

# Multiple Developmental Stage-Specific Enhancers Regulate CD8 Expression in Developing Thymocytes and in Thymus-Independent T Cells

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## Summary

We and others have recently identified a CD8 locus enhancer (E8<sub>i</sub>) that directs expression in mature CD8 single-positive thymocytes and peripheral CD8<sup>+</sup> T cells and in extrathymically derived intestinal intraepithelial lymphocytes (IEL). In this study, we show that deletion of E8<sub>i</sub> by homologous recombination results in reduced CD8 $\alpha\alpha$  homodimer expression on IEL. Since CD8 expression on thymus-derived T cells was normal, other enhancers regulate CD8 expression in these cells. By exploiting a transgenic reporter expression assay, we identified three additional enhancers that directed expression in diverse thymocyte subsets and mature T cells but not in CD8 $\alpha\alpha$ <sup>+</sup> IEL. The results suggest that CD8 $\alpha$  expression is primarily regulated by E8<sub>i</sub> in IEL and by the novel enhancers in the thymus-dependent lineages.

## Introduction

Peripheral T lymphocytes of the helper or cytotoxic phenotype express either the CD4 or CD8 coreceptor molecules, respectively, and develop in the thymus from a common progenitor expressing both coreceptors. During a process known as positive selection, double-positive (DP) thymocytes that express a T cell receptor (TCR) specific for major histocompatibility complex (MHC) class I develop into CD8 single-positive (SP) thymocytes, whereas those specific for MHC class II develop into CD4 SP cells. After completing maturation, SP thymocytes leave the thymus and constitute the thymus-dependent peripheral T cell pool (Kisielow and von Boehmer, 1995; Zuniga-Pflucker and Lenardo, 1996; Murrack and Kappler, 1997).

The molecular mechanisms that regulate the developmental choice between helper and cytotoxic lineage are not known (Davis and Littman, 1994; von Boehmer, 1996). Recently, it was shown that transgenic overexpression of an activated form of Notch favors T cell development toward the CD8 lineage, suggesting that the Notch pathway might be involved in lineage commitment of DP thymocytes (Robey et al., 1996). However, the involvement of known molecular targets of Notch in thymocyte lineage commitment has not been shown. Because there is coupling of coreceptor expression and

the functional programs of T cells (Chan et al., 1994; Corbella et al., 1994; Robey et al., 1994), there is a strong likelihood that factors regulating the expression of CD4 or CD8 are also involved in directing DP thymocytes toward the helper or cytotoxic lineage, respectively. Therefore, it is important to understand how the CD4 and CD8 genes are transcriptionally regulated during T cell development and to identify *cis*- and *trans*-acting elements involved in their regulation.

Within the last several years, the major *cis*-regulatory elements directing expression of CD4 have been identified. A T cell-specific enhancer located approximately 13 kb upstream of the transcriptional start site (Sawada and Littman, 1991) directs expression of CD4 in both CD4<sup>+</sup>CD8<sup>-</sup> (CD4<sup>+</sup>) and CD4<sup>-</sup>CD8<sup>+</sup> (CD8<sup>+</sup>) T cells, while subset specificity is achieved through silencing of CD4 transcription in CD8<sup>+</sup> T cells by elements located in the first intron of the CD4 gene (Sawada et al., 1994; Siu et al., 1994; Donda et al., 1996).

The characterization of *cis*-elements that regulate expression of CD8 is still at an early stage. Thymus-derived CD8<sup>+</sup> T cells express heterodimers of CD8 $\alpha$  and CD8 $\beta$  on their surface, while extrathymically derived intestinal intraepithelial lymphocytes (IEL) and a subset of human NK cells express CD8 $\alpha\alpha$  homodimers (Jarry et al., 1990; Lefrancois, 1991; Moebius et al., 1991). IEL that express CD8 $\alpha\alpha$  homodimers are of both the TCR $\alpha\beta$  or TCR $\gamma\delta$  lineage, whereas IEL that express CD8 $\alpha\beta$  heterodimers are exclusively of the TCR $\alpha\beta$  lineage. Thus, the expression of the CD8 $\alpha$  and CD8 $\beta$  genes, which are linked at a distance of about 36 kb on mouse chromosome 6 (Gorman et al., 1988), must be regulated both coordinately and independently. As an approach toward identifying regulatory elements for the CD8 genes *in vivo*, Hostert et al. (1997a) generated transgenic mice with a P1 clone containing 80 kb of genomic DNA encompassing the entire murine CD8 locus. Proper developmental stage- and lineage-specific expression of the transgenic CD8 reporter was achieved, indicating that all major elements are localized within this large genomic fragment. Similar results were reported for the human CD8 $\beta$  gene in transgenic mice containing a 95 kb genomic fragment surrounding the human CD8 $\beta$  locus (Kieffer et al., 1997). By performing DNase I hypersensitivity (DH) studies on the CD8 locus and testing genomic fragments containing DH sites for enhancer activity, we and others have recently identified *cis*-regulatory elements, within a 7.6 kb genomic BamHI fragment, that are involved in the regulation of murine CD8 gene expression *in vivo* (Ellmeier et al., 1997; Hostert et al., 1997b). It was shown that the E8<sub>i</sub> enhancer (initially designated E<sub>8SP</sub>) directs expression of a transgenic reporter gene in peripheral CD8<sup>+</sup> T cells and mature CD8 SP thymocytes but not at earlier stages of T cell development (e.g., DP thymocytes). Furthermore, the onset of enhancer activity correlated with the final steps of positive selection, indicating a developmental stage-specific regulation of its activity. In addition, we showed that E8<sub>i</sub>, which is located approximately 16 kb upstream of the CD8 $\alpha$  gene, directs expression of the reporter gene also in

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extrathymically derived IEL, thus indicating its specificity toward the CD8 $\alpha$  gene (Ellmeier et al., 1997). However, the identity of enhancer(s) that regulate expression of CD8 in DP thymocytes remained unknown.

In an effort to study E8<sub>i</sub> in more detail, we deleted the enhancer in embryonic stem (ES) cells by homologous recombination. This experimental approach was designed to confirm the proposed role of this enhancer in CD8 SP cells and IEL, and, additionally, to determine if it could also have a role in the regulation of CD8 expression in DP thymocytes (e.g., by interacting cooperatively with another *cis*-element that together with E8<sub>i</sub> forms a DP-specific enhancer). We show that E8<sub>i</sub> is the primary enhancer regulating the expression of CD8 $\alpha$  in the thymus-independent subsets of IEL of the gut. IEL from mice lacking E8<sub>i</sub> showed a dramatic decrease of CD8 $\alpha\alpha$  homodimer expression levels (both on TCR $\alpha\beta$  and TCR $\gamma\delta$  IEL) and the majority of TCR $\gamma\delta$  IEL lacked expression of any CD8 $\alpha\alpha$  homodimers. In contrast to thymus-independent T cells, the deletion of E8<sub>i</sub> had no appreciable effect on CD8 expression in thymus-derived lymphocytes. Neither the appearance of peripheral CD8<sup>+</sup> T cells nor the development of thymocytes was compromised in animals lacking E8<sub>i</sub>, indicating that other enhancers are involved in the regulation of CD8 expression in mature T cells. We identified such additional enhancers, including one specific for DP thymocytes, by analyzing reporter gene expression in transgenic mice. These enhancers directed expression of the human CD2 (hCD2) reporter at specific stages of T cell development and most likely compensate for E8<sub>i</sub> activity in E8<sub>i</sub>-null mice. However, none of these enhancers directed expression of the reporter in CD8 $\alpha\alpha$  homodimer-expressing IEL, thereby explaining the dramatic reduction of CD8 $\alpha$  expression in IEL of E8<sub>i</sub><sup>-/-</sup> mice.

## Results

### Deletion of the E8<sub>i</sub> Enhancer by Homologous Recombination

The mature and peripheral CD8<sup>+</sup> T cell enhancer E8<sub>i</sub> (Ellmeier et al., 1997; Hostert et al., 1997b) is located approximately 16 kb upstream of the CD8 $\alpha$  gene (Figure 1A). To delete the enhancer by homologous recombination, we prepared a targeting construct in which the 7.6 kb genomic BamHI fragment containing E8<sub>i</sub> (including the two DNase I hypersensitivity sites HS-1 and HS-2) was replaced by a neomycin resistance gene flanked by two loxP sites (Figure 1B). Homologous recombination was performed in E14.1 ES cells (Kuhn et al., 1991) and several targeted clones were isolated (designated +/N; Figure 1C). Two +/N ES cell clones were selected for injection into C57BL/6 blastocysts and chimeric mice obtained from both clones were backcrossed either to C57BL/6 or AKR1 mice. The targeted allele was transmitted to the offspring, which were subsequently interbred to obtain E8<sub>i</sub> +/+, +/N, and N/N mice. There were no significant phenotypic differences between F1 intercrosses from either of the two ES clones or from the AKR1 and C57BL/6 backcrosses, and therefore we will not distinguish between the clones and between the different strains.

To exclude that the expression of CD8 in the absence of E8<sub>i</sub> is influenced by the neomycin expression cassette due to transcriptional interference as observed by others (Sleckman et al., 1996), heterozygous E8<sub>i</sub> +/N mice were crossed with transgenic mice expressing Cre recombinase under the control of the CMV promoter (White et al., 1997). Heterozygous E8<sub>i</sub> +/ $\Delta$  mice (with the deletion of the neomycin gene) were then interbred to obtain E8<sub>i</sub> +/+, +/ $\Delta$ , and  $\Delta$ / $\Delta$  mice.

### Normal T Cell Development in Mice Lacking E8<sub>i</sub>

It has been shown that in transgenic mice the 7.6 kb genomic BamHI fragment was sufficient to drive expression of a reporter gene in mature CD8 SP thymocytes and peripheral CD8<sup>+</sup> T cells (Ellmeier et al., 1997; Hostert et al., 1997b). This suggested that the enhancer regulates the expression of CD8 after the final maturation of the CD8 lineage. However, as shown in Figure 2B, the absence of E8<sub>i</sub> had no effect on the expression of CD8 (both CD8 $\alpha$  and CD8 $\beta$  expression levels were normal; see Table 1) and on the appearance of CD8<sup>+</sup> T cells in the periphery. Lymph node or splenic (data not shown) CD8<sup>+</sup> T cells from the knockout mice displayed normal levels of CD8 expression, and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells was unchanged ( $2.6 \pm 0.5$  and  $3.1 \pm 0.5$  in wild-type littermates and E8<sub>i</sub>-deficient mice, respectively;  $n = 6$ ). In addition, loss of E8<sub>i</sub> activity did not interfere with the development of DP and CD8 SP thymocytes, indicated by the normal proportion of DP and CD4 or CD8 SP thymocytes (Figure 2A). Although the expression level of CD8 on peripheral T lymphocytes and DP thymocytes was normal, CD8 SP thymocytes (TCR $\alpha\beta^h$ ) from the knockout animals displayed a 10%–20% decrease in the level of CD8 expression compared to wild-type littermate controls (Table 1). The expression pattern of CD8 in the knockout mice did not differ between animals with or without the deletion of the neomycin expression cassette ( $\Delta$ / $\Delta$  and N/N mice in Figure 2, respectively). Therefore, the absence of E8<sub>i</sub> does not influence the expression of CD8 in thymus-derived peripheral T cells. This clearly indicates that other *cis*-regulatory elements (either specific for CD8 $\alpha$  or CD8 $\beta$  or both) compensate for loss of E8<sub>i</sub> enhancer activity and/or are involved in the regulation of CD8 gene expression in mature CD8 SP thymocytes and peripheral CD8<sup>+</sup> T cells.

### CD8 $\alpha\alpha$ Homodimer Expression on Gut Intraepithelial Lymphocytes Is Dramatically Reduced in Mice Lacking E8<sub>i</sub>

Intraepithelial lymphocytes from the gut generally belong either to the TCR $\alpha\beta$  or TCR $\gamma\delta$  T cell lineage. IEL of TCR $\alpha\beta$  lineage express either CD8 $\alpha\beta$  heterodimers or CD8 $\alpha\alpha$  homodimers, whereas TCR $\gamma\delta$ -positive IEL express CD8 $\alpha\alpha$  homodimers only. It is believed that IEL that express CD8 $\alpha\alpha$  homodimers (either of TCR $\alpha\beta$  or TCR $\gamma\delta$  lineage) develop extrathymically, whereas the origin of CD8 $\alpha\beta$  heterodimer-expressing IEL remains a matter of some controversy (Poussier and Julius, 1994; Rocha et al., 1995; Klein, 1996). We have shown previously that E8<sub>i</sub> directs expression of a transgenic reporter gene in intraepithelial lymphocytes. Expression

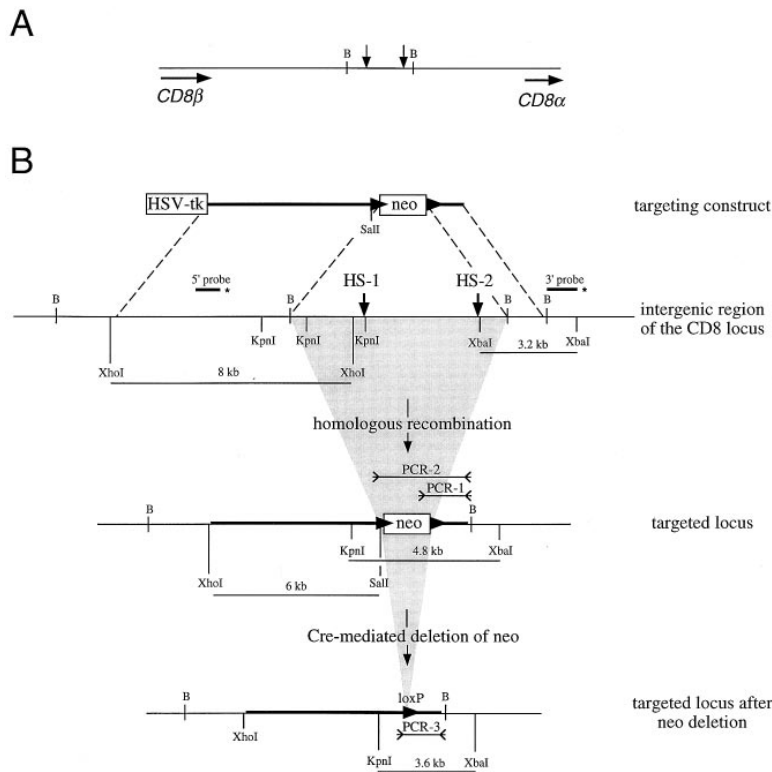
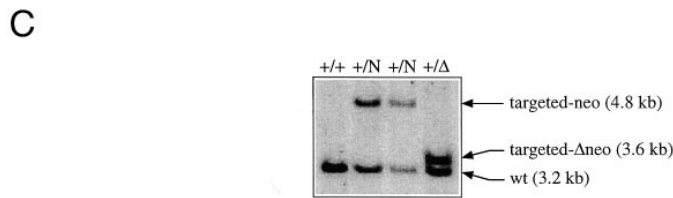


Figure 1. Deletion of the E8, Enhancer by Homologous Recombination

(A) Schematic representation of the CD8 locus. Horizontal arrows indicate the transcriptional orientation of the CD8 $\beta$  and CD8 $\alpha$  genes. Vertical arrows indicate the two DNase I hypersensitivity sites constituting the E8, enhancer (Ellmeier et al., 1997; Hostert et al., 1997b). BamHI sites (B) representing the border of the targeted 7.6 kb genomic fragment are shown.

(B) Schematic map of the targeting construct (top part), the CD8 intergenic region before and after homologous recombination (upper and lower middle part), and the genomic locus after Cre-mediated deletion of the neomycin resistance gene (bottom part). Only restriction sites important for the targeting strategy are shown, except for BamHI (B). The horizontal thick black line (in the top part, lower middle part, and bottom part) indicates the region of homology between the targeting construct and the endogenous locus. The bars with asterisks (in the upper middle part) represent the 5' and 3' probes used for Southern blotting to detect homologous recombination. Triangles in the top, lower middle, and bottom part indicate loxP sites. Solid lines labeled PCR-1, PCR-2 (lower middle part), and PCR-3 (bottom part) indicate the expected PCR fragments after homologous recombination (PCR-1) and before or after neo deletion (PCR-2 or PCR-3, respectively). Horizontal bars with numbers (indicating the size in kb) represent the expected genomic fragments after digestion with the appropriate restriction enzymes (XhoI and Sall for the 5' targeted region; KpnI and XbaI for the 3' targeted region).

(C) Southern blot of KpnI/XbaI-digested DNA isolated from a wild-type ES cell clone (+/+), from ES cell clones after homologous recombination (+/N), and from an ES cell clone after Cre recombinase-mediated deletion of neomycin (+/Δ). Deletion of the neomycin gene in mice was detected by PCR (PCR-3 fragment) and by Southern blotting with a probe specific for neomycin (data not shown). Only the blot hybridized with the 3' probe is shown. The origin of the detected fragments is indicated in (B).



of the hCD2 reporter was observed in all subpopulations of IEL, including CD8 $\alpha\alpha^+$  IEL of both the TCR $\alpha\beta$  and TCR $\gamma\delta$  lineage (Ellmeier et al., 1997). Therefore, IEL from either wild-type or homozygous knockout mice (either

N/N or Δ/Δ) were isolated and analyzed for the expression of CD8 $\alpha$  on subpopulations expressing either TCR $\alpha\beta$  or TCR $\gamma\delta$ . In contrast to thymocytes and lymph node T cells, IEL displayed a dramatic reduction in CD8 $\alpha$

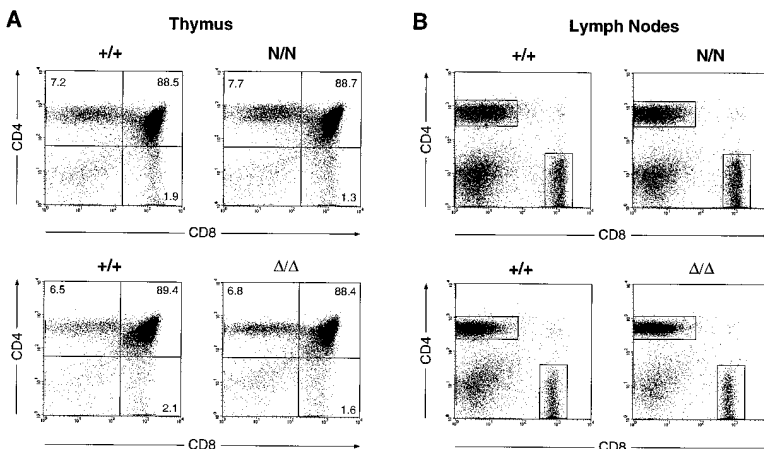


Figure 2. Normal CD8 SP T Cell Development and CD8 Expression in Mice Lacking E8<sub>1</sub>

(A) Two-color flow cytometry analysis of CD4 and CD8 expression on thymocytes isolated either from +/+ and N/N littermates (upper panel) or from +/+ and Δ/Δ littermates (lower panel). Numbers in the dot plot quadrants indicate the percentage (of total gated thymocytes) of the corresponding thymocyte subpopulations.

(B) Lymph node T cells isolated from mice of the indicated genotype were analyzed by two-color flow cytometry analysis for expression of CD4 and CD8. Gates show the area of either CD4 $^+$  or CD8 $^+$  T cells. Littermates were analyzed both for +/+ and N/N (upper panel) and for +/+ and Δ/Δ (lower panel) genotypes.

Table 1. Relative Reduction of CD8 $\alpha$  Expression Levels on Different T Cell Populations in E8 $_i$ -Deficient Mice

CD8 $\alpha\alpha^+$ IEL (n = 7)		CD8 $\alpha\beta^+$ IEL (n = 7)		Thymocytes (n = 6)		CD8 $^+$ LN T Cells (n = 6)	
TCR $\gamma\delta^+$ <sup>a</sup>	TCR $\alpha\beta^+$	TCR $\alpha\beta^+$		DP	CD8 SP	CD8 $\alpha$	CD8 $\beta$
3.64 $\pm$ 0.7	2.26 $\pm$ 0.4	1.45 $\pm$ 0.3		1.02 $\pm$ 0.1	1.37 $\pm$ 0.1	1.02 $\pm$ 0.1	1.00 $\pm$ 0.1

Numbers indicate n-fold reduction of CD8 $\alpha$  expression levels on the indicated T cell populations in E8 $_i$  knockout mice relative to wild-type littermate controls. To determine the expression levels of CD8 $\alpha$  on DP or CD8 SP thymocytes, cells were gated on CD4 $^+$ TCR $\alpha\beta^-$  or CD4 $^-$ TCR $\alpha\beta^+$  populations, respectively. For CD8 $^+$  LN T cells, relative reduction of expression levels for both CD8 $\alpha$  and CD8 $\beta$  is shown.

<sup>a</sup>Only 25%–50% of TCR $\gamma\delta^+$  IEL in E8 $_i$ -deficient mice expressed CD8 $\alpha\alpha$  homodimers compared to 80%–90% in wild-type littermate controls.

expression upon deletion of the E8 $_i$  enhancer. As shown in Figure 3A, a 3- to 5-fold reduction in CD8 $\alpha$  expression levels was observed for TCR $\gamma\delta^+$  cells (Table 1). Furthermore, the percentage of TCR $\gamma\delta^+$  IEL that also expressed CD8 $\alpha\alpha$  homodimers was reduced from about 80%–95% in wild-type mice to 20%–50% in N/N and  $\Delta/\Delta$  mice (Figure 3A). Thus, deletion of E8 $_i$  resulted in a significant decrease in the level of CD8 $\alpha$  on TCR $\gamma\delta$  lineage IEL as well as a major reduction in numbers of CD8 $\alpha\alpha^+$ TCR $\gamma\delta^+$  T cells. A similar, although less dramatic, reduction (1.5- to 3-fold; Table 1) of CD8 $\alpha$  expression was observed on CD8 $\alpha\alpha^+$ TCR $\alpha\beta^+$  IEL (Figure 3B, upper panel). CD8 $\alpha\beta$  heterodimer-expressing IEL also displayed significantly lower levels of CD8 $\alpha$  on their surface (Figure 3B, lower

panel; Table 1). These results indicate that E8 $_i$  is a major *cis*-regulatory element for CD8 $\alpha$  expression in these diverse IEL populations.

### Identification of Additional T Cell-Specific Enhancers in the CD8 Locus

The results from the E8 $_i$  enhancer deletion indicated that other *cis*-regulatory elements are involved in the regulation of CD8. However, other enhancers are only able to fully compensate for the mature CD8 $^+$  T cell-specific enhancer in thymically derived T cells but not in extrathymically derived CD8 $\alpha\alpha$  homodimer-expressing IEL. To identify *cis*-regulatory elements involved in CD8 $\alpha$

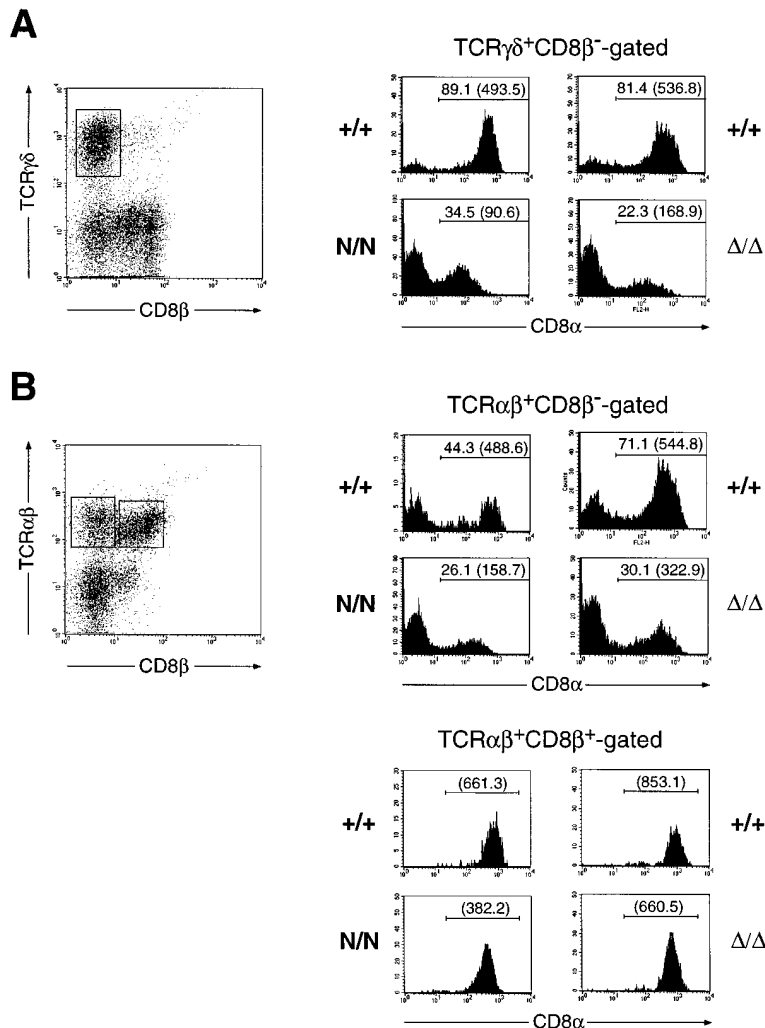


Figure 3. Reduced Expression of CD8 $\alpha$  on Intestinal IEL in the Absence of E8 $_i$

(A) Expression of CD8 $\alpha\alpha$  homodimers on IEL of the TCR $\gamma\delta$  lineage. A representative gate for the TCR $\gamma\delta^+$ CD8 $\beta^-$  population is shown. Bars in the histograms indicate IEL defined as being positive for CD8 $\alpha\alpha$  expression. The number above the marked regions indicates the percentage of CD8 $\alpha\alpha^+$  cells within the gated population and the number in brackets indicates the mean fluorescence of CD8 $\alpha\alpha$  expression levels. Wild-type littermate controls are shown above the histograms of N/N or  $\Delta/\Delta$  mice.

(B) Expression of CD8 $\alpha$  on IEL of the TCR $\alpha\beta$  lineage. Representative gates for the TCR $\alpha\beta^+$ CD8 $\beta^-$  (upper panel) and the TCR $\alpha\beta^+$ CD8 $\beta^+$  populations (lower panel) are shown in the dot plots. The numbers above the marked regions in the upper panel indicate the percentage of CD8 $\alpha\alpha$ -positive cells within the gated population and the mean fluorescence of CD8 $\alpha\alpha$  expression levels (in brackets), respectively. The number (in brackets) above the marked region in the lower panel indicates the mean fluorescence of CD8 $\alpha$  expression on CD8 $\alpha\beta$  heterodimer-expressing TCR $\alpha\beta$  IEL. Wild-type littermate controls are shown above the histograms of N/N or  $\Delta/\Delta$  mice.

and/or CD8 $\beta$  gene expression, we extended the experimental approach used to identify E8<sub>i</sub> (Ellmeier et al., 1997). In brief, a hCD2 reporter gene was cloned downstream of the CD8 $\alpha$  promoter and genomic fragments containing putative *cis*-regulatory elements were inserted upstream of the CD8 $\alpha$  promoter and tested for their ability to direct expression of the reporter gene in thymocytes and T cells of transgenic mice. We took advantage of the published hypersensitivity map of the CD8 locus (Hostert et al., 1997a) and generated a transgenic construct (TG-21) with a 15 kb genomic fragment containing several DH sites around the 3' end of the CD8 $\beta$  locus (see Figure 4D). This and other constructs described below were injected into (B6/D2) F2 mouse eggs to generate transgenic animals. Transgenic founders identified by Southern blotting were either analyzed directly or backcrossed to C57BL/6 mice to generate transgenic lines that were then analyzed.

The expression pattern of the hCD2 reporter in TG-21 transgenic founders or lines reflected the developmental subset- and lineage-specific expression pattern of the endogenous CD8 gene. During thymocyte development, hCD2 was expressed both in the DP and in the CD8 SP thymocyte population but not in CD4 SP thymocytes (Figure 4A). Furthermore, hCD2 expression was observed on peripheral CD8<sup>+</sup> T cells but not on CD4<sup>+</sup> cells (Figure 4B). No expression of hCD2 was found on B cells (data not shown). We observed position effect variegation of TG-21 expression, as shown previously for the E8<sub>i</sub> enhancer (Ellmeier et al., 1997; Hostert et al., 1997b). This is in agreement with the putative absence of a locus control region within the CD8 locus (Hostert et al., 1997a).

To determine whether this newly identified enhancer directs expression also in CD8 $\alpha\alpha$ <sup>+</sup> cells, IEL from the gut mucosa of TG-21 transgenic animals were isolated and analyzed for the expression of hCD2. As shown in Figure 4C, CD8 $\alpha\alpha$  homodimer-expressing IEL (of both TCR $\gamma\delta$  and TCR $\alpha\beta$  lineages) did not express hCD2. In contrast, hCD2 was detectable on CD8 $\alpha\beta$  heterodimer-expressing IEL, although the percentage of hCD2<sup>+</sup> cells within the CD8 $\alpha\beta$ <sup>+</sup> IEL was significantly lower than in CD8 $\alpha\beta$ <sup>+</sup> lymph node T cells (as shown in Figure 4B), indicating that the enhancer may have only weak activity in CD8 $\alpha\beta$ <sup>+</sup> IEL. These results therefore indicate that this enhancer functions specifically in T cells expressing CD8 $\alpha\beta$  heterodimers but not CD8 $\alpha\alpha$  homodimers.

#### Identification of a Double-Positive Thymocyte-Specific Enhancer

To narrow down the region within TG-21 that is involved in mediating the expression of hCD2 in CD8 $\alpha\beta$  homodimer-expressing cells, transgenic constructs (TG-23 and TG-31) containing smaller genomic fragments were generated (Figure 4D). TG-23, containing a 4.3 kb genomic BamHI fragment, mediated expression of hCD2 in a pattern indistinguishable from TG-21. Expression of hCD2 was observed in DP and CD8 SP thymocytes and in peripheral CD8<sup>+</sup> T cells (Figures 4A and 4B). Within the IEL T cell population, hCD2 was detected only on a small percentage of CD8 $\alpha\beta$ <sup>+</sup> but not on CD8 $\alpha\alpha$  homodimer-expressing cells (Figure 4C). Thus, TG-23 contains an

enhancer (designated E8<sub>ii</sub>) that is active in both immature DP thymocytes and mature CD8 SP thymocytes and T cells.

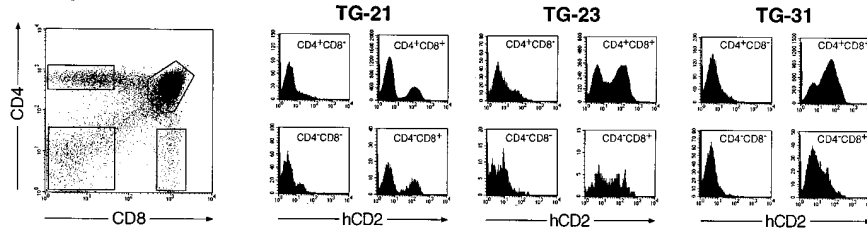
Because TG-23 displayed the same expression pattern as TG-21, we expected that TG-31 (generated with a 4.1 kb genomic EcoRI/BamHI fragment) would not contain any regulatory activity. Surprisingly, TG-31 directed hCD2 expression only on immature DP thymocytes and not on CD4 or CD8 SP thymocytes (Figure 4A). To determine the developmental stage at which hCD2 expression ceased during the development of CD8 SP thymocytes, TCR $\alpha\beta$  expression levels were compared between hCD2-positive and hCD2-negative cells within the intermediate CD4<sup>lo</sup>CD8<sup>+</sup> population (Figure 6C; see Figure 6A for gating areas). The majority of hCD2-positive CD4<sup>lo</sup>CD8<sup>+</sup> cells displayed intermediate levels of TCR $\alpha\beta$  (only 21.3% of cells expressed high levels of TCR $\alpha\beta$ ), indicating that they were still immature (Bendelac et al., 1992). In contrast, hCD2-negative cells showed a higher percentage of cells (44.5%) expressing high levels of TCR $\alpha\beta$  (Figure 6C). Since expression of hCD2 was variegated in TG-31 (approximately 75% of DP cells expressed hCD2; see Figures 4A and 6A), some hCD2-negative cells within the CD4<sup>lo</sup>CD8<sup>+</sup> population are of immature phenotype. This might explain why not more than 44.5% of thymocytes within the hCD2-negative CD4<sup>lo</sup>CD8<sup>+</sup> population expressed high levels of TCR $\alpha\beta$ . Consistent with the downregulation of hCD2 expression at the TCR<sup>int/hi</sup> to TCR<sup>hi</sup> transition during CD8 SP thymocyte development, no hCD2 expression was observed on lymph node CD8<sup>+</sup> T cells (Figure 4B). The enhancer activity within this fragment was designated E8<sub>iii</sub>.

During the process of mapping enhancers around the CD8 $\beta$  locus we also analyzed TG-22 and TG-25, which contained additional 3.2 kb of genomic sequence 5' to the TG-31 fragment (see Figure 5D). Expression of hCD2 in transgenic mice generated with these constructs was observed not only in CD8<sup>+</sup> T cells but also in the CD4<sup>+</sup> T cell lineage (Figure 5A). The percentage of hCD2<sup>+</sup> cells within the CD4<sup>+</sup> T cell compartment was always lower than in the CD8<sup>+</sup> T cell population in all transgenic founders and established lines (Figure 5A). However, the number of CD4<sup>+</sup> T cells expressing hCD2 corresponded proportionally to the percentage of hCD2-expressing CD8<sup>+</sup> T cells. Since five hCD2-expressing founders for TG-22 and three founders for TG-25 (see Table 2) displayed this broader expression pattern (directed by an enhancer activity designated E8<sub>iv</sub>), it is unlikely that this is mediated by position effect variegation due to the site of transgene integration. Expression of hCD2 was also observed in DP thymocytes in TG-22 mice (Figure 5B; Table 2) and in CD8 $\alpha\beta$  heterodimer-expressing IEL but not in CD8 $\alpha\alpha$ <sup>+</sup> IEL (Figure 5C).

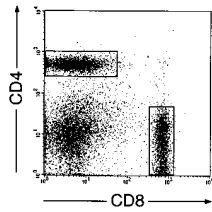
#### Summation of Enhancer Functions during Development

In mice prepared with TG-22, the hCD2 reporter was consistently expressed at higher levels on DP than on CD4 or CD8 SP thymocytes (Figures 5B and 6A). This suggests that there is a developmental stage-specific change or transition in the activities of the E8<sub>iii</sub> and/or

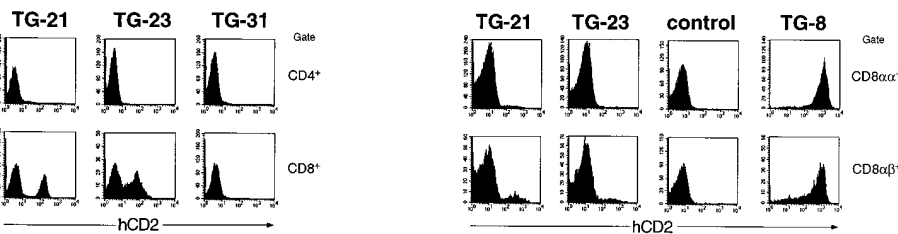
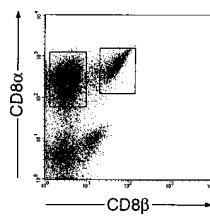
**A Thymus**



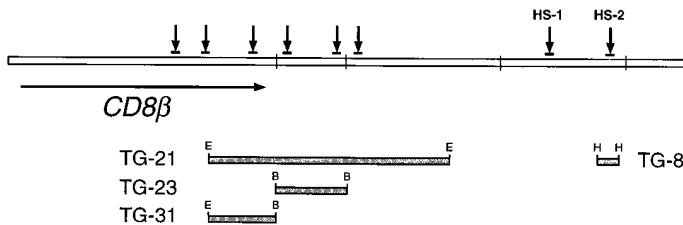
**B Lymph Nodes**



**C Intraepithelial Lymphocytes**



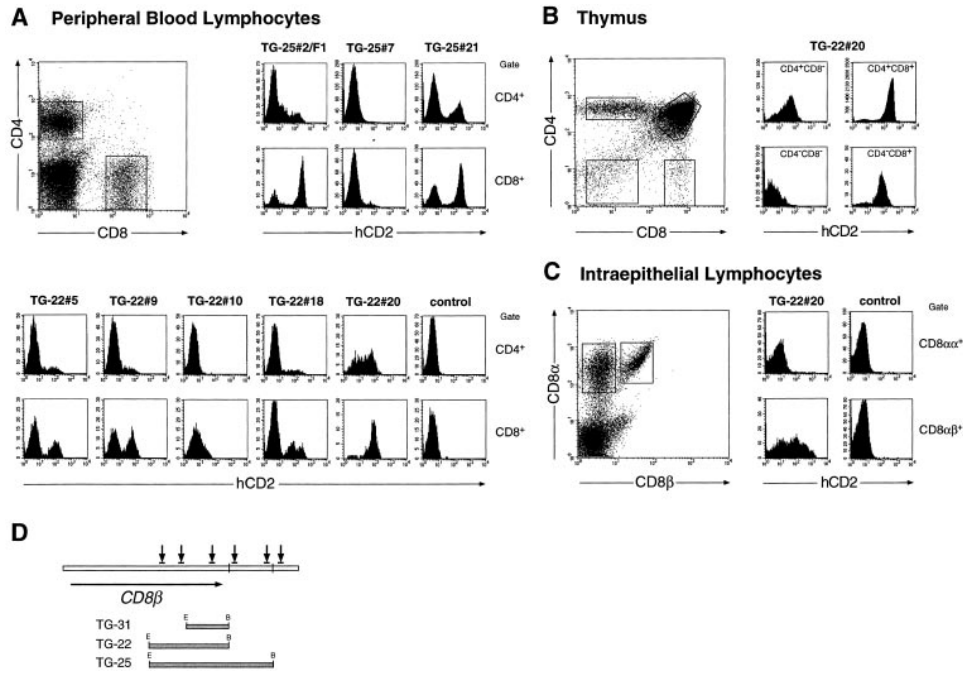
**D**



**Figure 4.** Expression of the hCD2 Reporter in T Lymphocyte Subsets of Transgenic Mice Generated with Constructs TG-21, 23, and 31  
 (A) Three-color flow cytometry analysis on thymocytes isolated from the transgenic mice indicated. Dot plot shows representative gates for the CD4<sup>-</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> thymocyte populations. Individual histograms show hCD2 expression on gated thymocyte populations (gates are indicated in the upper right of each histogram).  
 (B) Lymph node T cells were isolated from the indicated transgenic mice and analyzed by three-color flow cytometry for expression of CD4, CD8, and hCD2. Dot plot shows representative CD4<sup>+</sup> and CD8<sup>+</sup> T cell gates used for the histograms shown below.  
 (C) Intestinal intraepithelial lymphocytes isolated from transgenic mice TG-21, TG-23, a nontransgenic control mouse, and a transgenic positive control mouse generated with construct TG-8 (containing HS-2 of the E8<sub>β</sub> enhancer) were analyzed for expression of CD8α, CD8β, and hCD2. Representative gates for CD8αα homodimer- and CD8αβ heterodimer-expressing cells are shown in the dot plot. Histograms below show hCD2 expression on gated CD8αα<sup>+</sup> or CD8αβ<sup>+</sup> IEL.  
 (D) Schematic map of the genomic fragments used for the generation of the transgenic constructs TG-21, TG-23, TG-31, and TG-8. The location of DH sites (indicated by vertical arrowheads) is according to Hostert et al. (1997a). HS-1 and HS-2 represent the CD8<sup>+</sup> T cell lineage-specific DH sites constituting the E8<sub>β</sub> enhancer described earlier (Ellmeier et al., 1997). The transcriptional orientation of the CD8β gene is indicated by the horizontal arrow. Restriction enzyme sites are BamHI (B), EcoRI (E), and HindIII (H).

E8<sub>β</sub> enhancers. It was previously shown that DP thymocytes undergoing positive selection partially downmodulate surface expression of CD8 and CD4. Then, in the presence of MHC class I ligand, they progress through a CD4<sup>lo</sup>CD8<sup>+</sup> stage toward the mature CD4<sup>-</sup>CD8<sup>+</sup> stage in which they display high levels of TCR and low levels of HSA (van Meerwijk and Germain, 1993). The expression

levels of hCD2 were examined within these different CD8<sup>+</sup> developmental stages. As shown in Figure 6A, a progressive decrease to lower levels of hCD2 expression was observed during CD8 lineage development. To examine this further, the developmental stage of thymocytes expressing high or low levels of hCD2 within the intermediate CD4<sup>lo</sup>CD8<sup>+</sup> population was determined by



**Figure 5.** A T Cell-Specific Enhancer from the CD8 $\beta$  Locus Directs Expression of hCD2 in Both CD4<sup>+</sup> and CD8<sup>+</sup> T Lymphocytes  
(A) Three-color flow cytometry analysis of peripheral blood lymphocytes isolated from TG-22 and TG-25 founders or lines. Representative gates for CD4<sup>+</sup> and CD8<sup>+</sup> T cells are shown in the dot plot. Histograms show expression of hCD2 on either CD4<sup>+</sup> or CD8<sup>+</sup> T cells.  
(B) Thymocytes from a TG-22 mouse (founder #20) were analyzed by three-color flow cytometry. Gates for the CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>-</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> thymocyte populations are shown in the dot plot. Histograms show expression of hCD2 on different thymocyte subsets (indicated in the upper right of each histogram).  
(C) Intestinal intraepithelial lymphocytes isolated from TG-22 (founder #20) were analyzed for expression of CD8 $\alpha$ , CD8 $\beta$ , and hCD2. Gates for CD8 $\alpha\alpha$  homodimer- and CD8 $\alpha\beta$  heterodimer-expressing cells are shown in the dot plot. Histograms show hCD2 expression on gated CD8 $\alpha\alpha$ <sup>+</sup> or CD8 $\alpha\beta$ <sup>+</sup> IEL.  
(D) Schematic map of the genomic fragments used for transgenic constructs TG-22, TG-25, and TG-31. The location of DH-sites (indicated by arrowheads) is according to Hostert et al. (1997a). The transcriptional orientation of the CD8 $\beta$  gene is indicated by the horizontal arrow. Restriction enzyme sites are BamHI (B) and EcoRI (E).

analyzing the expression levels of HSA, TCR $\alpha\beta$ , and CD69 (Figure 6B). Thymocytes expressing higher levels of hCD2 were of the immature phenotype (HSA<sup>hi</sup>TCR $\alpha\beta$ <sup>int</sup>) and some of the cells expressed high levels of CD69, indicating that they already received the signal for selection (Bendelac et al., 1992; Swat et al., 1993; Merckenschlager

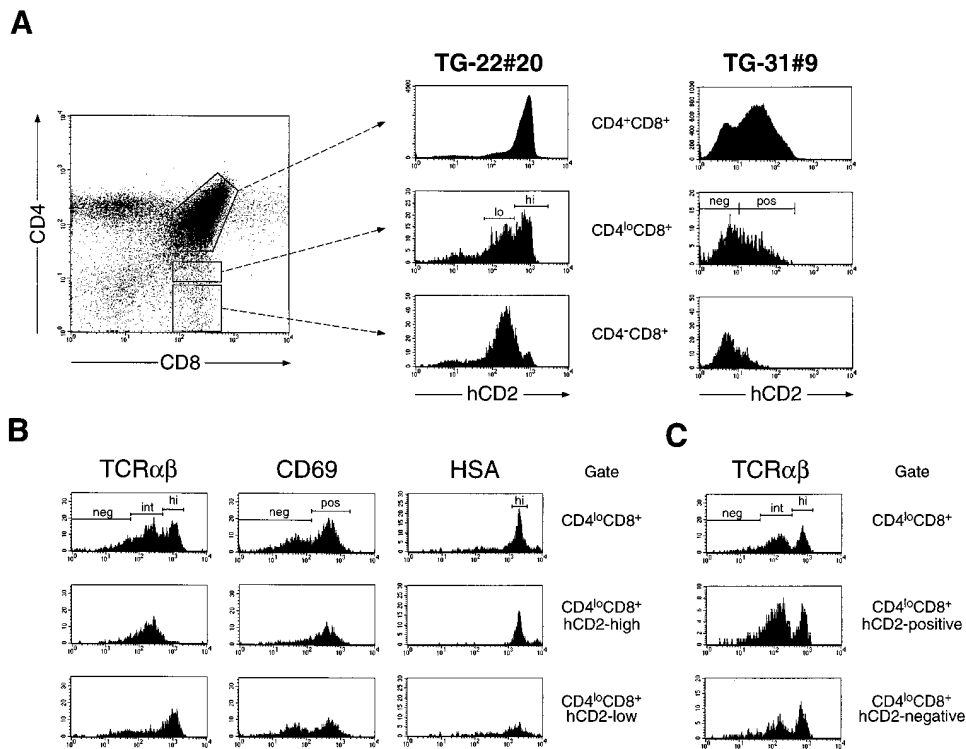
et al., 1997). In contrast, CD4<sup>lo</sup>CD8<sup>+</sup> cells expressing lower levels of hCD2 displayed the HSA<sup>lo</sup>TCR $\alpha\beta$ <sup>hi</sup> phenotype that characterizes mature cells. These results suggest that the expression of hCD2 in TG-22 mice is governed by the summation of the activities of E8<sub>III</sub> (high expression in DP cells that ceases at the HSA<sup>hi</sup>TCR $\alpha\beta$ <sup>int/hi</sup>

**Table 2.** Expression of hCD2 on Different T Cell Subsets in Transgenic Mice

TG	Founders	Peripheral Lymphocytes		Thymocytes <sup>a</sup>			Intestinal IEL <sup>a</sup>	
		CD4 <sup>+</sup>	CD8 <sup>+</sup>	DP	CD4 SP	CD8 SP	CD8 $\alpha\alpha$	CD8 $\alpha\beta$
21	6 (8)	-	+	+	-	+	-	+/-
22	5 (5)	+	+	+	+	+	-	+
23	4 (9)	-	+	+	-	+	-	+/-
25	3 (4)	+	+	nd	nd	nd	nd	nd
31	0 (3)	-	-	+	-	-	nd	nd

Summary of hCD2 expression in mice generated with the different transgenic constructs (TG-21, 22, 23, 25, and 31). The first number next to the transgenic construct indicates how many founders were expressing hCD2 in CD8<sup>+</sup> PBLs, while the second number (in brackets) indicates the total numbers of founders obtained. +, +/-, or - below the different T lymphocyte populations indicates expression, weak expression, or no expression of hCD2, respectively, on this particular subset in transgenic founders or lines. nd, not determined.

<sup>a</sup>Only three founders generated with TG-21 and two founders from TG-22 or 23 were analyzed for expression of hCD2 on thymocytes or IEL. For TG-31, only thymocytes isolated from two founders were analyzed for hCD2 expression.



**Figure 6. Developmental Stage-Specific Regulation of Enhancer Function during CD8 SP Thymocyte Development**

Thymocytes isolated from mice generated with TG-22 (founder #20) or TG-31 (founder #9) were analyzed by four-color flow cytometry for the expression of CD4, CD8, hCD2, and TCRαβ, CD69, or HSA.

(A) Histograms showing hCD2 expression levels on different subpopulations of CD8-expressing thymocytes. Representative gating areas for the CD4<sup>+</sup>CD8<sup>+</sup>, CD4<sup>lo</sup>CD8<sup>+</sup>, and CD4<sup>-</sup>CD8<sup>+</sup> populations are shown in the dot plot on the left. Human CD2-high (hi) and hCD2-low (lo) regions in the CD4<sup>lo</sup>CD8<sup>+</sup> histogram for TG-22#20 indicate representative gates used for further analysis in Figure 6B, while hCD2-negative (neg) and hCD2-positive (pos) regions in the CD4<sup>lo</sup>CD8<sup>+</sup> histogram for TG-31#9 indicate gates used for further analysis in Figure 6C.

(B) Expression of TCRαβ, CD69, and HSA (left, middle, and right column, respectively) on total CD4<sup>lo</sup>CD8<sup>+</sup> thymocytes (upper panel) and subsets of this population from TG-22#20 that express either high (middle panel) or low (lower panel) levels of hCD2, as defined in Figure 6A. (C) Expression of TCRαβ on total CD4<sup>lo</sup>CD8<sup>+</sup> thymocytes (upper panel) and subsets of this population from TG-31#9 that are either positive (middle panel) or negative (lower panel) for hCD2 expression, as defined in Figure 6A.

to HSA<sup>lo</sup>TCRαβ<sup>hi</sup> transition; see also Figure 6C) and E8<sub>IV</sub> (lower expression in SP cells) during thymocyte differentiation. Alternatively, it is possible that E8<sub>IV</sub> is active in DP cells as well and that the observed reduction of hCD2 expression during maturation of the CD8 lineage is due to extinguishing E8<sub>III</sub> enhancer activity (Figure 6C). Additional studies with transgenic constructs containing E8<sub>IV</sub> alone are required to determine if E8<sub>IV</sub> is also active in DP thymocytes.

## Discussion

In this study, we have used gene targeting to show that the CD8 enhancer E8<sub>I</sub> has a major role in regulating CD8αα homodimer expression in IEL. Previous studies had shown that E8<sub>I</sub> directs transgenic reporter gene expression both in mature CD8 SP thymocytes and CD8<sup>+</sup> T cells (Ellmeier et al., 1997; Hostert et al., 1997b) and in extrathymically derived IEL (Ellmeier et al., 1997). However, targeted deletion of this enhancer had no effect on development of the mature thymus-derived T cells, yet resulted in a dramatic reduction in the expression of CD8α in CD8αα<sup>+</sup> IEL of both TCRαβ and TCRγδ

lineages. Because expression of the closely linked CD8α and CD8β genes is likely to be coordinately regulated in thymus-dependent T cells, we believe that E8<sub>I</sub> represents an enhancer specific for CD8α expression in IEL. Additional T cell-specific enhancers identified near and within the 3' end of the CD8β gene, designated E8<sub>II</sub>, E8<sub>III</sub>, and E8<sub>IV</sub>, displayed developmental stage- and lineage-specific activities within the thymic-dependent T cell compartment but had no activity in the CD8αα<sup>+</sup> IEL. These novel enhancers are likely to function specifically in thymocyte differentiation and may regulate expression of CD8α, CD8β, or both. The results of this study thus reveal a remarkable diversification in lineage-specific regulation of CD8 gene expression by differential utilization of several closely linked *cis*-elements.

## The Role of E8<sub>I</sub> in Thymus-Derived T Cells

It has been shown that E8<sub>I</sub> is sufficient to direct expression of a reporter gene in mature CD8 SP thymocytes and in CD8<sup>+</sup> T cells, although these studies did not reveal whether E8<sub>I</sub> is the only enhancer regulating expression of CD8 in mature T cells. However, the tight correlation between onset of E8<sub>I</sub> activity with the final

steps of positive selection indicated a developmental stage-specific regulation of the enhancer and suggested an important role for E8<sub>i</sub> in the maturation of CD8 lineage thymocytes (Ellmeier et al., 1997; Hostert et al., 1997b). Therefore, it was surprising that the deletion of the enhancer did not affect the expression of CD8 and the appearance of thymus-derived CD8<sup>+</sup> T cells in the periphery. This might indicate that the behavior of the enhancer in transgenic mice did not reflect E8<sub>i</sub> function in the context of the endogenous chromosomal location. This possibility cannot be ruled out, although we consider this as rather unlikely since a defined developmental stage-specific induction (at the HSA<sup>hi</sup>TCR<sup>int/hi</sup> to HSA<sup>lo</sup>TCR<sup>hi</sup> transition) of enhancer activity and CD8 subset-specificity of E8<sub>i</sub> were observed in at least 19 independent transgenic founders or lines (generated with five different transgenic constructs). Instead, a more favorable explanation would be that additional *cis*-regulatory elements are involved in regulating CD8 expression in thymus-derived T cells and that these enhancers are able to compensate for loss of E8<sub>i</sub> activity (see below). This notion is supported by the observation that mature CD8 SP thymocytes from E8<sub>i</sub>-deficient mice (either N/N or Δ/Δ) exhibited a 10%–20% reduction in the level of surface CD8.

#### The Role of E8<sub>i</sub> in Intestinal IEL

CD8αα<sup>+</sup> IEL (of both the TCRαβ and TCRγδ lineage) are primarily of extrathymic origin, whereas CD8αβ<sup>+</sup> IEL (exclusively of the TCRαβ lineage) may require the thymus for development (Poussier and Julius, 1994; Rocha et al., 1995; Klein, 1996). The expression of CD8αα homodimers on thymus-independent IEL was severely compromised in E8<sub>i</sub>-deficient mice, and there was also a modest decrease of CD8α levels in CD8αβ<sup>+</sup> IEL. TCRγδ<sup>+</sup> IEL displayed reductions in both CD8αα expression levels (about 3- to 5-fold) and the percentage of cells expressing CD8αα. The reason for loss of CD8αα expression on most, but not all, E8<sub>i</sub>-null IEL is not known. It is possible that there are sublineages of TCRγδ IEL that differ in their ability to employ one or more enhancers other than E8<sub>i</sub> for CD8α gene expression. Other enhancers may thus be able to partially compensate in the CD8αα<sup>low</sup>TCRγδ<sup>+</sup> sublineage. Future comparative studies of the Vγ and Vδ usage by these IEL subsets in E8<sub>i</sub>-knockout mice might help to reveal whether they represent different sublineages. Alternatively, E8<sub>i</sub> may be involved in establishing an open chromosomal configuration of the CD8α locus during extrathymic development of TCRγδ<sup>+</sup> IEL, and its absence would result in variegated expression of CD8α.

TCRαβ IEL also displayed lower levels of CD8αα homodimers, although to a lower extent (2- to 3-fold). A decrease in the proportion of TCRαβ<sup>+</sup> cells expressing CD8αα homodimers was observed in some E8<sub>i</sub>-deficient mice. Nevertheless, the IEL phenotype in E8<sub>i</sub>-deficient mice clearly indicates that E8<sub>i</sub> contains major *cis*-regulatory elements required for CD8α expression in this presumptive extrathymically derived lymphocyte population.

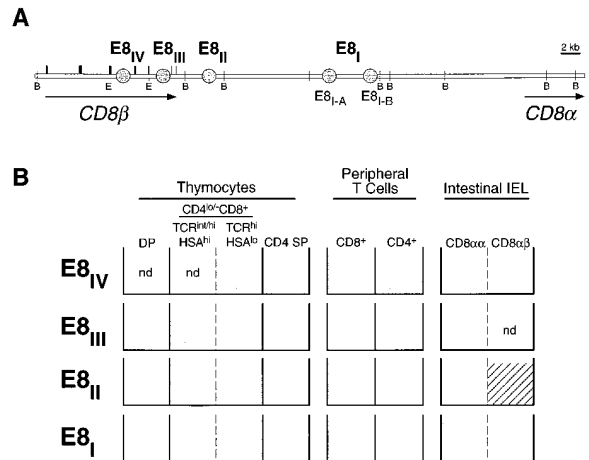


Figure 7. Function of Multiple Enhancers in CD8 Gene Regulation (A) Schematic map of the CD8 locus and the location of the individual enhancers relative to the CD8α and β genes (arrows indicate the transcriptional orientation). All BamHI (B), but only relevant EcoRI (E) restriction sites are shown. The exact location of E8<sub>IV</sub>, E8<sub>III</sub>, or E8<sub>II</sub> within the B/B, B/E, or E/E fragments, respectively, is not known. Closed and open bars around the CD8β gene indicate CD8β coding and noncoding exons, respectively (Nakayama et al., 1989). The mature CD8<sup>+</sup> T cell enhancer E8<sub>i</sub> can be divided into two enhancer subregions designated E8<sub>i</sub>-A and E8<sub>i</sub>-B (based on the location of the DNase I hypersensitivity sites and on transgenic reporter expression studies; Ellmeier et al., 1997; Hostert et al., 1997b). (B) Diagram summarizing the developmental stage-, subset- and lineage-specificity of the individual enhancers in the indicated T lymphocyte populations. The developing CD8 single-positive thymocyte lineage (CD4<sup>lo</sup>-CD8<sup>+</sup>) is subdivided into TCR<sup>int/hi</sup>HSA<sup>hi</sup> and TCR<sup>hi</sup>HSA<sup>lo</sup> populations. The shaded areas indicate enhancer activity, while the hatched area indicates only weak enhancer activity, nd, not determined.

#### Multiple Subset-Specific *Cis*-Regulatory Elements in the CD8 Locus

The phenotype observed in E8<sub>i</sub> enhancer knockout mice indicated that there must be other *cis*-regulatory elements that direct CD8 expression not only in immature thymocytes but also in peripheral CD8<sup>+</sup> T cells. It has recently been shown (Hostert et al., 1997a) that several DH sites are clustered (in a region designated cluster IV) around the CD8β gene. Transgenic mice prepared with a construct containing the DH sites from cluster IV (in a 15 kb genomic fragment) directed reporter gene expression in both immature DP thymocytes and mature CD8<sup>+</sup> T cells. This result is consistent with a recent finding that a 95 kb genomic fragment spanning the human CD8β gene could direct expression of CD8β on DP thymocytes and mature CD8 SP T cells in transgenic mice (Kieffer et al., 1997).

In the process of mapping the *cis*-acting elements within the 15 kb genomic fragment, we were able to separate an enhancer activity (E8<sub>III</sub>) specific for immature thymocytes from another enhancer activity (E8<sub>i</sub>) that mediated expression both in DP and in CD8 SP thymocytes (see summary in Figure 7). E8<sub>III</sub> directed expression of the reporter gene in DP thymocytes, but its activity ceased during positive selection as indicated by a gradual loss of hCD2 surface expression on thymocytes at

a later developmental stage (i.e., at the  $\text{TCR}\alpha\beta^{\text{int/hi}}$  to  $\text{TCR}\alpha\beta^{\text{hi}}$  transition) and by the absence of hCD2 expression on peripheral T lymphocytes. It remains to be determined whether there are physically separable DP and SP enhancer activities within  $\text{E8}_{\text{II}}$ . Further transgenic expression studies will help to clarify this issue.

An additional level of complexity was revealed by the finding that a 3.2 kb genomic region from the CD8 $\beta$  locus contains an enhancer activity ( $\text{E8}_{\text{IV}}$ ) that directs expression of the hCD2 reporter not only in CD8 SP thymocytes and CD8 $^+$  T cells but also in the CD4 lineage. This observation is unlikely to be explained by position effects due to transgene integration, since six independent founders generated from two different constructs (TG-22 and TG-25) containing the 3.2 kb fragment (in addition to other genomic regions, see map in Figure 5D) displayed this expression pattern of the hCD2 reporter (Table 2).

It has recently been shown that CD8 $\beta$  is expressed at low levels in a subset of CD4 $^+$  T cells, which would be consistent with the possibility that  $\text{E8}_{\text{IV}}$  represents an enhancer specific for expression of CD8 $\beta$  in both CD4 $^+$  and CD8 $^+$  T cells. Expression of a CD8 $\alpha$  transgene in the CD4 subset resulted in the appearance of about 25% mature CD4 $^+$  T cells that also expressed CD8 $\alpha\beta$  heterodimers, while the majority of the other transgenic CD4 cells expressed only CD8 $\alpha\alpha$  homodimers (Hostert et al., 1997a). The enhancer driving CD8 $\beta$  expression in the CD4 lineage appears to be weak, since only a subset of CD4 $^+$  T cells expressed low levels of the CD8 $\beta$  protein. This may explain why hCD2 expression was consistently observed in a smaller proportion of CD4 SP cells than in cells of the CD8 lineage, although the possibility remains that there are additional *cis*-elements required for expression of CD8 $\beta$  in CD4 lineage cells.

#### Enhancer Activities and Lineage Commitment

An implication of the finding of the mature CD8 enhancer ( $\text{E8}$ ) was that there must exist other enhancers directing CD8 expression in immature thymocytes. Therefore, a model was proposed suggesting that different *cis*-acting elements are involved in regulating CD8 expression in immature and mature thymocytes and that a switch in enhancer usage and activities occurs during the latest stages of positive selection and CD8 lineage development. A prediction of this model is that different sets of transcription factors may be involved in regulating CD8 expression in immature and mature thymocytes, unless the accessibility of the different enhancers changes during development. The identification of additional CD8 locus enhancers that display developmental stage-specific activity supports this model, although some enhancers may be active both in immature and mature thymocytes.

Our studies have not addressed the specificity of the enhancers toward the CD8 $\alpha$  and/or  $\beta$  genes and the close proximity of the four different enhancers raises the question as to how T cell lineage-specificity of CD8 $\alpha$  or CD8 $\beta$  expression is achieved. In contrast to  $\text{E8}_{\text{I}}$ , which directed expression in CD8 $\alpha\alpha^+$  IEL (Ellmeier et al., 1997) and hence is likely to function in conjunction with the CD8 $\alpha$  promoter,  $\text{E8}_{\text{II}}$ ,  $\text{E8}_{\text{III}}$ , and  $\text{E8}_{\text{IV}}$  function in CD8 $\alpha\beta^+$

cells and may therefore regulate expression of CD8 $\alpha$ , CD8 $\beta$ , or both. Studies on the regulation of the murine HoxB cluster have recently demonstrated that some promoters can either share or compete for the same enhancer (Sharpe et al., 1998). Analyses on promoter and enhancer interactions in *Drosophila* have also shown that compatibility between the interactions of these *cis*-regulatory elements, regulated by properties of the promoter region, is one way to achieve selectivity of gene expression in "gene-dense" areas (Li and Noll, 1994; Merli et al., 1996; Ohtsuki et al., 1998).

Since the transgenic constructs in our studies utilized the CD8 $\alpha$  promoter, it is possible that some enhancers identified in this study (if they are specific for CD8 $\beta$ ) do not function properly in conjunction with the CD8 $\alpha$  promoter. Incompatibility of enhancer and promoter combinations could also explain why the  $\text{E8}_{\text{I}}$  enhancer, which is specific for CD8 $\alpha$  expression in extrathymically derived IEL, does not act upon the CD8 $\beta$  gene and its promoter, indicated by the absence of CD8 $\beta$  expression in these T cells. Alternatively, boundary or insulator elements might be localized between the  $\text{E8}_{\text{I}}$  enhancer and the CD8 $\beta$  gene, thereby preventing  $\text{E8}_{\text{I}}$  and CD8 $\beta$  promoter interaction and subsequent expression of CD8 $\beta$  in IEL. Therefore, in future studies it will be important to generate transgenic constructs in which identified enhancers are combined with a reporter gene driven by the CD8 $\beta$  promoter. In addition, single or combinatorial deletions of the enhancers by homologous recombination should help to determine the specificity of the individual enhancers.

The identification of several *cis*-acting elements from the CD8 locus provides us now with the opportunity to identify *trans*-acting factors that bind to these enhancers. Since CD8 expression correlates with the cytotoxic program of T cells, this could also lead to the identification of factors involved in directing a DP thymocyte into the cytotoxic CD8 lineage.

#### Experimental Procedures

##### Generation of the $\text{E8}_{\text{I}}$ Targeting Construct

A genomic clone containing the enhancer region was isolated from a 129 genomic library (Stratagene) and subcloned into pBluescript (pBS; Stratagene) (Ellmeier et al., 1997). The short and the long arms of the targeting construct were isolated as a 1.1 kb BamHI/NotI fragment (representing one end of the isolated genomic clone) or as a 5.6 kb AflIII (blunted)/BamHI fragment, respectively. The short and the long arms were sequentially cloned into a pBS-based vector containing a polylinker with suitable cloning sites and the thymidine kinase gene driven by the herpes simplex virus enhancer/promoter elements (Thomas and Capecchi, 1990). A polylinker containing a Sall site was then cloned between the short and the long arm of the targeting construct and a 1.4 kb XhoI/Sall fragment containing a neomycin resistance gene (driven by the thymidine kinase enhancer/promoter) flanked by two loxP sites (Gu et al., 1993) was subsequently inserted into the Sall site. All cloning steps were performed according to standard procedures (Sambrook et al., 1989).

E14.1 ES cells (Kuhn et al., 1991) were transfected with 30  $\mu\text{g}$  NotI-linearized targeting vector and cultured on mitomycin C-treated murine embryonic fibroblasts. Two days after transfection, G418 (Geneticin, Gibco) was added to a final concentration of 350  $\mu\text{g}/\text{ml}$  and after another 2 days gancyclovir (GANC) was added (2  $\mu\text{M}$  final concentration) for negative selection. There was approximately 10-fold enrichment between plates with and without GANC. Nine days post-transfection, individual ES cell colonies were isolated and half

of each colony was initially screened in pools of six by PCR analyses (PCR product 1 in Figure 1). ES cell clones of PCR positive pools were expanded, and correct targeting (both for the 5' and 3' end) was confirmed by Southern blotting. Two targeted ES cell clones were injected into E3.5 C57BL/6 blastocysts and transferred into (B6/D2) F1 pseudopregnant females. Chimeric mice obtained were then backcrossed to either C57BL/6 or AKR1 mice and transmission of the targeted allele was confirmed by PCR and Southern blot analyses of tail DNA.

To obtain mice that had deleted the neomycin expression cassette, heterozygous enhancer knockout mice were crossed to transgenic mice expressing Cre recombinase under the control of the CMV promoter (White et al., 1997). The deletion of neomycin was confirmed by PCR analysis (PCR products 2 and 3 in Figure 1) and by Southern blotting.

#### Generation of Transgenic Constructs

A genomic 129 library (Stratagene) was screened with probes from the CD8 locus and several clones were isolated. The inserts were subcloned into pBS and subfragments containing DH sites were cloned into the CD8 $\alpha$  promoter-hCD2 reporter construct (transgene TG-a in Ellmeier et al., 1997). A detailed protocol can be obtained upon request.

#### Generation of Transgenic Mice

F2 eggs of (B6/D2) mice were injected with the transgenic constructs according to standard procedures (Hogan et al., 1994). Founders were identified by Southern blotting of tail DNA with a probe from the CD4 splicing module (Sawada et al., 1994). Transgenic animals were either analyzed directly or backcrossed to C57BL/6 to generate transgenic lines. All animals analyzed were of age between 4 and 16 weeks.

#### Flow Cytometric Analysis and Antibodies

Thymus, lymph nodes, and spleen were removed from euthanized animals and placed into 60 mm tissue culture dishes containing staining buffer (phosphate-buffered saline supplemented with 2% FCS and 0.1% sodium azide). Single cell suspensions were made by passing the tissue through a 70  $\mu$ m nylon cell strainer. The cell suspensions were washed once with staining buffer and  $1-5 \times 10^5$  cells were incubated on ice with Fc-block (Pharmingen) for 5 min and subsequently with the appropriate antibodies for 30 min. Afterward, the cells were washed once with staining buffer and analyzed or incubated with secondary antibodies on ice for 30 min. The following antibodies were used for the stainings: FITC- or biotin-conjugated (bio) anti-hCD2 (clone G11), PE-anti-mCD8 $\alpha$  (CT-CD8 $\alpha$ ), FITC-anti-mCD8 $\beta$  (CT-CD8 $\beta$ ), TC-anti-mCD4 (CT-CD4), PE- or bio-anti-mCD3 (Clone 500-A2), PE-anti-B220 (RA3-6B2) and TC-streptavidin from Caltag, APC-anti-mCD4 (RM4-5), FITC- or bio-anti-HSA (M1/69), bio-anti-CD69 (H1.2F3), bio-anti-mTCR $\gamma\delta$  (GL3), bio-anti-mTCR $\alpha\beta$  (H57-597), and bio-anti-mCD3 $\epsilon$  (145-2C11) from Pharmingen. Cells were analyzed using Becton Dickinson FACScan flow cytometer and cell quest software.

#### Isolation of Intestinal Intraepithelial Lymphocytes

IEL were isolated as described (Ellmeier et al., 1997). In brief, the gut was removed from euthanized animals and the gut lumen was washed by flushing with IEL isolation medium (20 mM HEPES-buffered RPMI supplemented with 10% serum, L-glutamate, and antibiotics). The gut was turned inside-out over a polyethylene tubing and incubated in 100 ml IEL isolation medium for 45 min at 37°C in a shaker with low agitation to release the lymphocytes from the gut epithelium into the medium. The IEL were pelleted by centrifugation (at 2000 rpm for 10 min at room temperature), resuspended in IEL isolation medium, and purified by 37% Percoll centrifugation (at 1750 rpm for 30 min at room temperature). Cells were washed twice with staining buffer, incubated for 5 min with Fc-block (Pharmingen), and subsequently stained with antibodies.

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