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*J Immunol* 2005; 174:1513-1524; doi: 10.4049/jimmunol.174.3.1513

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Functional and Molecular Analysis of the Double-Positive Stage-Specific CD8 Enhancer E8III during Thymocyte Development

Nicholas Feik,* Ivan Bilic,* Johanna Tinhofer,2* Bernd Unger,*† Dan R. Littman,‡ and Wilfried Ellmeier3*

Several developmental stage-, subset-, and lineage-specific Cd8 cis-regulatory regions have been identified. These include the E8III enhancer, which directs expression in double-positive (DP) thymocytes, and E8H, which is active in DP cells and CD8+ T cells. Using a transgenic reporter expression assay, we identified a 285-bp core fragment of the E8H enhancer that retains activity in DP thymocytes. In vitro characterization of the core enhancer revealed five regulatory elements that are required for full enhancer activity, suggesting that multiple factors contribute to the developmental stage-specific activity. Furthermore, deletion of E8H in the mouse germline showed that this enhancer is required for nonvariegated expression of CD8 in DP thymocytes when E8H is also deleted. These results indicate that E8III is one of the cis-elements that contribute to the activation of the Cd8a and Cd8b gene complex during T cell development. The Journal of Immunology, 2005, 174: 1513–1524.

The development of thymocytes that express the αβ TCR provides an ideal model system for the molecular analysis of regulatory mechanisms involved in cell-fate decisions and lineage differentiation in vertebrates. Based on the expression of a number of surface molecules, particularly the CD4 and CD8 coreceptors, distinct developmental stages can be defined. The earliest committed T cells do not express CD4 or CD8 and are referred to as double-negative (DN) thymocytes. These cells develop into double-positive (DP) cells that express both CD4 and CD8. DP thymocytes that express TCRs with appropriate avidity for self-peptide/MHC complexes develop during a process known as positive selection into either CD4+CD8+ or CD4−CD8+ single-positive (SP) T cells. The CD4+ T cells constitute the T cell lineage and express TCRs that together with CD4 recognize foreign peptides presented by MHC class II molecules, while the CD8+ T cells form the MHC class I-restricted cytotoxic T cell lineage. Both coreceptors serve an important role in the recognition of Ag/MHC complexes by the TCR not only in mature T cells, but also during T cell development and positive selection, as highlighted by the absence of the cytotoxic T cell lineage or the severely reduced Th cell subset in CD8− or CD8-deficient mice, respectively (1).

The mechanisms of helper or cytotoxic lineage differentiation are still a subject of debate, and the molecular details are only beginning to be elucidated (2). Nevertheless, a number of studies indicate a correlation between coreceptor expression and the functional phenotype of the T lymphocyte lineage. Thus, common factors may regulate the expression of the Cd4 and Cd8 genes and the specification of the appropriate T cell lineage during the DP to SP transition. Understanding the transcriptional regulation of coreceptor gene expression may also provide insight into the developmental choice between helper and cytotoxic T cell fate.

DNase I hypersensitivity (DH) site studies combined with transgenic reporter expression assays have been previously used to identify several major CD8 cis-regulatory elements. DP thymocytes and most of the peripheral CD8+ T cells express CD8α as a heterodimer formed by the CD8α and CD8β molecules, while intestinal intraepithelial lymphocytes (IEL) express only CD8αα homodimers. Four clusters (I, II, III, and IV) of DH sites were described within a 80-kb murine genomic fragment covering the Cd8a and Cd8b loci (3). Dissection of these clusters with transgenic reporter expression assays led to the identification of at least five genomic fragments that individually or in combination directed expression in the T cell lineage. These results revealed a complex regulatory network with developmental stage-, subset-, and lineage-specific use of multiple closely linked cis-regulatory elements in lineage-specific regulation of CD8α and CD8β expression during T cell development (4). To study the function of the various cis-regions in more detail, single or combinatorial deletions of cis-elements in the mouse germline have been initiated. Initial studies have focused on three regulatory regions: 1) enhancer E8I (overlapping with DH cluster III, sites 1 and 2 and therefore also named CIII-1,2 or CIII-1,2(E8I)) that is active in mature CD8+ T cells and IEL; 2) enhancer E8II (or CIV-4,5(E8II)), active both in DP thymocytes and CD8+ T cells; and 3) DH site cluster II, which in combination with cluster CIII-1,2 contributes to DP-specific expression (see also Fig. 1A for the location of the
various cis-elements and DH clusters and sites). Individual deletions of either E8III or E8III did not cause any alterations in the expression pattern of CD8 in the various T cell subsets (5–7). In contrast, deletion of both enhancers had a major effect on the expression of CD8 during thymocyte development (6). A population of “CD8-negative” DP thymocytes appeared that was indistinguishable from DP thymocytes by surface marker expression and by functional phenotype. Remarkably, a very similar phenotype with even a higher proportion of “CD8-negative” DP thymocytes was observed in mice with a deletion of DH cluster II (8). The concurrent appearance of “CD8-negative” DP thymocytes and DP was observed in mice with a deletion of DH cluster II (8). The variegation of expression of CD8 in the absence of either cluster II or enhancers E8III and E8III. These results revealed novel functions of the CD8 cis-regulatory elements and indicated that the CD8 enhancers may also function as recruitment factors or for factors that govern the accessibility of adjacent genomic fragment, was down-modulated during positive selection. (5). The activity of this enhancer, initially localized on a 4-kb trans-acting factors that bind to the enhancer. This study describes a more detailed characterization of the DP-specific enhancer E8III.

An additional enhancer identified in previous studies, enhancer E8III (or CIV-3(E8III)), directed expression only in DP thymocytes (5). The activity of this enhancer, initially localized on a 4-kb genomic fragment, was down-modulated during positive selection. Because enhancer function was restricted to immature DP thymocytes in transgenic reporter mice, it is likely that this element functions either as a target for developmental-stage-specific transcription factors or for factors that govern the accessibility of adjacent chromatin regions to transcriptional regulators during the DP to CD8SP transition. To understand the molecular basis of the DP-specificity of enhancer E8III and to understand its regulatory activity during T cell development, it is important to isolate the trans-acting factors that bind to the enhancer. This study describes a more detailed characterization of the DP-specific enhancer E8III.

We have identified a core 285-bp fragment (designated as E8III core enhancer) that is sufficient for directing expression in DP thymocytes. In vitro transactivation studies revealed five elements that are required for full enhancer activity of the core fragment, which suggests that multiple factors contribute to the DP stage-specific activity of E8III. Because other cis-elements from the Cd8a and Cd8b loci, either individually or in combination, also direct expression in DP thymocytes, it was not clear from previous transgenic studies whether the DP-specific enhancer is necessary for CD8 expression. Generation of E8III and E8III double-knockout mice revealed that E8III is indeed required in DP thymocytes to ensure nonvariegated expression of CD8 in the absence of E8III. The results implicate E8III as one of the multiple cis-elements involved in the activation of the Cd8ab gene complex during T cell development.

Materials and Methods

Generation of E8III reporter constructs

The luciferase reporter constructs used in this study were based on the pGL3-basic vector (Promega). The minimal Cd8a promoter (nt 1–391; (13)) was amplified with CD8αF-P (F for forward) and CD8αR-P (R for reverse) primers (5′-ATATATCTGGTACCTGACAAGCTGGAAGGCGCAGGAGGTCCGGTGGGAGG, respectively; underlined sequences indicate homology to Cd8a promoter region) and subcloned into pBlueScript (pBS; Stratagene). Afterward, the Cd8a promoter was inserted as a SacI-Sall fragment into a SacI-Xhol cut modified (HindIII and BamHI sites were deleted) pGL3-basic vector. Subsequently, multiple cloning sites (MCS; Xhol-Spel-EcoRI-BamHI-EcoRV) were introduced upstream of the Cd8a promoter between SacI and HindIII sites (generated at the 5′ end of the Cd8a promoter; see CD8αF-P primer). This plasmid constitutes the basic reference reporter construct L1 (Cd8a promoter only). The 4-kb EcoRI/BamHI CIV-3 (E8III) genomic fragment (L2 and L2) and various subfragments (L3-L11) were inserted via the MCS. The various genomic fragments had the following size and restriction sites: L2 (3946 bp; EcoRI/BamHI, genomic restriction sites), L3 (2485 bp; BamHI-SacI), L4 (1467 bp; SacI-BamHI), L5 (1130 bp; SacI-MscI), L6 (670 bp; SacI-HindIII), L7 (209 bp; SacI-Spel), L8 (1263 bp; Spel-BamHI), L9 (804 bp; HindIII-BamHI), L10 (285 bp; AvrII-BamHI), and L11 (525 bp; HindIII-AvrII).

The various 5′ (L2-L12) and 3′ (L16-L18) deletion constructs of the 285-bp enhancer were generated by PCR and cloned as EcoRI-BamHI fragments in the MCS upstream of the Cd8a promoter in plasmid L1. All deletion constructs were verified by sequencing. Underlined nucleotides within the primer sequence indicate nucleotides added for cloning purposes. 5′ deletion constructs (L2-L12) were generated by PCR using the same BamHI-reverse primer (5′-ATATATCGACCGGCCAGCACTGACCTGGCA TTTTTT; with the following forward primers: L12F, 5′-ATAGAATCTTCTAGCCCGTATAGACCTGCGAC; 3′ deletion constructs (L16-L18) were generated by PCR using the same EcoRI-forward primer (5′-ATATATCGACCGGCCAGCACTGACCTGGCATTTTTT; with the following reverse primers: L18R, 5′-TG TCGAGCTCGTCTAAGCGGTAGTATAGACCTGCGAC; and L15F, 5′-TATAGAATCTTCTAGCCCGTATAGACCTGCGAC). Internal deletion constructs (L19–L21) and site-specific mutations (L24, L24) were generated via overlap PCR. Deletion-specific primer pairs that were used for the first-step reaction are indicated, while all second-step overlap PCR were performed with the EcoRI-forward and BamHI-reverse primer pair. Primer pairs for the first-step products were: for the deletion of regulatory element (RE) 2 (RE2, 5′-ATATATCGACCGGCCAGCACTGACCTGACCTG; and Del25R, 5′-AGAATCTTCTAGCCCGTATAGACCTGCGAC) and 5′-BamHI-reverse primer pair. For the deletion of nt 159–184 (L19); EcoRI-forward and Del5 5′-TG TCGAGCTCGTCTAAGCGGTAGTATAGACCTGCGAC; and L18R, 5′-TATAGAATCTTCTAGCCCGTATAGACCTGCGAC).

Generation of transgenic constructs

The generation of the basic Cd8a promoter-human CD2 (hCD2) reporter construct (transgene Tg-a in Ref. 14) and of TG-31 (containing the 4-kb EcoRI/BamHI genomic fragment) (5) has been previously described. Shorter genomic fragments used for the generation of TG-32 (a 2.7-kb CD2 promoter only) were cotransfected to normalize for transfection efficiency. The total amount of transfected DNA was equal for each transfection and adjusted by adding pBS. After electroporation, cells were cultured in 15 ml of RPMI 1640) were electroporated by guest on April 18, 2017 from http://www.jimmunol.org/ Downloaded by guest on April 18, 2017

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1514 CHARACTERIZATION OF A DP-SPECIFIC CD8 ENHANCER
ES cell transfection and generation of enhancer-deficient mice

E14.1 ES cells (17) were transfected with 30 μg of NotI-linearized targeting vector and cultured on mitomycin c-treated murine embryonic fibroblasts. Two days after transfection, G418 (Geneticin, Invitrogen) was added to a final concentration of 350 μg/mL and after another 2 days, gancyclovir was added (2 μM final concentration) for negative selection. At this time, there was ~5-fold enrichment between plates with and without gancyclovir. Nine days posttransfection, individual ES cell colonies were isolated and half of each colony was reseeded into a well of a 48-well plate. Two days afterward, one plate was used for freezing the ES cell clones, while the other one was used for the isolation of DNA to perform Southern blotting for the detection of homologous recombination. Targeted ES cell clones were expanded into E3.5 C57BL/6 blastocysts and transferred into B6(D2) F1 pseudopregnant females. Chimeric mice obtained were then backcrossed to C57BL/6 mice, and transmission of the targeted allele in agouti offspring was confirmed by PCR and Southern blot analyses of tail DNA.

To obtain mice with a deletion of the neomycin expression cassette, heterozygous enhancer knockout mice were crossed to transgenic mice expressing Cre recombinase under the control of the CMV promoter (18). The deletion of neomycin was confirmed by PCR analysis with the following primers: PCR-1, 5′-CTGTTGATGACAGCTTATTGG; PCR-2, 5′-CATTGGGCGACGGTTGTT; and PCR-3, 5′-CAAAGAGAAGGAAAGGACACATT.

Expression of transgenic mice

F2 eggs of (B6/D2) mice were injected with the various transgenic constructs according to standard procedures. Transgenic founders were identified by Southern blotting for the detection of homologous recombination. Targeted ES cell transfection and generation of enhancer-deficient mice

Generation of transgenic mice

Thymus, lymph nodes, and spleen were removed from euthanized animals and placed into 60-mm tissue culture dishes containing staining buffer (PBS supplemented with 2% FCS and 0.1% sodium azide). Single-cell suspensions were made by passing the tissue through a 70-μm nylon cell strainer. The cell suspensions were washed once with staining buffer and 1–5 × 10^6 cells were incubated on ice with Ab-block (BD Pharmingen) for 5 min and subsequently with the appropriate Abs for 30 min. Afterward, the cells were washed once with staining buffer and analyzed or incubated with secondary Abs on ice for 30 min. The following Abs were used for the stainings: FITC- or biotin-conjugated anti-CD2 (clone G11), FITC- or PE-anti-mCD8α (CT-CD8αa), FITC-anti-mCD8β (CT-CD8βb), PE- anti-mCD4 (CT-CD4), PE- or biotin-anti-mCD3ε (clone 500-2A2), and TC-streptavidin from Caltag, APC-anti-mCD4 (RM4-5), bio-anti-mTCRγδ (GL3), bio-anti-mTCRαβ (H57-597), bio-anti-mCD3ε (145-2C11), PE-anti-mCD11c (HL3), PE-anti-mCD5 (53-73) from BD Pharmingen. Cells were analyzed using BD Biosciences FACSCalibur flow cytometer and CellQuest pro software.

Isolation of intestinal IEL

IEL were isolated as described (14). Briefly, the gut was removed from euthanized animals and placed into 60-mm tissue culture dishes containing staining buffer (PBS supplemented with 2% FCS and 0.1% sodium azide). Single-cell suspensions were made by passing the tissue through a 70-μm nylon cell strainer. The cell suspensions were washed once with staining buffer and 1–5 × 10^6 cells were incubated on ice with Ab-block (BD Pharmingen) for 5 min and subsequently with the appropriate Abs for 30 min. Afterward, the cells were washed once with staining buffer and analyzed or incubated with secondary Abs on ice for 30 min. The following Abs were used for the stainings: FITC- or biotin-conjugated (bio) anti-β2CD2 (clone G11), FITC- or PE-anti-mCD8α (CT-CD8αα), FITC-anti-mCD8β (CT-CD8ββ), PE- or biotin-anti-mCD4 (CT-CD4), PE- or biotin-anti-mCD3ε (clone 500-2A2), and TC-streptavidin from Caltag, APC-anti-mCD4 (RM4-5), bio-anti-mTCRγδ (GL3), bio-anti-mTCRαβ (H57-597), bio-anti-mCD3ε (145-2C11), PE-anti-mCD11c (HL3), PE-anti-mCD5 (53-73) from BD Pharmingen. Cells were analyzed using BD Biosciences FACSCalibur flow cytometer and CellQuest pro software.

Flow cytometric analysis and Abs

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FIGURE 1. Identification of the E8III core enhancer fragment. A, Schematic map of the Cd8a and Cd8b gene loci after Gorman et al. (41). Horizontal arrows indicate the transcriptional orientation of the Cd8a and Cd8b genes. Vertical arrows indicate the localization of DH sites that constitute clusters II, III, and IV (3). The vertical thick bars below DH sites indicate the genomic fragment used to define enhancers E8I, E8II, E8III, E8IV, and cluster II (5, 14). All BamHI (B) but only relevant EcoRI (E) sites are shown. B, Genomic fragments (and their length) used for the reporter assays are shown at the left. Fragments were inserted upstream of a Cd8a promoter-luciferase reporter vector and constructs were transfected into 1200M cells. Representative bar diagrams at the right indicate the luciferase activities of the various reporter constructs (L2-L11) relative to the Cd8a promoter construct (L1). All transfections were performed at least twice and in duplicates. Values are normalized for transfection efficiencies.

As described above, the E8III core enhancer was mapped to a 285-bp genomic fragment downstream of the Cd8b gene. Although the core enhancer is sufficient to direct expression in DP thymocytes, it cannot be revealed by the transgenic reporter expression assay whether E8III is necessary for CD8 expression. Furthermore, it is not possible to distinguish whether E8III acts on Cd8a, Cd8b or on both genes. To overcome the limitations of the transgenic reporter expression system, we decided to generate mice with a deletion of E8III. Another enhancer from the Cd8ab gene loci that is active in DP thymocytes (and also in CD8+ T cells) - designated E8II (or CIV-4.5) - maps adjacent to E8III (5). In previous studies we showed that CD8α and CD8β expression is normal in E8II-deficient mice (6). Because E8III and E8II are both active in DP thymocytes, a targeting vector was designed to allow the combined deletion of both cis-elements. Therefore, a 804-bp HindIII-BamHI genomic fragment (as defined in Fig. 1B, construct L9) immediately downstream of the Cd8b 3‘ untranslated region that contains the E8III core enhancer (see Fig. 3A, lower panel, for the location of the E8III core enhancer within the 804-bp fragment) and an adjacent 4.4-kb genomic BamHI fragment that contains E8III (5) were deleted in ES cell by homologous recombination. Heterozygous +/-N2N3 ES cell clones (Fig. 3C) were selected for injection into C57BL/6 blastocysts and chimeric mice obtained from +/-N2N3 ES cells were back-crossed to C57BL/6 mice for germ-line transmission. To delete the neomycin expression cassette, heterozygous +/-N2N3 mice were crossed with Cre recombinase transgenic mice (18) to obtain +/-23 mice. These mice were intercrossed to obtain +/-, +/-D3 and D3/D3 mice (Fig. 3D). Mild variegation of CD8 expression in the absence of E8III and E8II

Even though genomic fragments containing either E8III or E8II (or both enhancers) were sufficient to direct expression of a transgenic hCD2 reporter gene in DP thymocytes, D2D3/D2D3 mice had relatively normal thymic and peripheral CD8 expression patterns (Fig. 4A). T cell development appeared normal and there was also no significant change in the expression levels of CD8α and CD8β in double-deficient thymocytes or T cells (data not shown).

We noticed however a small difference in the relative abundance of the different T cell subsets in enhancer-deficient mice (Fig. 4, A
FIGURE 2. The E8III core enhancer directs expression in DP thymocytes. A, Schematic map of the Cdsb and Cdsb gene loci (as described in Fig. 1A) and of the genomic fragments used for the generation of transgenic mice (TG-30, -31, -32, -40, and -42). The length of the genomic TG inserts is shown at the right. The vertical bar below DH site CIV-3 indicates the genomic fragment (TG-31) used to define enhancer E8III. TG-31 has been already reported previously (5). B, Schematic map of the transgenic reporter construct used for the generation of transgenic mice. The basic construct contains part of the first Cdsb intron as a splicing module (19) between the Cdsb promoter and the hCD2 reporter gene. Genomic fragments shown in A were inserted upstream of the Cdsb promoter. C, Three-color flow cytometry analysis of thymocytes and lymph node cells isolated from TG-40 transgenic mice (founder no. 19). Dot plots show CD4 vs CD8 expression and representative gates (gates are indicated in the upper right of each histogram) or lymph node cells. D, Table showing a summary of the results from the transgenic reporter expression assay. For each transgenic founder, the copy number and the percentage of hCD2-positive cells within the DP thymocyte subset is shown. —, <0.5% hCD2-positive cells (background staining); n.a., not analyzed.

Another group of cells that express CD8αα homodimers are the IEL within the intestine (28, 29). Deletion of E8III and E8IV did not alter the expression of CD8αα homodimers on IELs (Fig. 5B).

Multiple regulatory elements are required for full E8III core enhancer activity

The results from the transgenic reporter assays and from enhancer-deficient mice indicated that the DP thymocyte-specific enhancer contributes to the proper regulation of CD8 expression. Furthermore, there was a good correlation between the enhancer function of the various E8III genomic fragments in both in vitro and in vivo reporter assays. Thus, transient reporter gene expression assays in 1200M cells are a suitable and reliable approach to further characterize and dissect the E8III core enhancer. Therefore, we performed additional transient expression assays to identify regulatory elements (RE) within the E8III core enhancer fragment. 1200M cells were transiently transfected with reporter constructs containing 5’, 3’, or internal deletions of the 285-bp fragment and the luciferase activity was determined (Fig. 6A). These deletion experiments led to the identification of five regulatory elements (RE-1, -2, -3, -4, and -5; Fig. 6B, boxed areas) required for full enhancer activity of the 285-bp core fragment. Deletion of RE-1 (Δ1–29; construct L12) led to an ~3- to 5-fold reduction of enhancer activity, a similar activity also observed with L14 (Δ1–134). This indicates that no additional important factor binding sites are lost due to the further deletion of nt 29–134. However, additional deletion of nt 135–158 (Δ1–158; L15) caused an abolition of enhancer activity, indicating the presence of another regulatory element (designated RE-2) within this 26-bp of DNA. Deletion of RE-3 (Δ252–285; Fig. 6A, middle panel, L16) at the 3’ end of the E8III core enhancer caused a 3- to 5-fold reduction of
FIGURE 3. Targeting strategy for the deletion of the CD8 enhancers EbH1 and EbH2. A, upper panel, Map of the Cd8a and Cd8b gene loci showing the location of the deleted CD8 enhancers relative to the Cd8a and Cd8b genes (indicated by horizontal arrows). Lower panel, Map showing the location of the 285-bp EbH2 core enhancer (indicated as a gray area) within the deleted regions. B, Schematic map of the targeting construct (top part), the Cd8ab gene loci before and after homologous recombination (upper and lower middle part), and after Cre recombinase-mediated deletion of the neomycin resistance gene (bottom part). Thick vertical arrows indicate DH sites CIV-3 and CIV-4,5 that overlap with EbH1 and EbH2, respectively. Only restriction sites important for the targeting strategy are shown. Open arrowheads (1, 2, and 3) indicate the location of the PCR primers used to detect deletion of neomycin cassette (D). The horizontal thick black line (in the top, lower middle, and bottom parts) 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’ probe used for Southern blotting. Horizontal bars with numbers (indicating the size in kilobases) show the expected genomic fragments after digestion with the appropriate restriction enzymes (XhoI and BamHI for the 5’ targeted region). C, Southern blot of XhoI/BamHI-digested DNA isolated from a wild-type ES cell clone +/+ and from an ES cell clone after homologous recombination (+/N2N3). The origin of the detected fragments is indicated in B. D, PCR genotyping of the targeted locus after the deletion of the neomycin expression cassette. The approximate location of the PCR primers is shown in B.

enhancer activity, similar to the reduction observed with a larger deletion (Δ229–285; L17) in the core enhancer. Further deletion of nt 185–228 (Δ185–285; L18) caused a drop in enhancer activity to baseline levels (i.e., Cd8a promoter only construct). Thus, the region encompassing nt 185–228 has been designated RE-4. We also generated two constructs to delete the region between RE-2 and RE-4 (Δ159–184 and Δ159–177; L19 and L20, respectively). Both constructs caused a similar 3- to 5-fold reduction of enhancer activity (Fig. 6A, lower panel and data not shown), thus defining the region between nt 159–177 as RE-5. To determine the individual contribution of RE-2, nt 135–158 (L21) were deleted. This caused, similar to other single deletions, a 3- to 5-fold reduction of enhancer activity (Fig. 6A, lower panel). Taken together, the deletion analysis of the EbH2 core enhancer fragment indicates that five regulatory elements are required for full enhancer activity. Combined deletion of RE-1 and RE-2 (L15) or RE-3 and RE-4 (L18) caused a complete loss of enhancer activity, while the enhancer core with a deletion of RE-1 (L12), RE-2 (L21), RE-3 (L16) or RE-5 (L20) retained some activity.

An E-box motif within RE-2 contributes to EbH2 core enhancer activity

We wished to determine whether the REs are able to interact with nuclear factors isolated from thymocytes or 1200M cells. Therefore, EMSAs with radiolabeled oligonucleotides corresponding to each RE were performed. Various thymocyte or 1200M nuclear protein/RE complexes were detected, and competition experiments with oligonucleotides containing unrelated sequences revealed that the observed protein/DNA interactions are specific (see Fig. 7B for RE-2/5, and data not shown). One of the binding sites identified by TESS database searches (<www.cbil.upenn.edu/tess>; Ref. 30) within RE-2 was an E-box motif (consensus sequence CANNTG; sequence within RE-2: CAGGTG) that is recognized by basic helix-loop-helix (bHLH) factors, a family of proteins with multiple functions during T cell development (31). EMSA with a radiolabeled oligonucleotide (nt 135–177; containing both RE-2 and RE-5) revealed four major nuclear protein/DNA complexes (I, II, III and IV) in 1200M cells (Fig. 7B, filled arrows). Competition experiments with oligonucleotides containing point mutations that destroy the E-box (CAGGTG to AAGGAA) and therefore the binding of bHLH factors showed that complex I is formed by bHLH/RE-2 interactions (Fig. 7C, open arrow). To identify the binding regions of complexes II, III, and IV, additional competition experiments were performed. As shown in Fig. 7D (left panel), an oligonucleotide (Comp A) covering RE-2 was not able to interfere with protein/DNA complex formation, indicating that RE-2 alone is not sufficient to interact with nuclear proteins. The E-box motif within RE-2 is probably too close (3 nt) to the end of competitor oligonucleotide A to compete with complex I formation. In contrast, complex II formation was inhibited with oligonucleotides B and C (Fig. 7D, middle and right panel, respectively), suggesting that sequences around the border of RE-2 and RE-5 are required. Complex III and IV formation appears to be dependent on both RE-2 and RE-5, because binding could not be competed with oligonucleotides A, B, or C.

To test whether the E-box within RE-2 contributes to EbH2 core enhancer activity, two complementary strategies were used. First, the same point mutations used for the competition experiments in
Fig. 7C were introduced into the 285-bp fragment, and reporter
gene assays were performed. As shown in Fig. 8A, mutations in the
E-box (E-boxmut; construct L24) reduced enhancer activity to a
level similar to that observed in the RE-2 deletion (L21), suggest-
ing that the E-box is the functional site within RE-2. A similar
reduction of luciferase activity was observed upon overexpression
of Id-1, which pairs with bHLH proteins to inhibit their binding to
target DNA sequences (Fig. 8B). In contrast, the activity of the
E-boxmut construct (L24) was not further decreased upon expression
Id-1 (data not shown). Taken together, these data indicate that a func-
tional E-box within RE-2 and binding of bHLH factor(s) are required
for full E8III core enhancer activity.

FIGURE 4. T Cell development and CD8 expression in ΔΔΔ3/ΔΔΔ3 mice. A, Representative two-color flow cytometric analysis of CD4 and CD8 expression on thymocytes isolated from +/+ , +/ΔΔΔ3 , and ΔΔΔ3/ΔΔΔ3 littermates. The numbers in the dot plot quadrants indicate the percentage of the corresponding thymocyte subpopulations (of total gated thymocytes). The region in the upper left quadrant of +/+ mice indicates gating area for CD4 SP thymocytes to determine CD3 and CD5 expression as shown in C. B, Diagram showing the percentage of CD4 SP (left panel) and CD8 SP (middle panel) and DP (right panel) thymocytes of all analyzed wild-type (+/+), heterozygous (+/ΔΔΔ3), and homozygous (ΔΔΔ3/ΔΔΔ3) knockout mice. Each circle represents one mouse. Horizontal bars indicate average value of each genotype. For CD4 SP cells the values are: 7.4 ± 1.9 (+/+), 7.3 ± 1.3 (+/ΔΔΔ3), and 10.4 ± 3.1 (ΔΔΔ3/ΔΔΔ3); CD8 SP: 3.1 ± 0.8 (+/+), 2.8 ± 0.7 (+/ΔΔΔ3), and 1.9 ± 0.7 (ΔΔΔ3/ΔΔΔ3); DP cells: 86.9 ± 3.4 (+/+), 86.8 ± 2.2 (+/ΔΔΔ3), and 84.6 ± 3.8 (ΔΔΔ3/ΔΔΔ3). Values of p represent nonpaired Student’s t test (ΔΔΔ3/ΔΔΔ3 and +/+ mice were compared). C, Representative histograms showing the expression of CD3 (left panel) and CD5 (right panel) on CD4 SP thymocytes of the indicated genotype. The gating area for CD4 SP cells is shown in A. Numbers in the histograms indicate the percentage of CD3-high or CD5-high cells within the gated region. The CD3-low (CD3low) thymocytes to determine CD3 and CD5 expression as shown in C. B, Diagram showing the percentage of CD4 SP (left panel) and CD8 SP (middle panel) and DP (right panel) thymocytes of all analyzed wild-type (+/+), heterozygous (+/ΔΔΔ3), and homozygous (ΔΔΔ3/ΔΔΔ3) knockout mice. Each circle represents one mouse. Horizontal bars indicate average value of each genotype: 2.2, 0.9, 1.5 (+/+), 1.3 ± 0.9, 1.5 ± 0.8, and 3.8 ± 1.5 for +/+, +/ΔΔΔ3, and ΔΔΔ3/ΔΔΔ3 mice, respectively, and 1.3 ± 0.9, 1.5 ± 0.8, and 3.8 ± 1.5 for +/+, +/ΔΔΔ3, and ΔΔΔ3/ΔΔΔ3 mice, respectively. Values of p represent nonpaired Student’s t test (ΔΔΔ3/ΔΔΔ3 and +/+ mice were compared). E, left panel, Flow cytometric analysis of CD4 and CD8 expression on thymocytes isolated from littermates of the indicated genotype (+/+ × TCRα-null, +/ΔΔΔ3 × TCRα-null, ΔΔΔ3/ΔΔΔ3 × TCRα-null). Right panel, Flow cytometric analysis of CD4 and CD8 expression in ΔΔΔ3/ΔΔΔ3 × TCRα-null thymocytes as previously reported (6).
A c-myb binding site is required for enhancer activity

TESS database sequence analysis (30) of RE-5 indicated the presence of a c-myb binding site (consensus sequence: AACCG/TG; sequence within RE-5: AACCGG), suggesting that some of the protein/DNA complexes could be formed by the interaction of the c-myb site with protein complexes. However, a cold RE-2/RE-5 oligonucleotide with a mutated c-myb site could still compete with the formation of complex I, II, III and IV (data not shown). To test whether the c-myb is important for E8III core enhancer activity, point mutations were introduced that destroy the c-myb binding motif (AACCGG to TTCCGG). As shown in Fig. 8C, mutations in the putative c-myb site (c-mybmut; construct L25) caused a 5-fold reduction of enhancer activity, similar to the reduction observed upon deletion of RE-5 (L20). This indicates that the AACGG sequence is one of the functional sites within RE-5.

Discussion

Several developmental stage-, subset-, and lineage-specific cis-regulatory regions that are involved in the regulation of Cd8a and Cd8b gene expression have been identified during the last several years. In this study, we have performed a detailed functional analysis of one of these regions, E8III (or CIV-3), which has been shown to direct expression only in DP thymocytes. By performing a transgenic reporter expression assay, we were able to identify a 285-bp genomic subfragment (designated E8III core enhancer) from the E8III enhancer region that is still active in DP thymocytes. Combined deletion of a genomic fragment containing the E8III core enhancer region with another CD8 cis-regulatory element (enhancer E8III (or CIV-4,5)) in the mouse germ line revealed that E8III activity is required within the Cd8a and Cd8b loci to ensure nonvariegated expression of both genes in DP thymocytes. Finally, in vitro reporter expression assays led to the identification of five regulatory elements within the E8III core enhancer fragment, indicating that binding of multiple factors is required for full core enhancer activity. Taken together, our study demonstrates that E8III partially contributes to the regulation of CD8 expression and provides further evidence that CD8 cis-elements are required during T cell development for the proper activation of the Cd8ab gene complex during the DN to DP transition.

Studies have shown that the expression of CD8 is coupled to the functional cytotoxic T cell phenotype during the development of DP thymocytes into CD8 SP T cells (32–34). Insight into how the various cis-regions are functioning and knowledge of the factors that bind to regulatory elements within the enhancers will therefore also provide molecular details of lineage commitment and T cell development. As a strategy to isolate the binding factors, we have initiated a “bottoms-up” approach (35) by using the cis-elements as molecular baits. One prerequisite for this approach is the availability of small DNA fragments that can be used as bait. Because the CD8 enhancers were initially mapped on larger genomic fragments (4–8 kb), it is important as a first step to identify the core enhancers and regulatory regions within those fragments that are sufficient to direct expression of CD8. In this study, we focused on the DP-specific enhancer E8III, localized initially on a 4-kb EcoRI-BamHI genomic fragment (5). By performing in vitro luciferase reporter experiments and, more importantly, in vivo transgenic reporter gene expression assays, we identified a 285-bp E8III core enhancer fragment, localized immediately downstream of the Cd8b gene. Two of four transgenic founders generated with a reporter construct containing this 285-bp genomic fragment showed DP stage-specific expression of the hCD2 reporter gene in a fashion indistinguishable from that originally observed with the 4-kb genomic fragment (TG-31; Ref. 5). Furthermore, only those transgenic constructs that included this region (TG-42 and –40) and not others (TG-30 and TG-32) showed the same developmental stage-specific activity as TG-31. These data suggest that the 285-bp fragment indeed represents the enhancer core of E8III and that the core region is sufficient to direct expression in DP thymocytes. We also noted that the E8III core enhancer fragment (in TG-40) appears to be more susceptible to position effects than larger fragments (TG-31 and TG-42); however, the generation of more transgenic founders for each construct would be required to quantify this observation.

In addition to the enhancer E8III, several other fragments from the Cd8a and Cd8b gene loci direct expression in DP thymocytes. E8II, an enhancer localized on a 4.4-kb BamHI fragment, directs expression in DP thymocytes and also in CD8– T cells (6). Furthermore, the combination of DH-site cluster II (which alone has no enhancer activity) and cluster III (active in mature CD8SP thymocytes and CD8+ T cells) directs expression of a reporter gene in DP thymocytes as well (7). Although several elements are able to direct expression in DP cells in transgenic mice, based on the in vivo activity of the E8III fragment, it may be expected that the deletion of the DP-specific enhancer would result in at least some reduction of CD8 expression at the DP stage. Surprisingly, we found that a combined deletion of enhancer E8III and the adjacent enhancer E8II resulted in a very subtle phenotype in a small subset of “DP” cells that failed to up-regulate CD8 expression. This phenotype is reminiscent of that observed in Δ1Δ2/Δ1Δ2 mice, or in Cluster II-deficient mice, in which the deletion of the corresponding elements led to the appearance of “CD8-negative” DP thymocytes due to variegation of CD8 expression in DP thymocytes (6, 8). Because CD8-negative DP thymocytes are not observed in E8II single-deficient mice (6), it is clear that this phenotype is caused by the loss of E8III activity, either alone or in combination with the loss of E8II activity. Thus our results have identified E8III as another cis-element involved in the activation of the Cd8ab gene loci during T cell development. Previous transgenic expression studies showed variegated expression of reporter genes driven by CD8...
cis-regulatory elements (4). These studies indicated that the identified CD8 enhancers do not act as locus control regions (36) and, therefore, do not mediate position-independent and copy number-dependent expression of the transgene. The variegated CD8 expression phenotype in the various CD8 cis-regulatory element-deficient mice is in agreement with these observations. Together, the transgenic and enhancer deletion studies strongly suggest that the combined activity of E8I, E8II, E8III, and cluster II is required for high-level and nonvariegated expression of CD8/ H9251 and CD8/ H9252, although some cis-elements seem to be more essential than others for developing thymocytes to activate the Cd8ab gene complex.

CTLs express CD8αβ heterodimers on their surface, whereas intestinal IEL express surface CD8αα homodimers (28, 29). The observation that Δ2Δ3/Δ2Δ3 mice display a normal expression pattern of CD8α in IEL confirms previous results that showed that the major cis-acting element directing CD8α expression in IEL is enhancer E8i (or CIII-1,2), and that E8i is required for CD8α expression only in the absence of E8ii (5–7, 14). Another population of cells that express CD8αα homodimers is formed by a subset of murine splenic DC. According to the expression of CD4 and CD8, three subsets of DC have been described in the murine spleen: CD4−CD8α+, CD4+CD8α+, and CD4+CD8α− (26, 27). Δ2Δ3/Δ2Δ3-deficient mice showed a similar distribution of these three subsets in the spleen within the CD11c+ DC population compared with wild-type mice and, in addition, similar expression levels. CD8α expression on DC was also not affected in Δ1Δ2/Δ1Δ2 mice (B. Unger and W. Ellmeier, unpublished observation). Thus, the combined activity of either E8i and E8ii or E8ii and E8iii is not required for CD8α expression in DC, although it remains possible that the activity of one of the three enhancers is sufficient to direct CD8α expression in DC. Therefore, the major cis-acting sequences required for DC-specific expression of CD8α still remain to be identified.

There is accumulating evidence that several cis-elements spread over the Cd8ab loci are required to ensure proper opening of chromatin during the DN to DP transition, and thus permit nonvariegated expression of CD8α and CD8β, although some cis-elements seem to be more essential than others for developing thymocytes to activate the Cd8ab gene complex.
factor to the \( Cd8a \) and \( Cd8b \) gene loci. The identification of distinct regulatory elements (RE1–5) within the \( E8_{III} \) core enhancer is therefore an important first step toward the identification of chromatin remodeling factors and/or transcription factors that regulate CD8 expression. We also noted that the enhancers \( E8_5 \) and \( E8_{II} \) contain sequences with a homology to some of the RE present in \( E8_{III} \) that is higher than 77% over a stretch of 13–24 nt (N. Feik and W. Ellmeier, unpublished observation). Thus, factors that bind to \( E8_{III} \) may also

**FIGURE 7.** Multiple protein complexes bind to RE-2 and RE-5. **A,** Sequence of regulatory elements RE-2 and RE-5 and the nucleotide positions within the 285-bp core enhancer. The E-box and c-myb sites within RE-2 and RE-5, respectively, are underlined. Horizontal thick black lines below the sequence indicate the competitor oligonucleotides used in EMSAs shown in **C** and **D.** The competitor oligonucleotides RE-2/5 E-boxmut and Comp C contain CAGGTG to AAGGAA mutations within the E-box motif. **B,** EMSAs to detect specific protein/RE-2/5 interactions. Reactions were performed with a radiolabeled oligonucleotide containing RE-2/5 and 1200M nuclear extracts (Nx) without competitor oligonucleotide (lane 6), in the presence of increasing amounts of RE-2/5 competitor oligonucleotide (lanes 5, 4, 3, and 2), or in the presence of increasing amounts of nonspecific (unrelated) competitor oligonucleotide (lanes 10, 9, 8, and 7). Lane 1, radiolabeled RE-2/5 oligonucleotide only. The four major specific protein/DNA complexes I, II, III, and IV are indicated with the filled arrows at the left side. **C,** EMSAs to detect specific protein/E-box interactions. Reactions were performed with a radiolabeled RE-2/5 oligonucleotide and 1200M nuclear extracts (Nx) without competitor oligonucleotide (lanes 2 and 7), in the presence of increasing amounts of RE-2/5 competitor oligonucleotide (lanes 10, 9, 8, and 7), or in the presence of increasing amounts of RE-2/5-E-boxmut competitor oligonucleotide (lanes 5, 4, 3, and 2). Lane 1, radiolabeled RE-2/5 oligonucleotide only. The open arrow at the left indicates the protein/E-box complex I, while filled arrows indicate protein/DNA binding complexes that are still competed with an E-boxmut oligonucleotide (complexes II, III, and IV). The RE-2/5-E-boxmut oligonucleotide contains CAGGTG to AAGGAA mutations within the E-box motif. **D,** EMSAs to detect regions within RE-2 and RE-5 that are required for complex II, III and IV formation. Left panel, Reactions were performed with a radiolabeled RE-2 oligonucleotide and 1200M nuclear extracts (Nx) without competitor oligonucleotide (lanes 2 and 7) in the presence of increasing amounts of RE-2/5 competitor oligonucleotide (lanes 6, 5, 4, and 3) or in the presence of increasing amounts of Comp A competitor oligonucleotide (lanes 11, 10, 9, and 8). Lane 1, Radiolabeled RE-2 oligonucleotide only. The open arrow at the left indicates the competition of complex II. Middle panel, EMSAs with a radiolabeled RE-2/5 oligonucleotide and 1200M nuclear extracts (Nx) without competitor oligonucleotide (lane 1) and in the presence of increasing amounts of Comp A competitor oligonucleotide (lanes 10, 9, 8, and 7). Lane 1, Radiolabeled RE-2/5 oligonucleotide only. **Right panel,** EMSAs with a radiolabeled RE-2/5 oligonucleotide and 1200M nuclear extracts (Nx) without competitor oligonucleotide (lane 1) and in the presence of increasing amounts of Comp C-E-boxmut competitor oligonucleotide (lanes 5, 4, 3, and 2). The open arrow at the left indicates the competition of complex II.
FIGURE 8. The E-box and c-myc binding sites contribute to enhancer activity. A, Luciferase reporter constructs containing the E8III core enhancer with either a deletion of RE-2 (L21) or a mutation of the E-box (CAGGTG to AAGGAA) within RE-2 (L24) were generated. 1200M cells were transfected and the luciferase activities of the reporter constructs relative to the Cd8a promoter construct (L1) were determined. B, Luciferase reporter construct containing the E8III core enhancer (L10) or the Cd8a promoter only (L1) were cotransfected into 1200M cells with expression constructs containing Id-1 either in transcriptional sense or antisense orientation. The luciferase activities of the reporter constructs relative to the Cd8a promoter construct (L1) were determined. C, Luciferase reporter constructs containing the E8III core enhancer with either a deletion of RE-5 (L20) or a mutation of the c-myc binding site (AACGG to TTCGG) within RE-5 (L25) were generated. 1200M cells were transfected and the luciferase activities of the reporter constructs relative to the Cd8a promoter construct (L1) were determined. A—C, All transfections were performed in duplicates. Values are normalized for transfection efficiencies.

The requirement for functional E-box and c-myc sites within the E8III core enhancer sequence for full activity suggests that some of the relevant factors may not be specific for the Cd8a and Cd8b genes. Both bHLH proteins and c-myc have been implicated in the regulation of a large number of genes within the T cell lineage, including Cd4 (38, 39). Because the deletion analysis clearly shows that binding of multiple factors is required for full enhancer activity, it is likely that binding of a particular combination of generally expressed transcription factors determines the specificity of the enhancer, or, alternatively, that one of the binding factors is only expressed at a certain developmental stage. We also wished to determine whether the five identified regulatory elements are occupied by nuclear factors in developing thymocytes, and used DMS in vivo footprinting combined with ligation-mediated (LM)-PCR (40). However, the presence of repetitive DNA sequences in close proximity to the 285-bp region (both up-stream and down-stream) caused technical difficulties in amplifying this region (N. Feik and W. Ellmeier, unpublished observation), and we were unable to determine whether the regulatory elements interact with nuclear proteins in vivo. In future experiments, it will therefore be important to determine by chromatin immunoprecipitation assays whether identified candidate factors bind to target sites within the Cd8a and Cd8b gene loci at different stages of development.

In conclusion, the generation of E8III and E8IId double-knockout mice revealed that E8IId is required in DP thymocytes to ensure nonvariegated expression of CD8 in the absence of E8III, thus indicating that E8IId is another CD8 cis-element that is involved in the activation of the Cd8ab gene complex during T cell development. The identification of the 285-bp E8IId core enhancer will also facilitate the generation of a transgenic expression cassette to direct the expression of a gene of interest only at the DP stage during thymic differentiation.

Acknowledgments
We thank Farah Hatam for technical help at early stages of the project and Ruth Herbst for critical reading of the manuscript.

References


