ADVANCES IN IMMUNOLOGY, VOL. 83

The CD4/CD8 Lineage Choice: New Insights into Epigenetic Regulation during T Cell Development

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I. Introduction

Differentiation of progenitor cells into various progeny with distinct functional properties is achieved by the regulated activation and silencing of batteries of genes. The final differentiated state is thus defined by the expression pattern of these genes, which is heritably maintained as cells undergo subsequent cell divisions. Hematopoiesis offers an ideal developmental system to study how progenitors differentiate into cells of distinct lineage and function and how differentiated states are then stably maintained. A series of transcription factors required for differentiation of erythroid, myeloid, and lymphocyte lineages have been described (Cantor and Orkin, 2001; Singh *et al*., 1999). Within the lymphoid lineages, activation of the Notch signaling pathway in the thymus favors T cell development, at the expense of B lymphocytes (Izon *et al*., 2002). This is followed by a series of developmental choices as thymocytes differentiate into T cells with distinct antigen recognition properties and effector functions.

Differentiation of thymocytes and their subsequent selection results in export to peripheral secondary lymphoid organs of a large variety of cells: $CD4^+$ T helper cells (T_h), $CD8^+$ cytotoxic cells (CTL or T_c), $CD4^+$ regulatory or suppressor cells (T_{reg}), $CD8^+$ intestinal intraepithelial lymphocytes (IEL), NKT cells with restricted T cell antigen receptor (TCR) repertoires, and other T cells with restricted TCRs that localize to various peripheral organs and mucosal regions. The molecular events leading to the differentiation of each of these lineages remain largely unexplored. Expression of the CD4 and CD8 glycoproteins defines the major lineages of T lymphocyes, and is coupled to the transcriptional programs that characterize the helper and cytotoxic lineages, respectively (Killeen and Littman, 1996). Regulation of expression of the genes encoding these coreceptor molecules has been studied in some detail, with the expectation that such studies could elucidate the mechanisms involved in lineage commitment and establishment of differentiated states. These studies have revealed a complex network of positive regulatory elements in both genes, as well as an extensively characterized negative regulatory region within

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the CD4 gene. In addition, the silencer in the CD4 gene has been shown to be involved in epigenetic regulation, and there are hints that the enhancers in the CD8 gene may also function through epigenetic mechanisms that result in heritable "on" states of expression. The recent findings on CD4 and CD8 regulation and the general tractability of the T cell system render these genes highly attractive for studies of the general phenomenon of epigenetic regulation during development (Jaenisch and Bird, 2003; Li, 2002). This chapter discusses the role of the CD4 and CD8 co-receptors in T cell development and signal transduction, the regulation of expression of the genes that encode these proteins, and the outstanding questions that need to be addressed. We also emphasize how our understanding of the CD4 silencer can inform future studies on epigenetic regulation during mammalian development.

II. Role of Coreceptors in T Cell Development

The thymus is the site where lymphoid progenitors differentiate into T cells bearing a variety of specificities and functions (von Boehmer *et al*., 1989). Most cells differentiate along a lineage that gives rise to cytotoxic and helper T cells with TCRαβ receptors specific for MHC class I and class II molecules, respectively. Cells of this lineage can be readily classified according to their expression of the coreceptor molecules, CD4 and CD8. These cell surface glycoproteins bind to membrane-proximal domains of MHC class II and class I molecules, respectively, and their cytoplasmic domains recruit the Src family protein tyrosine kinase $p56^{lck}$ (Lck) (Weiss and Littman, 1994). The most immature cells express neither coreceptor and are classified as double negative (DN) cells. These cells can be subdivided into four stages, based on surface expression of CD25 and CD44. At the DN3 stage ($CD25^{+}CD44^{lo}$), there is initiation of VDJ rearrangement at the *TCR*β locus, and cells that undergo in-frame rearrangement and hence express a pre-TCR are selected for further maturation, marked by several rounds of cell division before CD25 is shut off (DN4 stage: $CD25^{-}CD44^{lo}$). Cells that have completed β-selection then turn on expression of both CD4 and CD8αβ heterodimers, marking the double positive (DP) stage of differentiation (von Boehmer and Fehling, 1997). These cells, which are the most abundant in the thymus, then undergo VJ rearrangement of the *TCR*α genes, and cells with productive rearrangements initiate expression of TCRαβ heterodimers. The DP thymocytes are then subjected to selection based on their aptitude to interact with MHC molecules (Goldrath and Bevan, 1999; Sebzda *et al*., 1999). Most cells undergo "death by neglect" because they fail to bind with sufficient avidity to self MHC–peptide complexes. The few cells with TCRs that bind with high affinity to MHC–peptide complexes are deleted, through an apoptotic program known as negative selection. Only the rare DP thymocytes with TCRs of intermediate affinity

for MHC transduce signals that ensure their survival and further differentiation, which is known as "positive selection." Those cells selected by interacting with MHC class I proceed to become $CD4^{-}CD8^{+}$ T cells (CD8 single positive or SP cells, bearing CD8αβ heterodimers) with cytotoxic function, whereas those selected on MHC class II become $CD4^{+}CD8^{-}$ cells (CD4 SP) with helper functions.

Other lineages branch off at different stages from this major pathway of differentiation. Cells that express TCRγδ differentiate from the DN precursors and migrate to various specialized mucosal regions (Hayday *et al*., 2001). Many of these give rise to IEL that express CD8αα homodimers (Guy-Grand *et al*., 2001; Lefrancois and Olson, 1994). A subset of cells with canonical TCRαβ rearrangements are selected at the DP stage by interacting with the class Ib molecule CD1d, and give rise to NKT cells that migrate to many tissues and make up a large proportion of T cells in the liver (Kronenberg and Gapin, 2002). In 2003, a subset of CD4 SP thymocytes was found to differentiate into regulatory T cells that keep autoreactive cells in check (Hori *et al*., 2003).

The process by which DP thymocytes give rise to CD4 or CD8 SP cells is still poorly understood, but it is known to involve multiple steps. First, the cells undergo positive selection independently of commitment to one of the two lineages. This is known from the phenotype of mutant *hd/hd* (*h*elper-*d*eficient) mice, which lack CD4 SP cells and mature T helper cells (Keefe *et al*., 1999). MHC class II-restricted thymocytes undergo positive selection in these mice, but they all differentiate into $CD8^{+}$ CTL. These animals thus have no defect in positive selection, but they have defective lineage commitment that results in lineage reversal of class II-restricted T cells.

How cells commit to the helper versus the cytotoxic lineage remains a matter of some controversy. Early studies suggested that DP thymocytes stochastically shut off either CD4 or CD8, and undergo further selection only if they retain expression of the coreceptor appropriate for the class of MHC recognized by the TCR. Thus, cells specific for class I would undergo apoptosis if they shut off CD8, which interacts with class I, but would be further selected if they retain expression of CD8 and shut off CD4, which interacts with class II (Davis and Littman, 1994). Later studies have provided strong evidence that the strength of the TCR-initiated signal governs the fate of DP thymocytes. Enhanced activation of the protein tyrosine kinase $p56^{lck}$, which interacts with both CD4 and CD8 cytoplasmic domains, resulted in biased commitment to the CD4 lineage, such that even class I-restricted thymocytes became CD4 SP cells (Hernandez-Hoyos *et al*., 2000). Conversely, inhibition of Lck activity resulted in deviation towards the CD8 lineage. Because the proportion of CD4 molecules with Lck bound to them is higher than that of CD8, it has been proposed that the number of Lck molecules

recruited to the TCR complex is the factor that determines the strength of the TCR-initiated signal and, hence, instructs the lineage choice.

Most recently, Singer and colleagues have proposed a modified version of the strength-of-signal instructional model, which they have termed the "coreceptor reversal" or "kinetic signaling" model (Brugnera *et al.*, 2000; Singer, 2002). This model is based on the finding that, following positive selection signals initiated by TCR interaction with either class I or class II molecules, thymocytes down-regulate expression of CD8, resulting in $CD4^{+}CD8^{lo}$ cells that are precursors of both the CD4 and CD8 SP lineages. These precursor cells have been posited to give rise to cells of either the CD4 or CD8 lineage on the basis of the strength of the TCR/coreceptor signal relative to the strength during earlier selection at the DP stage. Thus, cells selected by interaction with class II would retain a strong signal at the $CD4^{+}CD8^{lo}$ stage, since the CD4 coreceptor continues to be expressed. This would direct the cells towards the CD4 SP lineage. However, if cells were selected by interaction with class I, there would be a reduced signal upon transition to the $CD4^{+}CD8^{lo}$ stage, and this would result in subsequent differentiation to the CD8 SP cytotoxic T cell lineage. The reduced or interrupted signal would therefore initiate the extinction of CD4 expression and the reactivation of CD8 expression. The novelty of this model lies in the proposition that abrogation of a signal dictates a switch in developmental fate. Based on *in vitro* thymocyte culture experiments, Singer and colleagues have suggested that IL-7R signaling is then required in cells destined for the CD8 SP lineage. This hypothesis is supported by the finding that conditional inactivation in thymocytes of the *SOCS-1* gene, a negative regulator of γc (and hence IL-7R) signaling, results in a considerable increase in CD8 SP cells (Chong *et al.*, 2003).

Although the coreceptor reversal model has yet to be experimentally confirmed, its predictions fit well with our current understanding of CD4 and CD8 gene regulation. For example, there is evidence that there are multiple stage-specific enhancers that regulate CD8 expression, with some active at the DP stage and others at various stages of CD8 SP differentiation. A better understanding of how the coreceptor genes are regulated is, hence, likely not only to contribute to the elucidation of how lineage choice occurs in DP thymocytes, but also may reveal how complex programs of differentiation, to the helper and cytotoxic lineages, are regulated. In addition, other studies have revealed an unexpected novel function of a CD8 gene enhancer that selectively regulates expression of CD8α (Madakamutil *et al.*, 2004). This enhancer has been shown to have a key role in the generation of memory CTL, and its further characterization may provide important insight into the genetic program that governs how a small subset of effector cells is set aside to differentiate into memory cells.

III. Regulation of CD4 Gene Expression

A. Positive Regulatory Elements

The *CD4* locus is located in syntenic regions of murine chromosome 6 and human chromosome 12. It spans ~80 kb, and its exon/intron structure is conserved in mouse and human. The *lag-3* gene, which encodes a CD4-related molecule that also binds to MHC class II, is located approximately 20 kb upstream of *cd4* in the mouse genome (Baixeras *et al*., 1992; Triebel *et al*., 1990). LAG-3 is expressed on all newly activated $CD4^+$ and $CD8^+$ T cells and on some cycling memory cells and, hence, differs significantly in its regulation from the closely linked *cd4* gene. LAG-3 appears to contribute to the expansion of activated T cells by regulating their survival (Workman and Vignali, 2003). LAG-3 is also expressed on natural killer cells and contributes to the ability of these cells to kill certain target cells (Miyazaki *et al*., 1996).

1. The CD4 Promoter

Candidate *cis*-regulatory elements were initially idenfied using DNase hypersensitivity (HS) analyses of the mouse *cd4* locus (Gorman *et al*., 1987; Sands and Nikolic-Zugic, 1992; Sawada and Littman, 1991; Siu *et al*., 1994). One of the HS sites is immediately upstream of the transcription initiation site, and a 101-bp fragment containing this sequence had promoter activity in transient transfection assays in T cell lines. Two Myb-binding motifs were required for full promoter activity (Nakayama *et al*., 1993; Siu *et al*., 1992). Another mutatagenesis study suggested the involvement of Ets-binding sites for CD4 promoter (P4) activity (Salmon *et al*., 1993). All studies to date on P4 have been confined to transient expression analyses *in vitro*, and the promoter has not been analyzed in mice by transgenesis or targeted mutagenesis. This is particularly relevant because, for the activation of reporter genes *in vivo*, P4 is not sufficient and requires the help of enhancers. It will be important to determine, using chromatin immunoprecipitation (ChIP), whether Myb, Ets, and other transcription factors are associated with P4 in various subsets of thymocytes and in mature CD4 and CD8 lineage cells. Comparison of CD4 and CD8 lineage T cells may also provide valuable information as to whether the promoter is occupied by transcription factors even when the gene is silenced (in $CD8^+$ T cells). It should be noted that lineage specificity is unlikely to be conferred by P4, since it functions in transgenic mice in conjunction with enhancers from either the *cd4* or *cd8* genes (Ellmeier *et al*., 1997).

2. CD4 Enhancer Elements

Analysis of gene expression in T cell lines transfected with a reporter gene under the regulation of the CD4 promoter and various sequences corresponding to DNase hypersensitivity (HS) sites resulted in the identification of an

enhancer located approximately 13 kb upstream of the transcriptional start site. A 339 bp fragment from this region directed expression of the reporter in an orientation- and position-independent manner, and functioned only in T cells (Sawada and Littman, 1991). The same element and its human homologue, combined with the CD4 promoter and various lengths of the mouse or human CD4 gene, directed expression of CD4 or reporter genes in thymocytes and T cells, but not in other cell lineages (Gillespie *et al*., 1993; Hanna *et al*., 1994; Killeen *et al*., 1993; Sawada *et al*., 1994; Siu *et al*., 1994). This T cell-specific enhancer has been referred to as the CD4 proximal enhancer ($E4_p$). $E4_p$ contains three nuclear protein-binding sites that were identified by DNaseI footprinting with nuclear extracts from T cell lines (Sawada and Littman, 1991). One of these is a binding motif for the HMG box family proteins, TCF1/LEF1, and the other two contain E-box motifs that bind basic helix–loop–helix proteins. Mutation of tandem E-box motifs (CD4-3) at the 3′ end of the enhancer abrogated enhancer activity in transiently transfected T cell lines, whereas mutation of the TCF1/LEF1 binding motif (CD4-2) and the 5′ E-box (CD4-1) had only minor effects (Sawada and Littman, 1991). A heterodimer consisting of E2A and HEB, both basic HLH proteins, was found to bind to one of the E-boxes in CD4–3 and to be required for CD4 enhancer activity in the *in vitro* assays (Sawada and Littman, 1993). This is discussed in greater detail in a later section.

Another HS site, mapped approximately 24 kb upstream of the transcriptional start site in murine *cd4*, was first characterized as a CD4 distal enhancer ($E4_d$) (Wurster *et al*., 1994). However, combined with the CD4 promoter, $E4_d$ does not appear to have enhancer activity *in vivo*. In addition, transgenes containing both $E4_d$ and $E4_p$ were expressed not only in T cells, but also in B cells and macrophages (Siu *et al*., 1994). The location of $E4_d$ is adjacent to the 3′ end of the neighboring *lag-3*, which is expressed in activated T cells and in NK cells. It is possible that $E4_d$ is involved in regulation of *lag-3* expression rather than *cd4* expression.

The role of $E4_p$ in directing expression in all thymocytes and T lymphocytes has been challenged by two reports, which together indicate that positive regulation of CD4 expression at different stages of development is more complex than initially thought. First, von Andrian and colleagues showed that activation of mature T cells by antigen results in down-regulation of expression of a transgenic GFP reporter regulated by $E4_p$ and the CD4 promoter (Manjunath *et al*., 1999). Because endogenous CD4 expression is not affected by activation, this result suggests that regulatory elements other than $E4_p$ contribute to the expression of CD4 in antigen-stimulated cells. It is possible that a second enhancer, either a novel or a known one such as $E4_d$, functions alone or in cooperation with $E4_p$ to maintain expression of CD4 in effector and memory T cells. Mutation of these enhancers within the endogenous *cd4* locus will be required to reveal their physiological functions.

A second report has presented compelling evidence that the proximal CD4 enhancer may not be sufficient to direct expression of transgenic reporter genes in immature DP thymocytes (Adlam and Siu, 2003). These studies employed the 339 bp $E4_p$ and approximately 1 kb of promoter sequence 5′ to the transcriptional start site. In multiple transgenic mice, this combination of regulatory sequences resulted in expression only in SP thymocytes and mature T cells, but not in DP thymocytes. However, inclusion of a sequence corresponding to a distal DNase HS site, DH17, located approximately 40 kb 3′ to the *cd4* locus in the first intron of the *isot* gene, restored expression in DP thymocytes (Fig. 1). This sequence was shown to have enhancer activity in transient transfections of a DP thymoma, but it did not function *in vivo* in the absence of the proximal enhancer. DH17 was shown to contain both an enhancer, in a 1.0 kb fragment that was termed TE (thymocyte enhancer), and an adjacent 0.9 kb locus control region (LCR) that confers copy number-dependent and position-independent expression of transgenes (Adlam and Siu, 2003).

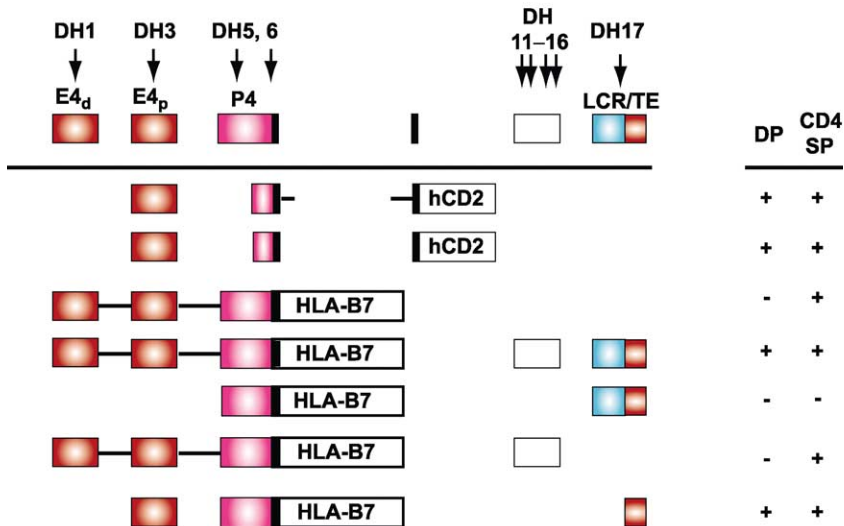


FIG 1 Enhancer and promoter elements in the *cd4* locus that contribute to reporter transgene expression in double positive thymocytes versus mature single positive T cells. Constructs that utilize the human CD2 transgene (hCD2) are from Sawada *et al.*, 1994, and unpublished studies of Zou and Littman. Constructs that utilize HLA-B7 are from Adlam and Siu, 2003. $E4_d$, distal enhancer; $E4_p$, proximal CD4 enhancer; P4, CD4 promoter; LCR/TE, locus control region and thymic enhancer located in first intron of *isot* gene. DH denotes DNase hypersensitive sites in the *cd4* locus. Sites within the *cd4* coding region have been omitted. We propose that DH5 may encode a negative DP thymocyte regulatory element that requires TE or another unidentified enhancer for expression in immature thymocytes.

The conclusion that the LCR/TE is essential, along with $E4_p$, for expression of CD4 in DP thymocytes is based on results with an impressively large number of transgenic mice. Similarly, many transgenic mice prepared with combinations of $E4_p$, the CD4 promoter (P4), and various parts of the first *cd4* intron have clearly demonstrated expression in DP thymocytes as well as in more mature T cells. For example, the human CD2 reporter was expressed in DP (and some DN) thymocytes in multiple transgenic lines prepared with $E4_p$ and P4 without inclusion of the LCR/TE from DH17 (Sawada *et al.*, 1994). Similarly, CD4-cre mice that have been widely employed to delete genes at the DP stage were prepared with the same combination of regulatory elements (Wolfer *et al.*, 2001). Use of $E4_p$/P4 also directed expression of GFP and of the chemokine receptors CXCR4 and CCR5 in DP thymocytes as well as in mature T cells in both mouse and rat (Ellmeier, Deng, and Littman, unpublished; Keppler *et al.*, 2002; Weninger and von Andrian, unpublished). The consistency observed within each of the studies that examined expression of the transgenes suggests that the discrepancy in results must be due to a subtle, but important, difference in the constructs used by the different groups (see Fig. 1). The discrepancy was discussed in an earlier review, in which it was suggested that the difference may lie in the length of the promoters used by the two laboratories (Ellmeier *et al.*, 1999). Siu and colleagues have used a promoter sequence that includes about 1 kb 5′ of the transcriptional start site, while our group has used only 0.5 kb (Adlam and Siu, 2003; Sawada *et al.*, 1994). We propose that a sequence immediately upstream of the CD4 promoter has negative regulatory activity in DP thymocytes and, hence, requires a specific enhancer activity at this stage of development. This enhancer activity most likely involves $E4_p$ functioning in conjunction with another element, possibly the LCR/TE within the *isot* intron (Fig. 1). Because the region containing *ISOT* is not required for expression of human CD4 transgenes in DP thymocytes, it is possible that other elements, adjacent to the CD4 coding region, provide this enhancer function in both the mouse and human genes. Alternatively, the mouse and human genes may differ in their requirement for enhancers directing expression in DP thymocytes.

The presence of yet another enhancer has been suggested by analysis of mice with targeted deletions of the silencer and adjacent sequences in intron I of *cd4*. In two separate studies aimed at analyzing silencer function (described in the following text), 6.5 kb or 1.6 kb intron sequences were deleted by homologous recombination in ES cells. In mutant mice generated from these cells, the level of cell surface CD4 expression was reduced in DP thymocytes and peripheral $CD4^+$ T cells (Leung *et al.*, 2001; Zou *et al.*, 2001). Therefore, a putative positive regulatory element may have been deleted along with the CD4 silencer. The low CD4 expression was not due to residual silencer activity, since the level on $CD4^+$ T cells from another mutant strain, in

which only 429 bp of the CD4 silencer was deleted, was similar to that from wild-type mice (Taniuchi *et al*., 2002b). Taken together, these results are compatible with the existence of a positive regulatory element, a CD4 intronic enhancer ($E4_i$), adjacent to the CD4 silencer. This enhancer is not necessary for directing expression of reporter transgenes in DP thymocytes, since the combination of $E4_p$ and P4 was functional in these cells even in the absence of intron I (Ellmeier *et al*., 1999; Zou and Littman, unpublished).

The functions of the various enhancers previously described ($E4_p$, $E4_d$, LCR/TE, and $E4_i$) have only been inferred from examination of expression patterns in transgenic mice. Because of the complexity of the positive regulation of CD4, an understanding of the roles of the individual elements awaits an analysis following their systematic deletion in the murine germ line.

B. Negative Regulatory Elements Involved in CD4 Silencing

Early attempts to generate mice in which expression of human CD4 transgenes would faithfully reproduce the pattern of endogenous mouse CD4 expression provided the first clue that the gene is negatively regulated in CD8 lineage cells. Human CD4 transgenes that contained $E4_p$ were expressed appropriately in DP thymocytes and T helper cells (Donda *et al*., 1996; Gillespie *et al*., 1993; Hanna *et al*., 1994; Killeen *et al*., 1993; Sawada *et al*., 1994; Siu *et al*., 1994). In contrast, when the murine E4p element was combined with the human promoter fused to a cDNA/genomic minigene in which exons 1–6 were derived from the cDNA, expression of CD4 was observed in all thymocytes and in both $CD4^+$ and $CD8^+$ T cells of transgenic mice (Crooks and Littman, unpublished). This suggested that a negative regulatory element, located within the first 5 introns of the human CD4 gene, had been omitted in the transgene. Subsequent studies showed that lineage-specific expression of transgenes requires sequences corresponding to a DNase HS site located 1.6 kb downstream from the first exon, within the first intron of the murine *cd4* locus (Sawada *et al*., 1994; Siu *et al*., 1994). A similar negative regulatory sequence in the first intron of human *CD4* was subsequently described (Donda *et al*., 1996). Inclusion in transgenic constructs of the murine CD4 silencer (S4), which was narrowed down to a 434 bp fragment from intron 1, resulted in repression of reporter gene expression in DN and CD8 SP thymocytes and in mature CD8 lineage T cells (Sawada *et al*., 1994; Siu *et al*., 1994). The activity of the silencer was independent of its orientation or location within the transgene, and it functioned with heterologous enhancers and promoters (Sawada *et al*., 1994). Importantly, expression of reporter transgenes in which the 434 bp CD4 silencer was combined with $E4_p$ and the 0.5 kb murine promoter recapitulated the normal developmental pattern of CD4 expression. Repression was observed not only in CD8 SP thymocytes, but also in a subset of immature $CD4^-CD8^-$DN thymocytes

(Sawada *et al.*, 1994). Based on these observations, a two-stage model was proposed for the temporal activation of the silencer during thymocyte differentiation (Sawada *et al.*, 1994; Siu *et al.*, 1994). In this model, the CD4 silencer is first turned on in immature DN thymocytes followed by inactivation at subsequent stages in which both CD4 and CD8 are expressed, then turned on again specifically in those thymocytes that become cytotoxic-lineage cells. The CD4 silencer was thus proposed to be the key *cis*-regulatory element regulating lineage specificity of CD4 expression.

Gene-targeting studies in mice have confirmed that the CD4 silencer is essential for shutting off CD4 at both the DN and CD8 SP stages of thymocyte development. Following deletion of intronic segments that included the entire silencer, CD4 was de-repressed in a subset of thymocytes corresponding to immature $CD4^{-}CD8^{-}$ DN thymocytes in wild-type mice, in mature (HSA^{low}) CD8 SP thymocytes, and in peripheral $CD8^{+}$ T cells (Leung *et al.*, 2001; Zou *et al.*, 2001). Because the level of CD4 expression in the silencer-deleted mice was lower in immature $CD4^{-}CD8^{-}$DN thymocytes than in mature cytotoxic-lineage T cells (Leung *et al.*, 2001; Zou *et al.*, 2001), we cannot rule out the existence of another negative regulatory element operating specifically in immature DN thymocytes. However, as the 434 bp CD4 silencer is sufficient to completely repress transgene expression in DN thymocytes, it is likely that enhancer activity in these cells is somehow lower than that in the later developmental stages. These results therefore indicate that the CD4 silencer is essential and, most likely, sufficient for full repression of CD4 expression in both DN thymocytes and mature cytotoxic-lineage T cells, and demonstrate it to be the key *cis*-regulatory element that regulates lineage-specific activation of the CD4 locus.

The search for functional sites within the CD4 silencer, which could potentially explain its selective activity in cytotoxic lineage T cells, has relied on a combination of transfection studies and analysis of transgenic mice, as well as on DNase footprinting analysis with nuclear extracts from T cells. A murine $CD4^{-}CD8^{+}$ thymoma, 1200M, was found to be ideally suited for functional studies of the 434 bp CD4 silencer in transient transfection assays. When the silencer sequence was present in triplicate, reporter gene expression was effectively repressed. This permitted further whittling of the silencer to a 101 bp core fragment (165–265) that retained full activity (Taniuchi *et al.*, 2002b). However, the minimal sequences required for silencer activity differed between transfection assays and transgenic reporter gene assays in mouse. A 147 bp fragment containing the core CD4 silencer was inactive in transgenic mice in the absence of sequences from either the 5′ or 3′ flanking regions (Sawada and Littman, unpublished). This suggested that the transient repression observed in the T cell line is fundamentally different from the repression or silencing observed *in vivo*, possibly because changes in chromatin organization depend on specific sequences in these flanks (see following text).

DNase I footprinting assays were employed by several groups to identify functionally relevant regions within the murine and human CD4 silencer. Using nuclear extracts from mouse T cell lines, Siu and colleagues identified three regions (site I, site II, and site III) within the 434 bp murine CD4 silencer (Duncan *et al.*, 1996) (Fig. 2). Analysis of a 5′ 190 bp human CD4 silencer revealed two sites (site I_h and site II_h), one of which (I_h) partially overlapped with the homologous site I in the murine silencer (Donda *et al.*, 1996). The requirement of these sites for silencer function was examined by mutational analyses in transgenic mice. Although single deletion of any site (site I, site II, or site III) from the 434 bp mouse CD4 silencer did not affect silencer activity, combined deletions of site II with either site I or site III resulted in the loss of activity in transgenic mice (Donda *et al.*, 1996; Duncan *et al.*, 1996). Another study using transient transfection assays identified two functional sites (site 1 and site 2) in the 101 bp core sequence (Taniuchi *et al.*, 2002b). Site 1 partially overlapped with site II of Siu and colleagues, but site 2 differed from the previously described sites (Fig. 2). Mutation at site 1 or site 2 in the context of the core sequence abrogated silencer function in transfection assays. Similarly, these mutations, placed in the context of the core CD4 silencer with either 5′ or 3′ flanking regions, resulted in complete loss of silencer activity in transgenic mice. A third site (site 3), located between sites 1 and 2, was first identified by footprinting analysis (Sawada and Littman, unpublished). Although deletion of site 3 in the context of the core silencer resulted in only partial loss of silencer activity in the transfection assay, the same deletion in the context of the silencer lacking the 5′ flanking region (131–434) resulted in complete loss of silencer activity in transgenic mice (Mahanta and Littman, unpublished). Thus, the functional requirement for different sites within the silencer differed according to the assay system used and the context in which various mutations were analyzed. Resolution of this problem required development of a reliable system in which the physiological roles of various sequences within the CD4 silencer could be compared.

Comparison of the functions of different motifs was accomplished by gene targeting, using homologous recombination in ES cells to introduce numerous mutations within the murine *cd4* locus (Taniuchi *et al.*, 2002b). This *in vivo* mapping study clearly demonstrated a physiological requirement for several sites or subregions within the CD4 silencer. Because the *cd4* gene is expressed from both alleles, loss of silencer function in one allele resulted in CD4 derepression that was easily monitored by flow-cytometry, thus allowing analysis in chimeric mice generated with ES cells harboring single mutant alleles.

Silencer mutations within the endogenous *cd4* locus had full, partial, or no effect on CD4 silencing in DN thymocytes and CD8 lineage T cells (Fig. 2). Importantly, there were differences in the consequence of the mutations at the different stages of T cell development, and this will be discussed in greater

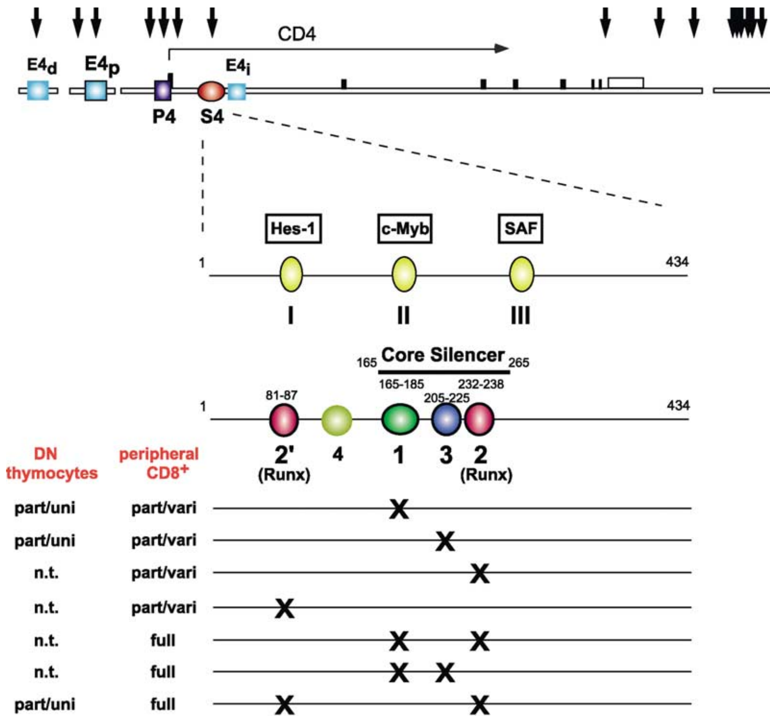


FIG 2 Transcriptional cis-regulatory elements in the murine *cd4* locus and functional sites within the murine CD4 silencer. The coding exons are shown as closed bars and the noncoding 5′ exon is shown as an open bar. The vertical arrows indicate DNaseI hypersensitive sites. The transcriptional direction of the CD4 gene is shown by the horizontal arrow. Proximal ($E4_p$) and distal ($E4_d$) enhancers (located approximately 13 kb and 24 kb upstream of the transcriptional start site, respectively) and a putative intronic ($E4_i$) enhancer (located immediately 3′ to the silencer) are shown as blue square boxes. The promoter (P4) and the silencer (S4) are shown as a purple square box and orange circle, respectively. The expanded region below the map represents the 434 bp murine CD4 silencer and putative factor binding sites. The top bar shows three footprint sites (yellow circles) defined by Siu and colleagues (Duncan *et al*., 1996) along with putative trans-acting factors. The second bar shows five sites defined by transient transfection assays, transgenic reporter assays, and targeted mutagenesis at the *cd4* locus (Taniuchi *et al*., 2002b). The core silencer (sequence 165–265) was sufficient for silencer activity in transfection assays. Site 2 and site 2′ are identical to the binding motif for Runt domain transcription factor (Runx) family members. The effects of targeted mutations on CD4 gene silencing in either immature DN thymocytes or peripheral $CD8^+$ mature T cells are shown at the bottom: "Part/uni" denotes partial CD4 de-repression in a uniform pattern. "Part/vari" denotes partial CD4 de-repression in a variegated pattern. "Full" represents CD4 de-repression in all of the $CD8^+$ T cells. n.t.: not tested.

detail in a later section. Deletion of the entire silencer (1–429) or of 90 bp within the core (167–257) resulted in complete CD4 de-repression in all mature CD8-lineage cells and in DN thymocytes. This result clearly showed that the core silencer is indispensable for silencer function. Mutations of single functional sites (site 1, 2, or 3) within the core resulted in CD4 de-repression in only a proportion of mature CD8-lineage cells. However, a combination of mutations at site 1 with mutations at either site 2 or site 3 resulted in CD4 de-repression in all of the mature $CD8^{+}$ T cells, demonstrating a functional redundancy of these sites (Taniuchi *et al.*, 2002b).

Analysis of flanking sequences, shown to have variable requirements in transgenic reporter studies, provided additional valuable information. Deletion of site III (260–281), which includes a putative binding motif for SAF (silencer associated factor), a factor reportedly localized in nuclei of $CD8^{+}$ but not $CD4^{+}$ T cells, had no effect on CD4 silencing. Deletion of the 3′ flanking region (279–429) affected CD4 silencing in only a minor proportion (~7%) of mature $CD8^{+}$ cells. In contrast, deletion of the 5′ flanking region (1–130) resulted in CD4 de-repression in a large proportion (~80%) of mature $CD8^{+}$ cells. Deletion of sequence 1–95 resulted in CD4 de-repression in only 10% of $CD8^{+}$ cells, suggesting the presence of another functional site in 95–130 (referred to as putative site 4) (Taniuchi *et al.*, 2002b). Sequence 75–90 within the 5′ flanking region, identified by footprinting analysis as site I (Duncan *et al.*, 1996), contains a Runx binding motif (referred to as site 2′) overlapping with an E box motif, a binding site for basic helix–loop–helix proteins. A targeted mutation in which only the Runx binding motif was mutated, leaving an intact E box motif, resulted in CD4 de-repression in a small (6%) subset of mature $CD8^{+}$ T cells, similar to that observed upon deletion of sequence 1–95. However, a combination of the site 2′ mutation with a mutation of the Runx binding motif in the core (site 2) resulted in complete CD4 de-repression in $CD8^{+}$ T cells (Taniuchi *et al.*, 2002a). This demonstrated a key role for the Runx binding motif in the 5′ flanking region of the silencer (see following text). A physiological role for the E box sequence has not been demonstrated by this kind of analysis, although *in vitro* studies have suggested that it may be a binding site for HES, a target of the Notch signaling pathway (Kim and Siu, 1998).

C. Trans-acting Proteins Involved in CD4 Gene Regulation

1. *Proteins Regulating CD4 Expression through Enhancers*

Investigation of trans-acting factors involved in CD4 enhancer function has been confined to the proximal enhancer. A heterodimeric complex of two basic helix–loop–helix proteins, E2A and HEB, was shown to bind to one of the two E-box motifs in CD4-3 (Sawada and Littman, 1993). In the thymus of mice

with a targeted disruption of a functional domain of HEB, there was the appearance of an unusual subset of $CD4^{low/-}CD8^{+}TCR^{int}$ cells (Barndt *et al*., 2000; Zhuang *et al*., 1996). The kinetics of appearance of this subset in the fetal thymus suggested a delayed up-regulation of the *cd4* gene during T cell ontogeny in the absence of a functional HEB protein. A similar population of cells was also observed in mice harboring heterozygous mutations for both *heb* and *e2a* ($heb^{+/-}$ $e2a^{+/-}$ mice). These results suggest that activation of CD4 is sensitive to the gene-dosage of HEB and E2A, and they are consistent with the involvement of the HEB/E2A heterodimeric complex in CD4 gene activation. This interpretation must be tempered, however, by the observation that compromised HEB and E2A function results in developmental arrest at the β-selection stage in thymopoiesis. The apparent defect in CD4 up-regulation thus occurs in the setting of defective developmental progression, and may not be a direct consequence of reduced HEB/E2A.

Some thymocytes can migrate to the periphery in the HEB-deficient mice, and CD4 expression is maintained in splenic T cells in these animals. Therefore, the requirement for HEB in CD4 gene expression may differ between immature thymocytes and mature T cells. This could be explained by the use of different enhancers at the two stages: HEB-dependent elements in $E4_p$ may be required in immature (DN and DP) thymocytes, whereas a different enhancer may provide HEB-independent activation in mature T cells. Alternatively, E2A could efficiently compensate for HEB function in mature T cells. A third possibility is that an epigenetic mechanism is involved in keeping CD4 active. HEB might therefore be necessary only for opening the CD4 locus in the initiation phase of gene activation, but would not be required subsequently to keep the locus active, due to heritable changes in chromatin. Interestingly, a similar observation was made in mice harboring a compromised CD8 enhancer (discussed in more detail later), although, in that case, there was accumulation of $CD4^{+}CD8^{low/-}TCR^{int}$ thymocytes. Thus, compromised enhancer function at the CD4 and CD8 loci may result in inefficient gene activation in a proportion of cells, and lead to subsequent variegated expression. Further analyses, such as a conditional deletion of $E4_p$ or the CD8 enhancers, may provide further insight into epigenetic aspects of activation of CD4 or CD8.

2. *Trans-acting Proteins that Act on the CD4 Silencer*

Several transcription factor-binding sites in the CD4 silencer have been shown to be functionally important in transgenic and targeted mutagenesis studies. The basic helix–loop–helix protein HES-1, a target of the Notch signaling pathway, was shown *in vitro* to bind to one of these motifs, the E box sequence located in site I (Kim and Siu, 1998). In a T cell line, HES-1 repressed reporter gene expression through the E-box sequence in a

dose-dependent manner (Kim and Siu, 1998). This result was consistent with a proposed function of Notch in regulation of the CD4/CD8 lineage decision. However, inactivation of Notch1 at the DP stage had no effect on lineage choice, suggesting that Notch signaling may not be important in CD4 silencing (Wolfer *et al*., 2001). In RAG2-defective host mice reconstituted with HES-1-deficient progenitors, T cell development was arrested at the DN stages (Tomita *et al*., 1999). However, in irradiated host mice, HES-1-deficient progenitors gave rise to mature T cells, including normal mature $CD4^{-}CD8^{+}$ SP cells, suggesting no role for HES-1 in the lineage choice (Kaneta *et al*., 2000). Further studies are required to determine if the HES-1-binding E box has a role in CD4 gene regulation.

Two other factors proposed to have a function in CD4 silencing are c-Myb and SAF, shown *in vitro* to bind to site II and site III, respectively (Fig. 2) (Allen *et al*., 2001; Kim and Siu, 1999). Combined mutations of the c-Myb-bindingmotif and the SAF binding site led to the loss of silencer function in transgenic reporter mice (Duncan *et al*., 1996; Kim and Siu, 1999). Site II overlaps with site 1 identified by Taniuchi *et al*. (Fig. 2). However, the Myb-binding motif is not conserved between the mouse and human CD4 silencer, and the consensus sequence is different from the minimum functional sequence of site 1, characterized by fine mapping in transfection assays (Taniuchi *et al*., 2002b). It is therefore unlikely that Myb binding to this site is required for silencer function. A role for SAF is also difficult to assess at this stage. This homeobox protein was identified in a screen for factors binding to the site III sequence, and it was reported to localize to the nucleus in $CD8^{+}$, but not $CD4^{+}$, T cells (Kim and Siu, 1999). However, there has been no functional analysis of the role of SAF in silencing. CD4 silencing in mature $CD8^{+}$ cells was normal in the absence of the SAF binding motif (Taniuchi *et al*., 2002b), although it is possible that this site provides a redundant function that can only be revealed by analysis of compound mutations.

Whereas evidence for the involvement of many transcription factors in the regulation of CD4 expression remains circumstantial, recent genetic studies have identified two transcription factors that are clearly required for the negative regulation of CD4 (Taniuchi *et al*., 2002a). These proteins are members of the Runt domain transcription factor (Runx) family. There are three mammalian members of the Runt domain family, *Runx1–3*, that encode α subunits that bind to the Runx binding motif (5′-PuACCACA-3′) by making heterodimeric complexes with a common β subunit, Cbfβ/Pebp2β (Wheeler *et al*., 2000). Fine mapping analysis in 1200M cell transfection assays revealed that the minimal functional sequence of site 2 (^{232}GACCACA^{238}) in the silencer core corresponded to a consensus Runx binding motif (Taniuchi *et al*., 2002a). A second Runx binding motif (site2′, ^{81}AACCACA^{87}) was identified in the

5′ flanking region. Both of the Runx binding motifs were required for full CD4 silencer activity. Specific mutations of either site resulted in variegated CD4 de-repression in mature CD8$^+$ cells, while mutations of both Runx motifs resulted in full CD4 de-repression in all mature CD8-lineage T cells, indicating that Runx sites are indispensable for establishment of epigenetic CD4 silencing. However, in immature DN thymocytes, the level of CD4 expression (or de-repression) when both Runx sites were mutated was lower than that upon deletion of the entire CD4 silencer (Taniuchi *et al*., 2002a). Thus, the function of Runx sites is likely to be partially compensated by other functional elements in immature DN thymocytes, but not in thymocytes developing into cytotoxic lineage cells, in which epigenetic CD4 silencing is being established. This difference in mechanism of repression or silencing is discussed in greater detail in the next section.

Although transcripts for all three *runx* genes appear to be present in all thymocyte subsets, two of the three genes were shown to be required for regulating CD4 expression at distinct developmental stages (Taniuchi *et al*., 2002a). Runx1 was shown to be required for CD4 repression in immature DN thymocytes, while Runx3 was shown to be required for establishment of epigenetic CD4 silencing in CD8 lineage T cells. Because null mutations of *runx1* result in an early lethal phenotype, the role of this gene in thymocytes was studied by using conditional inactivation of *LoxP*-flanked *runx1* with Cre recombinase expressed at different stages of development. When *runx1* was inactivated early in DN thymocytes, using *Lck–Cre* transgenic mice, CD4 was de-repressed in DN cells to a level similar to that observed when both *Runx* binding sites were mutated (Taniuchi *et al*., 2002a). Mice homozygous for *runx3* mutations die perinatally, but fetal thymic development can be examined. In Runx3-deficient fetal thymi, CD4 repression in immature DN thymocytes was normal. However, *RAG2*-null host mice reconstituted with *runx3*-mutant progenitors displayed either full or variegated de-repression of CD4 in mature CD8-lineage cells, which differed from what was observed with the double *Runx* site mutations (Taniuchi *et al*., 2002a). The variegation in CD4 de-repression is likely due to a partial compensatory function of Runx1. Using a different strain of Runx3-deficient mice, in which *runx3* inactivation is tolerated beyond the perinatal stage, Groner and colleagues showed full de-repression of CD4 in CD8 lineage T cells of mice with the compound *runx1*$^{+/-}$ *runx3*$^{-/-}$ mutant genotype (Woolf *et al*., 2003). The relative contribution of Runx1 to the establishment of epigenetic CD4 silencing remains unclear. There was no apparent loss of CD4 silencing in CD8 lineage cells when *runx1* was inactivated at different stages of thymocyte differentiation (Taniuchi *et al*., 2002a). Runx1 inactivation in DN thymocytes of *runx1*$^{f/f}$/ *Lck-Cre* mice severely blocked the generation of DP and also mature thymocytes and resulted in a reduced number of peripheral mature CD4$^+$ T cells

(Taniuchi *et al*., 2002a). However, the efficiency of *runx1* excision in mature T cells was lower than that in thymocytes, consistent with an essential role for Runx1 during selection events in the thymus. When *runx1* was inactivated in DP thymocytes, in *runx1*$^{f/f}$/*CD4-Cre* mice, positive selection was compromised, but there was no CD4 de-repression in those CD8^{+} T cells that reached the secondary lymphoid organs, despite very efficient inactivation of *runx1* in these cells (Egawa, Taniuchi, and Littman, unpublished). It is possible that Runx1 and Runx3 have equivalent abilities to initiate silencing of CD4 in cells destined for the CD8/cytotoxic T cell lineage. Because Runx3 appears to be expressed primarily in CD8 SP medullary thymocytes, while Runx1 is expressed at highest levels in immature cortical thymocytes, the loss of Runx3 would therefore more readily result in CD4 de-repression.

A role for a mammalian SWI/SNF chromatin remodeling complex in CD4 silencing has also been proposed. CD4 de-repression was observed in immature DN thymocytes in which the function of the BAF complex, a mammalian counterpart of the yeast SWI/SNF complex, was compromised. Haploinsufficiency of Brg1 (ATPase subunit) compounded with expression of a dominant negative form of BAF57 (a DNA binding subunit containing an HMG-box) resulted in partial CD4 de-repression in immature DN thymocytes, and in CD4 de-repression in a minor proportion of mature CD8^{+} T cells (Chi *et al*., 2002). CD4 de-repression caused by impaired BAF function was enhanced in mice harboring a mutation in site 1 of the CD4 silencer, but this was observed only in immature DN thymocytes, not in mature CD8^{+} cells. Association of the BAF complex with the CD4 locus was demonstrated by chromatin immunoprecipitation (ChIP) assays in a T cell line, but it remains unclear whether the BAF complex directly binds to the CD4 silencer. Interestingly, the CD8 gene was poorly up-regulated in a proportion of cells corresponding to the DP stage when BAF function was compromised. Therefore, the BAF chromatin remodeling complex appears to function reciprocally at these two loci in early thymocyte differentiation: it contributes to repressing the CD4 locus and to activating the CD8 locus. Similarly, *runx1* inactivation at the immature DN stage resulted in impaired CD8 up-regulation, in addition to the partial defect in CD4 repression. Although further genetic and biochemical studies are required, these results suggest that Runx1 functions at target loci to direct either repression or activation, in part by recruiting BAF complexes.

D. Distinct Mechanisms of CD4 Silencing at Two Developmental Stages

A common *cis*-regulatory element, the CD4 silencer, is clearly required for repression of CD4 both in DN thymocytes and in CD8 SP thymocytes that give rise to mature cytotoxic CD8^{+} T cells. The silencing mechanisms at these

different stages of development are likely to share some general features, but they are also thought to be fundamentally different. The early silencing must be reversible, since thymocytes turn on CD4 following β-selection. In contrast, CD4 remains stably silenced in mature cytotoxic T cells (although there may be an exception in some activated human CD8^{+} T cells, in which CD4 can be re-expressed (Kitchen *et al*., 1998)). Stable or heritable states of gene expression are governed by alterations in the structure of chromatin. Regulation of chromatin structure is achieved largely through post-translational modification of histones, which results in recruitment of diverse protein complexes (Jenuwein and Allis, 2001). It has been proposed that CD4 silencing in DN thymocytes is the result of active repression, which is reversible and does not involve extensive remodeling of chromatin, whereas silencing in CD8 lineage thymocytes is irreversible, probably due to an imprint that alters the chromatin structure at the CD4 locus (Taniuchi *et al*., 2002b). The latter would result in formation of heterochromatin, which is inaccessible to the transcriptional activation machinery. Although there is as yet no direct evidence for differences in histone modification or chromatin structure at the repressed CD4 locus in thymocytes at different stages of development, there are several lines of evidence that support this model, and these are discussed here.

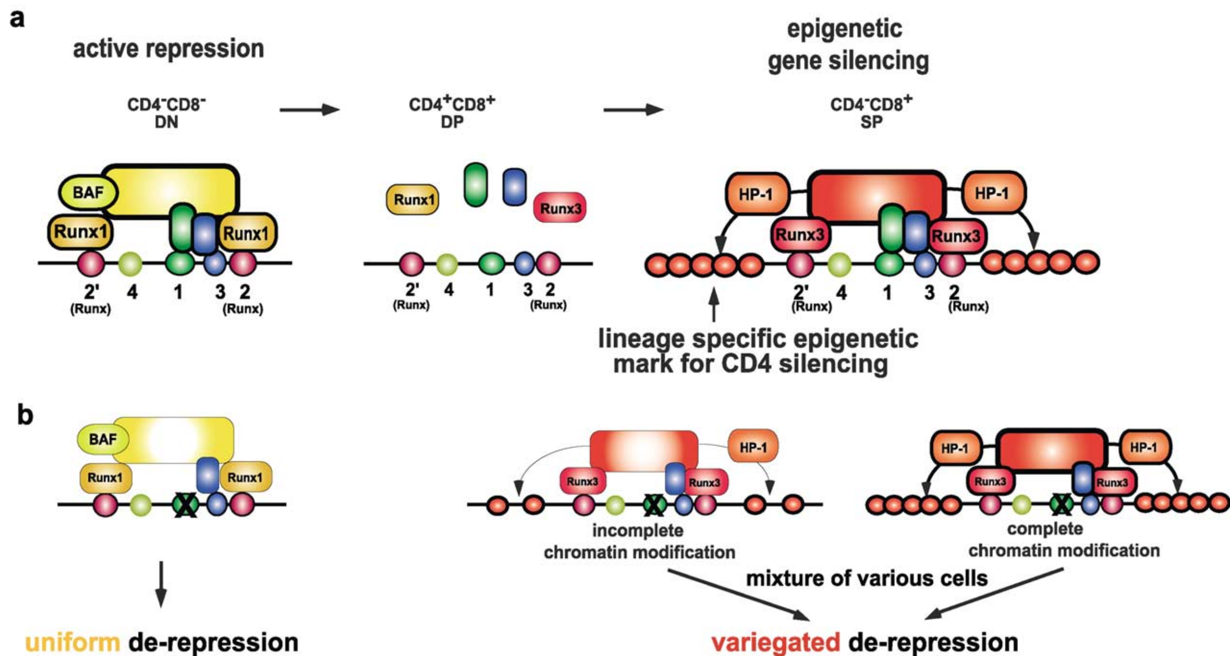
Targeted mutations within the core sequence of the silencer resulted in compromised CD4 silencing, manifested by partial de-repression of CD4 in both immature DN thymocytes and in mature CD8-lineage T cells. However, the patterns of CD4 de-repression were different at these stages: there was uniform de-repression in immature CD4^{-}CD8^{-} DN thymocytes, but variegated de-repression in mature CD8-lineage cells (Taniuchi *et al*., 2002b). Variegated gene expression, or position effect variegation (PEV), is usually observed in transgenic mice or at sites adjacent to chromosomal translocations, and it is thought to be due to modification of chromatin structure (Festenstein *et al*., 1999). The results suggested that, in DN thymocytes, CD4 is actively repressed by a combination of transcription factors. Loss of binding of any one of these factors would result in partial but uniform de-repression of CD4 expression (Fig. 3). In contrast, in cells committed to the CD8 lineage, loss of binding to any single site in the silencer would reduce the probability of recruiting a complex that modifies chromatin during a critical developmental window. If the complex is recruited, then silencing is established and maintained. However, if the complex is not recruited during this time frame, the locus would not be modified and CD4 would be de-repressed (Fig. 3).

Further analysis showed that CD4 silencing in mature cytotoxic T cells from mice with silencer mutations displayed the characteristic stochastic property attributed to heterochromatin-mediated gene silencing. Comparison of heterozygous and homozygous mice harboring a site 1 silencer mutation showed that the “on-or-off” status was determined stochastically on each allele.

The variegated CD4 de-repression was already observed in mature ($TCR^{high}HSA^{low}$) thymocytes, indicating that the on or off status was likely to be determined in developing thymocytes after positive selection. Once cells succeeded in silencing CD4 in the absence of a binding site within the silencer, the silenced status was maintained stably through subsequent mitoses (Taniuchi *et al.*, 2002b). This is a key feature indicating that the silencer functions epigenetically in CD8 lineage T cells.

Proof that the silencer is required for initiation, but not for maintenance, of CD4 silencing in $CD8^+$ T cells was provided by studies of mice in which the CD4 silencer could be deleted at different stages of development. Gene targeting in ES cells was used to flank a 1.6 kb segment of murine *cd4* intron 1, which includes the entire silencer, with two *loxP* sequences (Sil^{flox} allele) (Zou *et al.*, 2001). Expression of Cre recombinase resulted in excision of the *loxP*-flanked silencer. Similarly to the germline deletion, excision of the CD4 silencer in DP thymocytes, in *CD4-Cre* transgenic mice, resulted in de-repression of CD4 in all mature $CD8^+$ T cells. In contrast, *in vitro* deletion of the CD4 silencer in purified $CD4^-CD8^+$ SP cells from spleen, by using a Cre-encoding retrovirus, did not result in CD4 de-repression, even after several mitoses (Zou *et al.*, 2001). This clearly showed that the silencer is required for establishment, but not for the maintenance, of CD4 silencing. It is likely that a chromatin-modifying complex, recruited by silencer-binding factors during a developmental window as cells differentiate along the CD8 lineage, marks the CD4 locus with an epigenetic tag that is then inherited in the absence of the CD4 silencer.

Insight into the nature of the temporal window during which silencing is established comes from mice in which the silencer was excised after cells committed to the CD8 lineage. The Sil^{flox} mice were bred to transgenic mice in which Cre was regulated by an enhancer ($E8_1$, see following text) from the murine *cd8*αβ locus that was shown to direct expression specifically in mature (HSA^{low}) CD8-lineage thymocytes. Remarkably, CD4 was expressed in $CD8^+$ T cells in which the silencer was deleted, even though CD4 expression had initially been extinguished as the cells progressed to the $CD4^-8^+HSA^{hi}$ stage following lineage commitment (Zou *et al.*, 2001). This result suggested that a certain time window was necessary to complete chromatin modification for epigenetic CD4 silencing. It will be of considerable interest to determine if the reversible CD4 silencing observed at this early stage following lineage commitment is mechanistically similar to that observed in DN thymocytes. In yeast, a model of stepwise chromatin modification was proposed for heterochromatinization at the silent mating type loci. This is initiated by deacetylation of histone by a histone deacetylase (HDAC), followed by methylation of lysine 9 in histone 3 by histone methyl-transferase (HMT), and then recruitment of the yeast homologue of heterochromatin



a
active repression
epigenetic
gene silencing
CD4-CD8-
DN
CD4+CD8+
DP
CD4-CD8+
SP
BAF
Runx1
Runx1
Runx1
Runx3
HP-1
HP-1
Runx3
Runx3
2'
(Runx)
4
1
3
2
(Runx)
lineage specific epigenetic
mark for CD4 silencing
b
BAF
Runx1
Runx1
HP-1
Runx3
Runx3
HP-1
HP-1
Runx3
Runx3
incomplete
chromatin modification
complete
chromatin modification
mixture of various cells
uniform de-repression
variegated de-repression

protein-1 (HP-1), one of major heterochromatin structural components (Nakayama *et al.*, 2001). In mice, HP-1 was shown to be involved in gene silencing at centromeric regions, because overdosage of *HP-1*β by use of a transgene enhanced the silencing effect in the peri-centromeric regions, and suppressed PEV (Festenstein *et al.*, 1999). Taniuchi and colleagues showed that the HP-1β transgene suppressed CD4 de-repression in $CD8^+$ T cells from mice harboring a mutation at site 3 in the CD4 silencer. However, the HP-1β transgene did not alter the level of uniform CD4 de-repression in immature $CD4^-CD8^-$ DN thymocytes (Taniuchi *et al.*, 2002b). Therefore, overdosage of HP-1 enhanced CD4 silencing only in mature T cells, which is consistent with the involvement of this heterochromatin component in irreversible epigenetic silencing, at the late stage of thymocyte development, and not in reversible repression of CD4 in DN thymocytes.

The distinct roles of Runx1 and Runx3 in silencing at different stages of thymocyte development could also be interpreted as evidence that unique mechanisms are involved at each stage. However, it is also equally likely that the requirement for Runx1 in DN thymocyte active repression and for Runx3 (with some contribution from Runx1) in CD8 SP thymocyte silencing is simply due to the expression patterns of these transcription factors. Runx proteins are known to recruit HDACs by interacting with several corepressor molecules, including Groucho/TLE, which binds to the highly conserved C-terminal sequence found in all Runt domain family members, as well as Sin3A

Fig 3 A model for distinct mechanisms of silencer-mediated CD4 repression at two developmental stages. (A) In immature $CD4^-CD8^-$ DN thymocytes, several CD4 silencer binding factors, including the BAF chromatin remodeling complex, together recruit corepressor molecules, resulting in reversible transcriptional repression (active repression). Runx sites (site 2 and site 2′) would be occupied by Runx1 at this stage. At the transitional stage from $CD4^+CD8^+$ DP thymocytes to $CD4^-CD8^+$ SP cytotoxic-lineage T lymphocytes, lineage specific modifications of chromatin structure (small orange circle) are established through the function of distinct machinery (orange square) recruited by the CD4 silencer binding factor complex. We propose that this epigenetic modification is preceded by the recruitment of a reversible complex similar to that found in DN thymocytes. Altered chromatin structure would contain HP-1 molecules and serve as a heritable mark for epigenetic maintenance of the silenced status. Runx sites would be occupied mainly by Runx3 at this stage. (B) Compromised silencer function due to a mutation of site 1 results in partial uniform de-repression of CD4 in immature DN thymocytes (left). During the transition from the $CD4^+CD8^+$ DP stage to $CD8^+$ SP thymocytes, at which epigenetic silencing is established, the compromised silencer function results in one of two possible outcomes at the CD4 locus. In a fraction of the CD8-lineage T cells, modification of chromatin is complete enough for epigenetic inheritance of the silenced status. However, in the rest of these cells, modification of chromatin is not complete enough to shut off the CD4 gene. Following subsequent cell divisions, the amount of CD4 transcription would be dependent on the status of chromatin modification and would thus vary. The mixture of cells harboring silenced or activated CD4 results in variegated CD4 de-repression. HP-1 contributes to the successful establishment of the epigenetic mark.

(Levanon *et al*., 1998; Lutterbach *et al*., 2000; Wheeler *et al*., 2000). It is therefore possible that the Runx proteins contribute to active repression of CD4 by recruiting HDACs in both DN and CD8 SP thymocytes. In the CD8 SP thymocytes, following transition from the HSA^{hi} to the HSA^{lo} stage, there is likely to be the additional recruitment of the epigenetic machinery, which is dependent on the presence of Runx factors and induces locus heterochromatinization that is stably maintained even in the absence of the CD4 silencer by epigenetic mechanisms.

E. Link of CD4 Silencing to Lineage Specification

A major incentive for studying signaling pathways that regulate coreceptor expression is the likelihood that these are shared with pathways involved in cell fate determination of DP thymocytes. For this reason, Runx3 was a good candidate for a factor involved not only in CD4 silencing as cells committed to the CD8 lineage, but also in specification of the cytotoxic lineage. Indeed, there was a reduction in the number of CD8$^+$ T cells in secondary lymphoid organs of RAG2-deficient mice reconstituted with *runx3*$^{-/-}$ fetal liver cells (Taniuchi *et al*., 2002a). Similar results were observed in viable *runx3*$^{-/-}$ mice (Woolf *et al*., 2003). In the thymus, generation of mature (TCR^{high}HSA^{low}) CD8$^+$ thymocytes was normal in animals reconstituted with the mutant progenitors but was reduced in the viable mutant mice, although not to the extent observed in the periphery. This phenotype was not due to a defect in lineage commitment, however. Although CD8$^+$ T cells from secondary lymphoid organs of these mice had defective TCR-mediated proliferative responses, after activation with IL-2 they had normal cytotoxic activity and normal expression of perforin, a protein critical for CTL function (Taniuchi *et al*., 2002a). CD4$^+$ cells in Runx3-deficient mice had normal proliferative responses following TCR crosslinking or alloantigen stimulation. The reduction in number of CD8$^+$ but not CD4$^+$ T cells in secondary lymphoid organs is most likely due to defective homeostatic signaling in the former. Continuous interaction with MHC is required for survival of naive peripheral T cells (Goldrath, 2002), and loss of signaling in the absence of Runx3 would be expected to result in selective loss of the CD8$^+$ T cells. Studies of the half-life of CD8$^+$ versus CD4$^+$ T cells in mice deficient for Runx3 will be important to determine if this is indeed the mechanism accounting for the reduced cellularity.

Runx3 is therefore likely to be essential for the TCR-mediated response of CD8$^+$ T cells to antigen stimulation rather than for induction of cytotoxic functions. This may be a common feature of Runx function in T cells, since inactivation of *runx1* during thymocyte differentiation resulted in both defective β-selection and positive selection, which require pre-TCR and TCRαβ signal transduction, respectively. It will be of interest to identify genes, other

than CD4, that are involved in TCR signaling and are regulated by Runx1 and Runx3 at these different stages of T cell development.

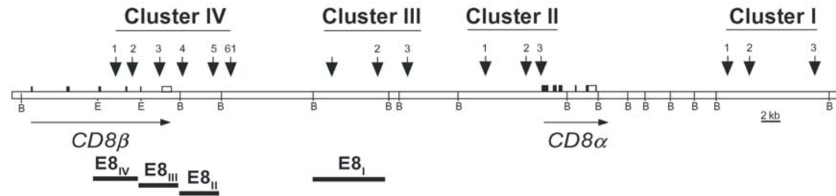
It remains unclear how lineage specificity of CD4 silencing is regulated. Although Runx3 mRNA was detected in CD4 SP thymocytes, albeit at a lower abundance than in CD8 SP thymocytes (Taniuchi *et al.*, 2002a), immunohistochemical analysis suggests that Runx3 protein is present only in CD8 SP and not in CD4 SP thymocytes (Woolf *et al.*, 2003). *Runx3* gene expression may therefore be sufficient to induce CD4 silencing. Activation of *runx3* transcription may be one component of the CD8 lineage program, and CD4 silencing would therefore be one of the consequences of this program. At this time, it is unclear if expression of Runx3 would be sufficient to impose silencing in developing thymocytes. It is possible that other components that bind to the silencer (e.g., to sites 1, 3, and 4) are constitutively present, and that expression of Runx3 is the key factor that results in silencing. Alternatively, the other factors may likewise be regulated differentially in the CD4 versus CD8 lineage, either at the transcriptional, translational, or post-translational level, and may thus also regulate silencing in the CD8 SP thymocytes. It will be important to identify the other silencer-binding factors and also to characterize the mechanism by which Runx3 expression is regulated, as this may hold the key to the pathway that specifies the cytotoxic T cell lineage.

IV. Regulation of CD8 Gene Expression

In contrast to the CD4 coreceptor molecule, which is expressed as a monomer at the cell surface, CD8 is expressed either as a CD8αα homodimer or as a heterodimer formed by the CD8α and CD8β polypeptides. DP thymocytes and conventional TCRαβ cytotoxic T cells express CD8αβ. The CD8α and CD8β genes are closely linked, which suggests that they are coordinately regulated in these cells (Gorman *et al.*, 1988). However, since intraepithelial lymphocytes (IEL) from the gut (Jarry *et al.*, 1990; Lefrancois, 1991) and $CD8^+$ dendritic cells (DC) (Vremec *et al.*, 1992) express only CD8αα homodimers, there must exist independent cis-regulatory elements specific either for CD8α and/or CD8β.

A. *Cis*-regulatory Elements Involved in CD8 Regulation

Early studies on the CD8α and CD8β promoters and on promoter-proximal regions failed to reveal CD8-specific cis-regulatory elements (for a review on CD8 promoters, see Ellmeier *et al.*, 1999). Subsequent DNase hypersensitivity (DH) analyses over the entire CD8αβ locus (referred to hereafter as the *cd8* locus in mouse) led to the identification of four clusters (I, II, III, and IV) of DH sites within an 80 kb murine genomic fragment (Hostert *et al.*, 1997a)



	Transgenic fragments	THYMUS DN	THYMUS DP	THYMUS CD4	THYMUS CD8	T CELLS CD4	T CELLS CD8	IEL-CD8+ αα	IEL-CD8+ αβ
T1		-	+	-	+	-	+	nd	
T2		-	-	-	-	-	-	nd	
T3		-	-	-	+	-	+	+	+
T4		-	-	-	+	-	+	nd	
T5	+	-	+	-	+	-	+	nd	
T6	+	-	+	-	+	-	+	nd	
T7		-	+	-	+	-	+	-	+/-
T8		-	+	-	+	-	+	-	+/-
T9		-	+	-	-	-	-	nd	
T10		-	+	+/-	+	+/-	+	-	+

	cis-Element deletions	THYMUS DN	THYMUS DP	THYMUS CD4	THYMUS CD8	T CELLS CD4	T CELLS CD8	IEL-CD8+ αα	IEL-CD8+ αβ
K1		-	+	-	+	-	+	+/-	+/-
K2		-	+	-	+	-	+	+	+
K3	+	-	var	-	+	-	+/-	+/-	+/-
K4		-	var	-	+	-	+/-	nd	

and six clusters (I to VI) within a 95 kb human genomic fragment (Kieffer *et al*., 1997). Transgenic mice generated with these large genomic fragments displayed appropriate developmental stage- and lineage-specific expression of the transgenic CD8α and β genes, demonstrating that the major cis-regulatory elements are located within these fragments. Interestingly, mosaic expression of the transgene was observed, most likely due to position effect variegation (Festenstein *et al*., 1996). This suggests that a locus control region (LCR, a cis-acting element that mediates position-independent and copy number-dependent expression of a transgene; for review, see Festenstein and Kioussis, 2000) is either not required to facilitate expression of CD8α and CD8β in their endogenous context or was omitted from the transgenic constructs.

Additional transgenic reporter expression assays were used to study and dissect the different DH clusters in more detail, and several genomic fragments involved in the regulation of CD8 expression were identified (Ellmeier *et al*., 1997, 1998; Hostert *et al*., 1998, 1997a,b; Kieffer *et al*., 1996, 1997; Zhang *et al*., 1998, 2001). At least four different genomic fragments isolated from the murine *cd8* locus were able to direct expression in a developmental stage-, subset-, and lineage-specific mode (Fig. 4). Since the genomic fragments used in the transgenic assay contained DH sites, it is likely that the enhancer activities co-localize with the hypersensitivity sites. The first enhancer identified, designated $E8_I$ or CIII-1,2, had activity only in mature CD8 SP thymocytes and in $CD8^+$ T cells (Ellmeier *et al*., 1997; Hostert

FIG 4 Summary of characterization of *cis*-regulatory regions in the murine *cd8* locus. Upper part: Schematic map of the *cd8*α and *cd8*β loci on mouse chromosome 6. Vertical arrows indicate individual DNase I hypersensitivity (DH) sites that have been grouped to DH clusters I–IV (Hostert *et al*., 1997a). Horizontal arrows indicate the location and transcriptional orientation of the *cd8*α and *cd8*β genes, while the open and closed bars indicate coding and noncoding exons, respectively. All BamHI (B), but only relevant EcoRI (E), sites are shown. The black horizontal bars show the genomic fragments used to define $E8_I$, $E8_{II}$, $E8_{III}$, and $E8_{IV}$. It is very likely that the enhancer activities within these genomic fragments overlap with some of the DH sites that map within the fragments. *Middle part*: Graphic representation of the genomic fragments used in transgenic reporter expression assays. The enhancer activity of the various fragments is shown at the right. The references reporting the activities are: T1 and T2 (Hostert *et al*., 1997a); T3 (Ellmeier *et al*., 1997; Hostert *et al*., 1997b); T4 (Hostert *et al*., 1997b); T5 (Hostert *et al*., 1998); T6 (Hostert *et al*., 1998; Zhang *et al*., 1998); T7–T10 (Ellmeier *et al*., 1998). + indicates strong enhancer activity, +/− weak activity, and – no enhancer activity. Nd: not determined. *Lower part*: The bars indicate the genomic region deleted in enhancer-deficient mice. The expression of CD8 in the absence of the enhancer is shown at the right. The references reporting the enhancer deletions are: K1 (Ellmeier *et al*., 1998; Hostert *et al*., 1998); K2 and K3 (Ellmeier *et al*., 2002); K4 (Garefalaki *et al*., 2002). + indicates normal CD8 expression, +/− reduced CD8 expression, and – no CD8 expression. "Var" indicates variegated expression of CD8. Nd: not determined.

et al., 1997b). Its up-regulation coincided with the transition of CD8 lineage thymocytes from the $TCR^{int}HSA^{hi}$ to the $TCR^{hi}HSA^{lo}$ stage, which marks the transition of selected cells from the thymic cortex to the medulla. Another enhancer, $E8_{III}$ (CIV-3), directed expression only in immature DP thymocytes. Enhancer $E8_{II}$ (CIV-4,5) directed expression both in DP thymocytes and in $CD8^+$ SP thymocytes and T cells (Ellmeier *et al.*, 1998). Finally, the cis-regulatory element $E8_{IV}$ (CIV-1,2) displayed low activity in $CD4^+$ SP and mature T cells in addition to DP and CD8 lineage cells (the significance of the low-level activity in helper T cells is currently not understood) (Ellmeier *et al.*, 1998). While all the *cis*-acting elements were active in thymic-derived T cells (at least at certain developmental stages), only $E8_I$ also directed expression in IEL (Ellmeier *et al.*, 1997), which have been proposed to be of extrathymic origin, although this remains controversial (Guy-Grand *et al.*, 2003; Leishman *et al.*, 2002). This suggested that $E8_I$ specifically regulates expression of CD8α (see following text). Furthermore, it was also shown that combinatorial interactions between cis-elements exist (Hostert *et al.*, 1998). The combined activity of DH site cluster II, which by itself has no enhancer activity, and cluster III directs expression of a reporter gene not only in $CD8^+$ T cells (as one would expect since cluster III, containing CIII-1,2($E8_I$), directs expression in the mature $CD8^+$ T cell lineage), but also in DP thymocytes. Taken together, these studies indicate that lineage-specific regulation of CD8α and CD8β gene expression during T cell development is achieved through a complex regulatory network that utilizes several closely linked cis-regulatory elements with distinct developmental stage-, subset-, and lineage-specific functions (Fig. 4).

The results from the transgenic reporter expression assays raise the question of why so many different, and probably partially redundant, *cis*-acting elements are required for the regulation of CD8 expression. One could argue that, in the context of the endogenous CD8 locus, the enhancers that show activity in the same subsets, such as $E8_I$ and $E8_{II}$ in CD8 SP and mature $CD8^+$ T cells and $E8_{II}$ and $E8_{III}$ in DP thymocytes, are together required for high-level expression of CD8α and CD8β. When analyzed in transgenic reporter assays isolated from their genomic context, they therefore display similar activities. Another possible reason for the requirement of multiple cis-elements is that some of the enhancers may direct specifically the expression of either CD8α or CD8β within the thymus-derived T cell lineage. Because it is difficult to experimentally test these possibilities in transgenic reporter assays, new insights have come from studies in mice with specific targeted mutations in *cd8* regulatory sites.

To investigate whether different elements in the *cd8* locus have unique regulatory functions and to overcome the limitations of the transgenic reporter system, a systematic deletional analysis of the various enhancers and

cis-elements in the mouse germ line has been initiated. Based on the results from transgenic reporter expression assays, one would have predicted that (1) individual deletions of enhancers should (at least) lead to a reduction of CD8 expression in the subsets in which the enhancers show activity, and that (2) combined deletions of enhancers with similar T cell subset activities should lead to a more dramatic reduction of CD8 expression compared to individual deletions. Dependent on whether some of the enhancers are specific for CD8α or CD8β, one may also expect a reduction in the expression of only one of these coreceptor genes. These predictions have been recently tested for three cis-elements: the mature $CD8^+$ T cell enhancer $E8_I$, the DP and CD8 SP specific $E8_{II}$, and DH site cluster II that contributes to DP-specific gene expression in transgenic mice.

In agreement with the predictions based on the transgenic reporter studies, mice homozygous for deletion of $E8_I$ (Δ1/Δ1) had a 3- to 5-fold reduction of CD8α expression levels on IEL, particularly on the $TCR\gamma\delta^+$ cells (Ellmeier *et al*., 1998; Hostert *et al*., 1998). This indicates that $E8_I$ is the major regulatory element for CD8α expression on IEL. In contrast, even though $E8_I$ alone is sufficient to direct expression in CD8 SP thymocytes and in mature $CD8^+$ T cells, expression of CD8αβ was largely normal in these thymus-derived cells in the Δ1/Δ1 mice. There was a very slight reduction (about 1.3-fold) in the CD8αβ level in mature thymocytes, but not in peripheral T cells (Ellmeier *et al*., 1998). This may be significant in the context of sequential functions of CD8 enhancers during thymocyte maturation, and will be discussed in greater detail.

Mice with targeted deletions of $E8_{II}$ (Δ2/Δ2) had normal expression of CD8 in thymocytes and in both thymus-derived $CD8^+$ T cells and IELs. Since, in transgenic mice, both $E8_I$ and $E8_{II}$ direct expression in mature $CD8^+$ T cells, it was possible that these two enhancers could compensate for each other in this subset. In addition, in $E8_{II}$-deficient mice, $E8_{III}$ or cluster II (in combination with other regulatory regions) could similarly compensate to direct CD8 expression in DP thymocytes. These possibilities were tested by analyzing mice double-deficient for $E8_I$ and $E8_{II}$ (Δ1Δ2/Δ1Δ2 mice). In contrast to individual deletions, deletion of $E8_I$ and $E8_{II}$ had a major effect on the expression of CD8 during thymocyte development, but not in mature CD8 lineage cells (Ellmeier *et al*., 2002). A population of "CD8-negative" DP thymocytes appeared that was indistinguishable from DP thymocytes by expression of other surface markers and by functional phenotype. Remarkably, a very similar phenotype with an even higher proportion of CD8-negative DP thymocytes was observed in mice with a deletion of DH cluster II (Garefalaki *et al*., 2002). The concurrent appearance of CD8-negative DP thymocytes and DP cells is consistent with variegation of expression of CD8 in the absence of either $E8_I/E8_{II}$ or cluster II, which suggests that precursor cells in the mutant mice undergo

stochastic establishment or loss of CD8 gene expression. As a consequence, fewer DP thymocytes were present and fewer mature $CD8^+$T cells developed. These results revealed a novel function of the *cis*-regulatory elements and enhancers that cannot be predicted by transgenic reporter analyses. They additionally suggested that there is partial redundancy of enhancers involved in initiation of CD8 gene expression in ISP or DP thymocytes. Even in the absence of either both $E8_I$ and $E8_{II}$ or cluster II, a majority of thymocytes expressed CD8 in the DP compartment, suggesting that after initiation of expression, an epigenetic mechanism keeps the CD8 locus in an "on" configuration. Surprisingly, expression of CD8 on those cells in which it was up-regulated (i.e., DP thymocytes and peripheral $CD8^+$T cells) was only slightly reduced in the mutant mice. It will be necessary to perform additional targeted mutational analyses in mice to determine if enhancers other than $E8_I$, $E8_{II}$, and cluster II are required to initiate and sustain expression of CD8 in these cells, or, indeed, whether there is an epigenetic mechanism that renders mature cells independent of further function of these *cis*-regulatory elements.

As has been mentioned, $E8_I$ directs reporter gene expression in $CD8\alpha\alpha^+$ IEL in transgenic mice (Ellmeier *et al.*, 1997) and its targeted deletion confirmed that it is the major enhancer that directs expression of CD8α in this lineage (Ellmeier *et al.*, 1998; Hostert *et al.*, 1998). However, a subset of CD8αα-expressing $TCR\gamma\delta^+$ IEL in $E8_I$-deficient mice continued to express surface CD8αα homodimers, albeit at lower levels (Ellmeier *et al.*, 1998; Hostert *et al.*, 1998). Because none of the other identified enhancers directed transgene expression in IEL, a possible explanation remained that unknown enhancer elements could compensate for loss of $E8_I$ in this particular $CD8\alpha\alpha^{low}TCR\gamma\delta^+$ IEL subset. Surprisingly, $E8_{II}$, which did not show any activity in $CD8\alpha\alpha^+$ IEL in transgenic mice (Ellmeier *et al.*, 1998), was found to compensate, at least partially, for the loss of $E8_I$. In Δ1Δ2/Δ1Δ2 mice, there was almost complete absence of CD8α expression in $TCR\gamma\delta^+$ IEL and a further reduction of CD8α expression in $CD8\alpha\alpha^+TCR\alpha\beta^+$ IEL compared to Δ1/Δ1 animals (Ellmeier *et al.*, 2002). $E8_I$ and $E8_{II}$ are therefore the *cis*-regulatory elements that direct expression of CD8α in CD8αα homodimer-expressing IEL of the TCRγδ lineage. In $CD8\alpha\alpha^+$ IEL of the TCRαβ lineage, additional elements must be able to direct low-level expression of CD8α.

As has been mentioned, CD8αα homodimers are also expressed on splenic DC. However, neither $E8_I$ nor $E8_{II}$ was able to direct the expression of a reporter gene in DC (Jung, Ellmeier, and Littman, unpublished). In addition, CD8α expression on DC was unaltered in $E8_I$ and $E8_{II}$ single knockout mice or in $E8_I$/$E8_{II}$ double-deficient mice (Ellmeier and Littman, unpublished). Thus, the regulatory elements required for CD8α expression in DC remain to be identified.

B. Trans-acting Factors and Chromatin Remodeling in CD8 Gene Regulation

The results from the CD8 enhancer- and cluster II-deficient mice suggested that the regulation of chromatin is an important step in the activation of CD8α and CD8β during T cell development (Kioussis and Ellmeier, 2002). More direct evidence that chromatin remodeling is indeed required in the regulation of CD8 expression comes from recent studies of the mammalian SWI/SNF-like BAF complex. In addition to its role in CD4 silencing (as has been described), the BAF complex also regulates the activation of CD8 and both BAF57 (an HMG box-containing factor) and Brg1 (an ATPase required for chromatin remodeling) have been implicated in this process (Chi *et al*., 2002). The first indication came from the analysis of CD8α expression levels in BAF57DN transgenic animals. Mice heterozygous for the BAF57DN transgene expressed lower levels of CD8α on DP thymocytes compared to non-transgenic littermates, and this phenotype was even more pronounced in mice homozygous for the transgene. Furthermore, haplo-insufficiency of Brg1 (achieved by generating *brg1*$^{+/-}$ mice) led to the appearance of a population of $CD4^+CD8^-CD3^-$ thymocytes (which was not caused by de-repression of CD4 in DN cells). Although it was not formally demonstrated whether these thymocytes are phenotypically (except with respect to CD8 expression) and physiologically similar to DP cells, this population was reminiscent of the CD8-negative DP cells that develop in mice with either cluster II or combined $E8_I$ and $E8_{II}$ deletions. These results suggest a link between some of the *cis*-regulatory elements and the BAF complex.

Another factor that has been implicated in the activation of the CD8α and CD8β loci is Ikaros, a transcription factor with multiple functions in hematopoiesis (Georgopoulos, 2002). A study in which antibodies specific for Ikaros were used to perform ChIP analysis revealed an association of Ikaros with sequences within the CD8 locus (Harker *et al*., 2002). Interestingly, the Ikaros-binding regions overlap with DH sites within DH clusters II and III. Furthermore, it was shown that haploinsufficiency of Ikaros causes an increase in the variegation of transgenic reporter genes that were driven by genomic fragments containing cluster II and/or cluster III. This effect seemed to be specific for CD8 regulatory elements, since the degree of variegation caused by control hCD2 regulatory elements was unaffected by Ikaros haploinsufficiency (Harker *et al*., 2002). In addition, the effect of Ikaros (and related family members such as Aiolos) was also seen at the endogenous *cd8*α and β loci. Compound mutations of the genes encoding Ikaros and Aiolos (*Ikaros*$^{+/-}$ *Aiolos*$^{-/-}$) led to the appearance of immature $CD4^+CD8^-$ cells (Harker *et al*., 2002), reminiscent of the CD8-negative DP cells observed in enhancer-deficient mice and in mice carrying mutations

in members of the BAF complex. A similar population of cells has also been observed in mice in which *runx1* was inactivated with the *Lck-Cre* transgene (Taniuchi *et al.*, 2002a). Together, these findings suggest that Ikaros and Runx1 may bind to various enhancers in the CD8 locus, and that they recruit the BAF chromatin remodeling complex, making the locus accessible to the transcriptional machinery. Alternatively, BAF-binding may precede the recruitment of Ikaros, Runx1, and other factors, and may thus remodel chromatin to permit sequence-specific binding of these factors. Elucidation of the relationship between the transcription factors and the BAF chromatin remodeling complex awaits ChIP analysis at different stages of thymocyte differentiation with strains of mice bearing mutations within the *cd8* locus or in genes encoding the various *trans*-acting factors.

C. $E8_I$ in Memory Cell Differentiation: Is $E8_I$ a Target of TCR Signaling?

A surprising new twist to the CD8 regulation story comes from the recent observation by Cheroutre and colleagues that a selected subset of conventional CD8αβ$^+$ T cells transiently expresses CD8αα homodimers not only upon *in vitro* TCR stimulation but, more importantly, also upon *in vivo* activation with antigen (Madakamutil *et al.*, 2004). This induction was revealed by staining with tetramers of the class Ib molecule TL1, which binds selectively to CD8αα homodimers and not CD8αβ heterodimers. The excess synthesis of CD8α after induction presumably results in preferential formation of cell surface CD8αα homodimers due to the limiting concentration of CD8β, which is only found in heterodimers. Remarkably, the induction of CD8α was found to be required for survival of the activated effector cells that subsequently differentiate into memory CD8$^+$ T cells. The induction of CD8αα homodimers upon activation of CD8αβ$^+$ T cells was abrogated in $E8_I$-deficient mice both *in vivo* and *in vitro*, indicating that $E8_I$ is not only the major enhancer regulating expression of CD8αα on IEL, but is also required for up-regulation of CD8αα on cells destined to become memory T cells (Madakamutil *et al.*, 2004). Although $E8_I$-deficient mice were able to mount a normal primary immune response against LCMV, they failed to develop a significant population of LCMV-specific memory T cells. Thus, activation of $E8_I$ by TCR signaling in CD8$^+$ CTL results in up-regulation of CD8αα, which is required for subsequent generation or survival of memory CTL. The up-regulation of CD8αα is consistent with the observation of reduced CD8β expression on human memory CTL (Konno *et al.*, 2002) and presumably occurs transiently in only a small subset of effector CD8$^+$ T cells that are destined to differentiate into memory T cells.

This surprising result calls for a reevaluation of the function of $E8_I$, at least beyond the DP stage in the thymus. Expression of CD8αα homodimers in IEL is clearly dependent on $E8_I$, and, as in memory CTL, this may require TCR-mediated signaling.

In the thymus, $E8_I$ appears to contribute to induction of CD8 expression in DP thymocytes, and it also appears to function late after positive selection to ensure that the level of CD8 is optimal on SP cells. It is possible that at this stage, when cells transit from the $TCR^{int}HSA^{hi}$ to the $TCR^{hi}HSA^{lo}$ phenotype, and presumably migrate from the cortex to the medulla, $E8_I$ also functions in response to TCR-mediated signaling. In the context of the "CD8 reversal model" for lineage specification (Brugnera *et al.*, 2000), it is expected that CD8 is regulated at different stages of development. Following positive selection signaling, DP expression is extinguished, presumably due to cessation of $E8_{III}$ activity (and of other enhancers that cooperate with $E8_{III}$). Cells selected by interaction with MHC class I would then undergo CD4 silencing, concomitant with activation of another set of CD8 enhancers, which has yet to be identified but which could include $E8_{II}$ and $E8_{IV}$, both of which direct expression in DP and CD8 SP thymocytes. This would restore high levels of CD8αβ heterodimer, and the potential for the TCR–coreceptor complex to resume signaling by interacting with self-MHC. We propose that this would result in a TCR-mediated signal that activates $E8_I$ (and, possibly, expression of genes required for migration of the cells from the cortex to the medulla).

Finally, future studies will have to focus on the analysis of the developmental stage-specific regulation of enhancer and DH cluster activities and on their interplay to ensure proper expression of CD8. One could imagine that the accessibility of the enhancers for *trans*-factors is developmentally regulated and therefore determines whether an enhancer is active or not in a particular subset. Thus, the enhancer would contain information for the recruitment of chromatin remodeling activities at the proper developmental stage. Alternatively, a different set of *trans*-acting factors that are either repressed or induced at the onset of positive selection would regulate CD8 expression in immature versus mature thymocytes, respectively. Whether an enhancer is functional or not would be determined by the presence of the *trans*-acting factor. The identification of the enhancer binding factors will certainly help to reveal which of the models (which are, of course, not mutually exclusive) is correct.

D. Enhancer Specificity for CD8α versus CD8β

A question that has not been directly addressed in the studies already described is how specificity of CD8α and β gene expression is achieved in different T cell lineages. Since the clusters of DH sites that contain at least five

different cis-regulatory elements are in close proximity to each other and since CD8α and CD8β display T cell lineage-dependent differences in their expression pattern, a tight regulatory interaction between the CD8α and CD8β genes and the enhancers must exist. One could speculate that $E8_I$, which is necessary and sufficient to direct expression in CD8αα$^+$ IEL (Ellmeier *et al.*, 1997, 1998; Hostert *et al.*, 1998), is likely to function only in conjunction with the CD8α promoter and may therefore be specific for regulating CD8α expression. However, additional transgenic expression assays indicate that $E8_I$ together with the CD8β promoter is able to direct expression of a reporter gene in IEL (Ellmeier and Littman, unpublished). Thus, incompatibility between $E8_I$ and the CD8β promoter does not seem to explain why $E8_I$, which is active in IEL, fails to direct CD8β gene expression in this T cell lineage. It is more likely that an insulator element (Bell *et al.*, 2001) may be localized between $E8_I$ and the CD8β promoter, thus preventing enhancer and promoter interactions.

V. Conclusion

Close examination of how the CD4 and CD8 genes are regulated during T lymphocyte development, not only in the thymus but also in secondary lymphoid organs, has provided us with valuable insight into mechanisms of lineage specification. Further studies in this area promise to uncover how helper versus cytotoxic T cells differentiate, and, possibly, even how effector T cells give rise to subsets of long-lived memory T cells with specialized activation properties. In a more general sense, examination of these loci has provided a unique opportunity to study epigenetic regulation in vertebrate development. The CD4 locus remains the only vertebrate gene currently shown to undergo developmentally regulated epigenetic silencing, and as such, it presents an ideal system for analyzing how heterochromatin is established during developmental processes in higher eukaryotes.

Acknowledgments

W. Ellmeier is supported by the Austrian Research Fund (FWF) and the START program of the Austrian Ministry of Education, Science, and Culture, and D. R. Littman is an Investigator of the Howard Hughes Medical Institute.

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