thymocytes into CD4⁺ 8⁺ TCR^{hi} thymocytes. *J. Exp. Med.* **181**, 1643–1651.
- Marrack, P., and Kappler, J. (1997). Positive selection of thymocytes bearing $\alpha\beta$ T cell receptors. *Curr. Opin. Immunol.* **9**, 250–255.
- Martin, D.I., Fiering, S., and Groudine, M. (1996). Regulation of β -globin gene expression: straightening out the locus. *Curr. Opin. Genet. Dev.* **6**, 488–495.
- Moebius, U., Kober, G., Griscelli, A.L., Hercend, T., and Meuer, S.C. (1991). Expression of different CD8 isoforms on distinct human lymphocyte subpopulations. *Eur. J. Immunol.* **21**, 1793–1800.
- Nakauchi, H., Tagawa, M., Nolan, G.P., and Herzenberg, L.A. (1987). Isolation and characterization of the gene for the murine T cell differentiation antigen and immunoglobulin-related molecule, Lyt-2. *Nucleic Acid Res.* **15**, 4337–4347.
- Robey, E., and Fowlkes, B.J. (1994). Selective events in T cell development. *Annu. Rev. Immunol.* **12**, 675–705.
- Robey, E., Itano, A., Fanslow, W.C., and Fowlkes, B.J. (1994). Constitutive CD8 expression allows inefficient maturation of CD4⁺ helper T cells in class II major histocompatibility complex mutant mice. *J. Exp. Med.* **179**, 1997–2004.
- Robey, E., Chang, D., Itano, A., Cado, D., Alexander, H., Lans, D., Weinmaster, G., and Salmon, P. (1996). An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* **87**, 483–492.
- Salmon, P., Boyer, O., Lores, P., Jami, J., and Klatzmann, D. (1996). Characterization of an intronless CD4 minigene expressed in mature CD4 and CD8 T cells, but not expressed in immature thymocytes. *J. Immunol.* **156**, 1873–1879.
- Sambrook, J., Fritsch, E., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).
- Sawada, S., and Littman, D.R. (1991). Identification and characterization of a T-cell-specific enhancer adjacent to the murine CD4 gene. *Mol. Cell. Biol.* **11**, 5506–5515.
- Sawada, S., Scarborough, J.D., Killeen, N., and Littman, D.R. (1994). A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. *Cell* **77**, 917–929.
- Scollay, R., and Shortman, K. (1985). Identification of early stages of T lymphocyte development in the thymus cortex and medulla. *J. Immunol.* **134**, 3632–3642.
- Siu, G., Wurster, A.L., Duncan, D.D., Soliman, T.M., and Hedrick, S.M. (1994). A transcriptional silencer controls the developmental expression of the CD4 gene. *EMBO J.* **13**, 3570–3579.
- Suzuki, H., Punt, J.A., Granger, L.G., and Singer, A. (1995). Asymmetric signaling requirements for thymocyte commitment to the CD4⁺ versus CD8⁺ T cell lineages: a new perspective on thymic commitment and selection. *Immunity* **2**, 413–425.
- Swat, W., Dessing, M., von Boehmer, H., and Kisielow, P. (1993). CD69 expression during selection and maturation of CD4⁺ 8⁺ thymocytes. *Eur. J. Immunol.* **23**, 739–746.
- Takahama, Y., and Singer, A. (1992). Negative selection of precursor thymocytes before their differentiation into CD4⁺ CD8⁺ cells. *Science* **258**, 653–656.
- van Meerwijk, J.P., and Germain, R.N. (1993). Development of mature CD8⁺ thymocytes: selection rather than instruction? *Science* **261**, 911–915.
- von Boehmer, H. (1996). CD4/CD8 lineage commitment: back to instruction? *J. Exp. Med.* **183**, 713–715.
- Wilkinson, M.F., Doskow, J., von Borstel, R.D., Fong, A.M., and MacLeod, C.L. (1991). The expression of several T cell-specific and novel genes is repressed by trans-acting factors in immature T lymphoma clones. *J. Exp. Med.* **174**, 269–280.
- Zheng, W., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* **89**, 587–596.
- Zuniga-Pflucker, J.C., and Lenardo, M.J. (1996). Regulation of thymocyte development from immature progenitors. *Curr. Opin. Immunol.* **8**, 215–224.