A Developmental Switch from TCRδ Enhancer to TCRα Enhancer Function during Thymocyte Maturation

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Summary

V(D)J recombination and transcription within the TCRδ locus are regulated by three characterized cis-acting elements: the TCRδ enhancer (Eδ), TCRα enhancer (Eα), and T early α (TEA) promoter. Analysis of enhancer and promoter occupancy and function in developing thymocytes in vivo indicates Eδ and Eα to be developmental-stage-specific enhancers, with Eδ “on” and Eα “off” in double-negative III thymocytes and Eδ “off” and Eα “on” in double-positive thymocytes. Eδ downregulation reflects a loss of occupancy. Surprisingly, Eα and TEA are extensively occupied even prior to activation. TCRδ downregulation in double-positive thymocytes depends on two events, Eδ inactivation and removal of TCRδ from the influence of Eα by chromosomal excision.

Introduction

Immature T lineage-committed cells develop through a series of intrathymic steps to form mature peripheral T lymphocytes that bear either an αβ or γδ T cell receptor (TCR). The regulated rearrangement and expression of TCR genes is an important component of the developmental program. CD4 CD8– double-negative (DN) thymocytes can be subdivided into four sequential maturation stages distinguished by CD44 and CD25 expression: CD44–CD25– (DN I), CD44 CD25– (DN II), CD44 CD25+ (DN III), and CD44 CD25+ (DN IV) (Pearse et al., 1989; Godfrey et al., 1993). TCRγ and δ rearrangements initiate at DN stage II, and extensive rearrangements of TCRγ, γ, and δ are all apparent at DN stage III (Godfrey et al., 1994; Wilson et al., 1994; Tourigny et al., 1997; Capone et al., 1998; Livak et al., 1999). The outcome of these rearrangement events influences further differentiation. Most thymocytes that differentiate along the αβ pathway (DN IV to immature single-positive [ISP] to CD4+ CD8+ double-positive [DP]) have a productive TCRβ rearrangement and express a β-pTα pre-TCR (Dudley et al., 1994; Fehling et al., 1995). Most of these cells carry out-of-frame rearrangements of either TCRγ or TCRδ, arguing that TCRγ expression diverges from this pathway (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995). Nevertheless, expression of a γδ TCR, or even a γ-pTα complex, can promote low level maturation to the DP stage in the absence of functional TCRβ rearrangement (Kersh et al., 1995; Buer et al., 1997; Livak et al., 1997; Passoni et al., 1997; Kang et al., 1998a, 1998b). Similarly, γδ cells typically display in-frame TCRγ and δ rearrangements and incompletely rearranged TCRδ genes; yet some γδ cells carry an in-frame TCRβ rearrangement (Dudley et al., 1995; Burtrum et al., 1996; Aifantis et al., 1998). Commitment to the αβ and γδ T cell pathways appears to depend upon both TCR-dependent and TCR-independent mechanisms (Kang and Raullet, 1997; Hedrick and Sharpe, 1998; MacDonald and Wilson, 1998).

In thymocytes differentiating along the αβ pathway, the DN to DP transition is associated with striking changes in TCR gene rearrangement and expression: TCRα gene rearrangement is initiated (Pearse et al., 1989; Wilson et al., 1994, 1996; Petrie et al., 1995) and TCRγ and TCRδ gene expression is inhibited (Wilson et al., 1994, 1996; Kang et al., 1998a, 1998b; MacDonald and Wilson, 1998). As a consequence of this inhibition, γδ and γ-pTα receptors are downregulated (Buer et al., 1997; Livak et al., 1997; Kang et al., 1998a, 1998b; MacDonald and Wilson, 1998), and any thymocytes whose DP maturation depends on these receptors do not survive. A developmentally regulated cis-acting silencer has been implicated in the repression of the TCRγ gene (Ishida et al., 1990; Kang et al., 1998b). However, the situation for TCRδ is less clear. Because TCRδ gene segments are nested between Vδ and Jα gene segments within the TCRα/δ locus (Figure 1A), the activation of Vα to Jα rearrangement in a DP thymocyte eliminates the rearranged TCRδ gene from the chromosome. Nevertheless, excised TCRδ genes are retained as stable extrachromosomal circles in a high percentage of thymocytes and resting peripheral αβ T cells (Livak et al., 1995; Nakajima et al., 1995; Livak and Schatz, 1996). Why extrachromosomal TCRδ genes are not expressed in DP thymocytes is unknown.

The developmentally distinct programs of TCRδ and TCRα gene rearrangement and expression are particularly striking given the organization of TCRδ and TCRα gene segments within a single locus (Figure 1A). These programs must be orchestrated by cis-acting elements that function in a region-specific and developmental-stage-specific fashion. Two well characterized T cell-specific transcriptional enhancers are separated by 90 kb within the locus, the TCRδ enhancer (Eδ) upstream of Cδ and the TCRα enhancer (Eα) downstream of Cα (Krangel et al., 1998). In addition to regulating transcription, enhancers impart developmental regulation to V(D)J recombination by modulating the accessibility of recombination signal sequences (RSSs) to RAG-1 and RAG-2 (Sliekman et al., 1996; Stanhope-Baker et al., 1996; McMurry et al., 1997). Eδ and Eα have been shown to be developmental regulators, as they impart lineage- and developmental-stage-specific control to the rearrangement of V(D)J recombination reporter substrates in transgenic mice: Eδ activates rearrangement at the

enhancer (Eδ) of Cδ and the TCRα enhancer (Eα) downstream of Cα (Krangel et al., 1998). In addition to regulating transcription, enhancers impart developmental regulation to V(D)J recombination by modulating the accessibility of recombination signal sequences (RSSs) to RAG-1 and RAG-2 (Sliekman et al., 1996; Stanhope-Baker et al., 1996; McMurry et al., 1997). Eδ and Eα have been shown to be developmental regulators, as they impart lineage- and developmental-stage-specific control to the rearrangement of V(D)J recombination reporter substrates in transgenic mice: Eδ activates rearrangement at the
DN stage and Eα at the DP stage (Capone et al., 1993; Lauzurica and Krangel, 1994a, 1994b). Moreover, elimination of Eδ from the endogenous TCRαβ locus results in partial inhibition of TCRβ rearrangement (Monroe et al., 1999), and elimination of Eα results in dramatic inhibition of TCRα rearrangement (Sleckman et al., 1997). Eα is therefore a critical regulator of accessibility for the entire 70 kb Jα region. Another regulatory element that also modulates Jα accessibility and TCRα rearrangement is the T early α (TEA) promoter. This promoter, immediately upstream of the Jα cluster, drives germline transcription through the Jα region (de Chasseval and de Villartay, 1993; Shimizu et al., 1993; Villey et al., 1997). Gene targeting showed TEA to be important for rearrangements involving the most 5’ Jα gene segments (Villey et al., 1996). Thus, accessibility of these Jα gene segments requires collaboration between Eα and TEA. By inference, accessibility of more 3’ Jα gene segments may require collaboration between Eα and undiscovered regulatory elements. Finally, although Eα does not regulate TCRβ rearrangement, it is required for optimal TCRβ expression in mature γδ T cells (Sleckman et al., 1997). Thus, the developmental program at the TCRα/β locus is mediated, at least in part, by activation of Eδ at the DN stage and activation of Eα subsequently, in both DP thymocytes and mature γδ cells.

Results

Eα Occupancy during T Cell Development
Eα activity is thought to be induced upon transit of thymocytes into the DP compartment. Because in vivo occupancy of the minimal Eα (Tα1 and Tα2 protein binding sites) within transgenic minilocus substrates is highly cooperative (Hernández-Munain et al., 1998), developmental activation of the endogenous Eα might reflect a transition from unoccupied in the DN compartment to fully occupied in the DP compartment. To address this issue, we sought to analyze in vivo occupancy of Eα in DN and DP thymocytes. As a source of DN cells, we used total thymocytes from RAG-2−/− mice (Shinkai et al., 1992), 90% of which are in DN stage III (Godfrey et al., 1994). As a source of DP cells, we used total thymocytes from RAG-2−/− mice carrying a functionally rearranged TCRβ transgene (RxB) (Shinkai et al., 1993). Essentially all thymocytes in these mice are DP.

We initially analyzed Eα occupancy by measuring its hypersensitivity to DNase I digestion in both DN III and DP thymocytes. DNase I digestion in both DN III and DP thymocytes (Figure 1B). DNase I hypersensitive sites (HSSs) result from local distortions of nucleosomal structure due to transcription factor binding (Gross and Garrard, 1988). We treated permeabilized thymocytes from RAG-2−/− and RxB mice with increasing amounts of
Figure 2. Eα Is Occupied in Both DN III and DP Thymocytes

Thymocyte DNA from RAG-2−/− (R) and Rxβ mice was methylated with DMS either as naked (N) DNA in vitro or as chromosomal (C) DNA in intact cells and processed for genomic footprinting. (A) Top strand analysis. Protected and hypersensitive guanines are indicated by arrows with open and closed dots, respectively. Asterisks identify guanines preferentially protected in the Rxβ thymocytes. (B) Bottom strand analysis. (C) Summary of protected (open dots) and hypersensitive (filled dots) guanines.
DNase I, purified genomic DNA, and then analyzed SacI digested DNA by Southern blot as previously described (Diaz et al., 1994). Although we could not detect DNase I HSs corresponding to the TCRα LCR (Diaz et al., 1994), we observed a strong HS at a position corresponding to Eα. Surprisingly, Eα was hypersensitive to DNase I digestion in both RAG-2αβ and Rαβ thymocytes, arguing that it is occupied in both DN III and DP thymocytes.

We then used genomic footprinting (Mueller et al., 1992) for a more detailed analysis of Eα occupancy (Figures 2A, 2B, and 2C). Genomic DNA was treated with dimethylsulfate (DMS) either as naked (N) DNA in vitro or as chromatin (C) in live cells to methylate accessible guanines at the N7 position, and a comparison of guanine methylation patterns in the two preparations was used to identify footprints indicative of protein binding sites occupied in vivo. Surprisingly, occupancy of Eα in DN III thymocytes of RAG-2αβ mice and in DP thymocytes of Rαβ mice was very nearly identical. Identically footprinted sites include an unidentified site and an E box (which replaces the GC-I box of human Eα [Hernández-Munain et al., 1998] upstream of Tα1; the GC-II box [Hernández-Munain et al., 1998] and CRE site in Tα1 [note a polymorphism between RAG-2αβ [strain 129] and Rαβ [strain C57B/6] in bottom strand analysis of the CRE site]; an unidentified site between Tα1 and Tα2; TCF/LEF, CBF/PEBP2, and Ets binding sites in Tα2; a GATA site, an unidentified site, and an E box in Tα3; and a previously unidentified site between Tα3 and Tα4 that includes potential binding sites for CACC-binding factors, Myb and Sp1.

The only apparent change in occupancy within Eα persisted was detected at an E box present in Tα4. Occupancy of this site in Rαβ DP thymocytes was detected as three protected guanines on both the top and bottom strands. However, in RAG-2αβ DN thymocytes, only partially protected top strand guanines and two protected bottom strand guanines were apparent. This suggests either a quantitative increase or a qualitative change in Tα4 E box occupancy on transition from DN III to DP. We also detected some apparent changes in occupancy at previously unidentified sites, including two CACC sites, that are 3′ of Tα4 and outside of the functionally defined Eα. These changes reflect gains of occupancy upon transit from DN III to DP. The significance of these relatively minor changes in occupancy within and downstream of Tα4 is unclear.

**TEA Promoter Occupancy during T Cell Development**

Early TCRα gene rearrangement events that involve the most 5′ Jα gene segments depend on both Eα and the TEA promoter (Villey et al., 1996; Sleckman et al., 1997). Given that Eα is occupied as early as DN stage III, it was possible that the developmental onset of Vα to Jα rearrangement depends on developmental regulation of TEA promoter occupancy. To test this, we analyzed TEA promoter occupancy in DN III and DP thymocytes. We detected a DNase I HS over the TEA promoter in thymocytes of both RAG-2αβ mice and Rαβ mice, arguing that the promoter is occupied in both populations (Figures 1C and 1E). Furthermore, promoter occupancy appeared to be independent of Eα, because a DNase I HS was detected in thymocytes from Eα−/− mice as well (Figure 1D). To address TEA promoter occupancy in greater detail, we analyzed the promoter by genomic footprinting in thymocytes of RAG-2αβ and Rαβ mice. We observed identical footprints over a long GC-rich sequence in both thymocyte populations (Figures 3A and 3B). Hence, the TEA promoter appears to be factor loaded and primed for activation as early as DN stage III.

**Eα and TEA Promoter Activity during T Cell Development**

Given that Eα and the TEA promoter are both occupied as early as DN stage III, we questioned whether there was any evidence for germline TCRα transcription or Vα to Jα rearrangement at this stage. Germline TCRα transcripts, as measured by hybridization with either a Cα or a TEA probe, were readily detected in Northern blot analysis of RNA from Rαβ DP thymocytes but were not detected in RNA from RAG-2αβ DN thymocytes (Figure 4A). Furthermore, consistent with previous results (Sleckman et al., 1997), these transcripts were not detected in RNA from Eα−/− thymocytes, indicating that they are critically dependent on Eα.

To investigate the initiation of Vα to Jα rearrangement, we used PCR to detect rearrangements involving one of the 5′ Jα gene segments in sorted thymocyte populations isolated from RAG-2αβ mice. Jα48 (nomenclature of Koop et al., 1992; jα57 in the nomenclature of Koop et al., 1994) is one of a group of Jα gene segments that rearrange early (Petrie et al., 1995), in a fashion that is dependent on both the TCRα promoter (Villey et al., 1996) and Eα (Sleckman et al., 1997). Rearrangement of Vα3 to Jα48 was undetectable in DN III and γδ thymocytes but was detected at a low level in DN IV thymocytes and at a much higher level in both DP and αβ thymocytes (Figure 4B). Identical results were obtained for rearrangement of several other Vα gene segments (data not shown). Thus, Vα to Jα rearrangement is initiated in DN IV thymocytes, even though the TEA promoter and Eα are occupied as early as DN stage III.

To confirm that initiation of Vα to Jα rearrangement at DN stage IV is determined by developmental activation of Eα, we analyzed the activation of V(D)J recombination within a human TCRβ gene minilocus in sorted thymocyte populations isolated from transgenic mice. This minilocus includes unrearranged Vα, Dα, Jα, and Cα segments (Figure 4D). Previous studies demonstrated that the V to D step of transgene rearrangement occurs in an enhancer-dependent fashion, whereas the V to J step of transgene rearrangement can be activated by inclusion of either Eβ or Eα in the jα3-Cα intron (Lauzurica and Kran gel, 1994a, 1994b). Both VD and VDJ recombination products can be detected in a PCR reaction using a V- and a J-specific primer, the former as a 1.2 kb product and the latter as a 0.3 kb product. Analysis of a minilocus that includes Eα showed the enhancer-independent V to D step of rearrangement to initiate in DN III thymocytes (Figure 4B). However, the enhancer-dependent VD to J step of rearrangement did not initiate until DN stage IV and increased dramatically in DP thymocytes. Furthermore, consistent with previous analyses (Lauzurica and Kran gel, 1994b), VD to J rearrangement was detected in αβ but not γδ thymocytes. Therefore, under the control of Eα, developmental activation of the enhancer-dependent step of transgene
rearrangement mimicked precisely the activation of endogenous Vα to Jα rearrangement.

Because Eα may require collaboration with additional cis-elements (i.e., TCRγδ promoters) within the TCRγδ minilocus to activate VD to J rearrangement, it remains possible that these elements, rather than Eα, determine the developmental onset of VD to J rearrangement. To eliminate this possibility, we analyzed the developmental activation of minilocus rearrangement under the control of Eα. In this case, fully rearranged VDJ products were detected at high levels in DN III thymocytes (Figure 4C). Moreover, consistent with previous experiments (Lauzurica and Krangel, 1994b), these products were detected at high levels in both αβ and γδ thymocytes. These results indicate that any minilocus cis-acting elements other than Eδ or Eα that are required for VD to J rearrangement are either active or available for activation as early as DN stage III. Hence, the DN IV-specific rearrangement are either active or available for activation at this stage. These experiments clearly dissociate Eα occupancy from Eα activity, as Eα is occupied but inactive in DN III thymocytes.

TCRγδ Gene Downregulation across the DN to DP Transition

The above studies indicate that Eδ is activated at DN stage III. It has been shown that TCRγδ transcripts present at high levels in late stage DN thymocytes are dramatically downregulated upon the transit to the DP stage (Wilson et al., 1994, 1996; MacDonald and Wilson, 1998). Because this downregulation is coincident with activation of Eα and excision of the TCRγδ gene from the chromosome, it is unclear to what extent downregulation reflects inactivation of Eδ, chromosomal excision, or some other process. To address this, we first analyzed TCRγδ gene transcription in DN III and DP thymocytes under circumstances in which the TCRγδ gene remains chromosomal. Northern blot analysis of germline TCRγδ transcripts in RAG-2−/− DN III thymocytes and Rxβ DP thymocytes revealed high levels of transcripts in both instances (Figures 5, lanes 2 and 4). Because Eδ and Eα are both potential regulators of chromosomal TCRγδ gene transcription (Sleckman et al., 1997), we also analyzed germline TCRγδ gene transcription on an Eα−/− background. In this case, TCRγδ transcripts were readily detected in DN III thymocytes but were essentially undetectable in DP thymocytes (Figure 5, lanes 3 and 5). This result indicates that Eα can promote chromosomal TCRγδ gene transcription in DP thymocytes and argues that Eα-induced chromosomal excision of the TCRγδ gene may be a critical event in TCRγδ downregulation because it serves to isolate the TCRγδ gene from the influence of Eα. In addition, because Eα-independent TCRγδ gene transcription in DN III cells is primarily dependent on Eδ (Monroe et al., 1999), the observed chromosomal TCRγδ gene downregulation on an Eα−/− background implies that another mechanism that is intrinsic to the TCRγδ gene likely plays a role in its downregulation as well.

To address whether developmental downregulation of TCRγδ transcription is associated with inactivation of Eδ, we compared occupancy of Eδ in RAG2−/− DN III thymocytes to that in Rxβ DP thymocytes by genomic footprinting (Figures 6A and 6B). Eδ function depends on CBF and c-Myb binding to adjacent sites within the δEα element (Hernández-Munain and Krangel, 1994; Hernández-Munain et al., 1996; Lauzurica et al., 1997). Occupancy of these sites could be readily detected in RAG2−/− DN III thymocytes; the CBF site displayed two partially protected guanines on the top strand, whereas the Myb site displayed a partially protected guanine on the top strand and a hypersensitive guanine on the bottom strand. Importantly, these sites appear to be unoccupied in Rxβ DP thymocytes. Hence, there is a loss of Eδ occupancy during transit from the DN to the DP compartment that correlates with a loss of TCRγδ germline transcription on an Eα−/− background. This loss of Eδ occupancy likely explains the absence of TCRγδ expression from TCRγδ genes present on extra-chromosomal circles in DP thymocytes and in resting peripheral αβ T cells.

Discussion

A Model for the Developmental Regulation of Transcription and V(D)J Recombination at the Endogenous TCRαβ Locus

Our data extend the results of previous experiments establishing roles for Eδ and Eα as developmental-stage-specific regulators of V(D)J recombination and transcription at the TCRαβ locus (Capone et al., 1993;
Figure 5. Eα-Independent Germline TCRδ Transcription Is Down-regulated in DP Thymocytes
Total RNA obtained from thymocytes and splenocytes of mice with the indicated genotypes was analyzed on a Northern blot sequentially hybridized with radiolabeled Cδ, CD3e, and GAPDH probes.

Lauzurica and Krangel, 1994b; Roberts et al., 1997; Sleckman et al., 1997; Monroe et al., 1999). In essence, we demonstrate a developmental switch from Eδ activity to Eα activity as thymocytes differentiate along the αβ pathway. We consider the data in terms of a model in which transcription and rearrangement are orchestrated by developmentally regulated interactions between these enhancers and specific TCRα/δ promoters (Figure 7).

In DN III thymocytes, an occupied Eδ can interact with (presumably occupied) upstream TCRδ promoters to drive germline TCRδ transcription and TCRδ gene rearrangement. Although our data do not address whether Eδ is occupied at an earlier stage, analysis of minilocus V(D)J recombination (Figure 4C) offers no evidence for human Eδ function in DN II thymocytes. The recently described initiation of endogenous murine TCRδ gene rearrangement as early as DN stage II (Capone et al., 1998; Livak et al., 1999; and confirmed by us: C. H. M., unpublished data) might reflect the activity of a regulatory element distinct from Eδ; this element could account for residual TCRδ rearrangement in Eδ-deficient mice (Monroe et al., 1999). Like Eδ, the TEA promoter and Eα are also occupied in the DN III compartment, yet neither Eδ nor Eα appears capable of interacting with TEA at this stage. The absence of Eδ-TEA interaction might reflect the presence of enhancer-blocking activity between these elements (Zhong and Krangel, 1997) or a functional incompatibility between Eδ and TEA. The absence of Eα-TEA interaction is considered in a subsequent section.

On transition into the DP compartment, Eδ is inactivated through loss of occupancy, whereas Eα becomes active and interacts with the TEA promoter to drive germline TCRα transcription and Vα to Jα rearrangement. As a consequence, previously rearranged TCRδ genes are excised from the TCRα/δ locus in the form of minilocus rearrangement. Analysis of endogenous Vα to Jα rearrangement in these samples gave results identical to those for line L (data not shown). (D) A schematic of the human TCRδ minilocus and the PCR strategy used to detect its rearrangement.
extrachromosomal circles. Although these extrachromosomal circles are stable and highly represented in DP thymocytes and resting peripheral αβ T cells (Livak and Schatz, 1996), transcription of extrachromosomal TCR genes does not occur because Eδ is inactive and Eα is no longer linked in cis. TCRδ (and for that matter, TCRγ) downregulation may be biologically important because a significant fraction of thymocytes differentiating along the αβ pathway carry an in-frame TCRδ (or TCRγ) rearrangement (Dudley et al., 1995; Kang et al., 1995; Livak et al., 1995; Wilson et al., 1996). Expression of a functional TCRδ or TCRγ polypeptide could result in intracellular competition for CD3 components that would interfere with assembly of TCR αβ+CD3 complexes. In addition, since a fraction of thymocytes can mature to the DP stage by expressing a γδ TCR or even a γ-pTα pre-TCR (Kersh et al., 1995; Buer et al., 1997; Livak et al., 1997; Passoni et al., 1997; Kang et al., 1998a, 1998b), TCR downregulation may be a mechanism to eliminate these TCR-lineage-mismatched cells.

Interestingly, Eδ, Eα, and the TEA promoter all appear to be occupied in mature peripheral γδ T cells. Eδ occupancy was shown by genomic footprinting in γδ T cell hybridomas (C. H. M., unpublished data), whereas Eα and TEA occupancy is assumed based on detection of Eα-dependent TEA transcripts in these cells (Sleckman et al., 1997). Hence, Eα is activated upon differentiation from DN to DP and from DN to γδ, in a developmental-stage-specific rather than lineage-specific way (Sleckman et al., 1997). The activated Eα in γδ T cells interacts not only with TEA but with upstream TCRδ promoters as well, thereby contributing to TCRδ transcription (Sleckman et al., 1997). It presumably does not activate Vα to Jα rearrangement due to a lack of RAG gene expression in mature γδ cells (Wilson et al., 1994).

The developmental switch from Eδ to Eα function in developing αβ lymphocytes described here is in some aspects analogous to and in other aspects distinct from regulatory events occurring at the IgH and Igκ loci during B cell development. Similar to the TCRα/δ locus, the IgH locus includes both an intronic enhancer and a 3′ enhancer complex that are activated at early and late stages of B cell development, respectively (Birshstein et al., 1997). The Igκ locus contains an intronic and a 3′ enhancer as well. However, although the intronic enhancer is relatively more important for early events and the 3′ enhancer relatively more important for late events (Gorman et al., 1996; Xu et al., 1996), it is not clear that the two κ enhancers are activated at different stages of B cell development. Further, no Ig enhancer is known to shut off once activated. As such, regulatory transitions at Ig loci are more similar to that at the TCRα/δ locus as thymocytes differentiate from DN to γδ than from DN to DP. We note, as well, that our data on Eα occupancy are rather similar to that described for the intronic Eκ, which appears to be equivalently occupied in pro- and pre-B cells, even though it is inactive in the former and active in the latter (Shaffer et al., 1997).
in which Eα is active. The event that converts the occupied but inactive minimal Eα at DN stage III to an active form at DN stage IV is unclear. Enhancer activity could be regulated by binding of a transcription factor that is undetectable by DMS genomic footprinting. Although this cannot be ruled out, we do detect binding at all sites previously shown to play a role in minimal Eα activity (Giese et al., 1995; Hernández-Munain et al., 1998). Another possibility is that related factors or factor isoforms with identical DNA contacts could differentially occupy the enhancer in DN III and DP thymocytes. Many Eα binding proteins are members of larger families, different members of which could have distinct functional consequences for Eα activity. For example, the CREB/ATF family includes several members with different isoforms that can function as either activators or repressors of transcription (Habener et al., 1995). Alternatively, coactivators and corepressors that do not directly contact the DNA but rather interact with enhancer-bound proteins to bridge to components of the basal transcriptional machinery or to modify chromatin structure could differentially regulate Eα activity in DN and DP cells. These could include ALY (Bruhm et al., 1997), CBP/p300 (Mayall et al., 1997), β-catenin (Clevers and Van de Watering, 1997; Hsu et al., 1998), and Groucho proteins (Levanon et al., 1998). Minimal Eα activity may also depend on posttranslational modifications such as phosphorylation or acetylation of previously bound factors. For example, phosphorylation of CREB at Ser-133 is necessary for the binding of coactivator CBP (Mayall et al., 1997).

Although the minimal Eα is sufficient to activate transcription and V(D)J recombination in a transgenic reporter (Roberts et al., 1997; Hernández-Munain et al., 1998), it is possible that areas outside the minimal Eα could be critical for enhancer function within the endogenous locus. In this regard, we detected a change of occupancy at the Tα4 E box and a gain of occupancy at sites 3′ of Tα4 upon transition from DN III to DP. The change in Tα4 E box occupancy seems more consistent with a qualitative rather than quantitative change in binding, because contacts with several guanines change, whereas others do not. Hence, different proteins may interact with this site in DN III and DP thymocytes, i.e., a suppressor in the former population and an activator in latter. Of note, because occupancy of the Tα4 E box clearly changes during the DN to DP transition whereas occupancy of E boxes within Tα3 and upstream of Tα1 does not, the factors that bind to these E boxes are likely different. Although bHLH regulation of Eα has been suggested, the endogenous regulators have not been identified (Bernard et al., 1998).

Eα activity could also be determined by elements mapping outside of the region examined by genomic footprinting in this study. This seems unlikely, because a murine Eα fragment of only 515 bp imparts appropriate developmental control to a V(D)J recombination reporter (Capone et al., 1993), and our footprinting analyses covered nearly the entire region. We also think it unlikely that Eα activation requires either relief from silencing as initially proposed (Winoto and Baltimore, 1989), or LCR activity (Diaz et al., 1994), as these elements are all excluded from the 515 bp Eα fragment (Capone et al., 1993). Moreover, targeted deletion of the putative LCR has a negligible effect on TCRα rearrangement and transcription (Hong et al., 1997).

TCRα Gene Downregulation in the Transition from DN to DP Thymocytes

Our data indicate that downregulation of TCRα gene expression on transition from the DN to the DP compartment is a consequence of two events: TCRα gene excision as a consequence of Vα to Jα rearrangement and inactivation of Eα through a loss of occupancy. One issue raised by our genomic footprinting analysis of Eα is that footprints are relatively weak even in DN III thymocytes. This could reflect Eα occupancy in only a fraction of DN III thymocytes. We consider this unlikely, because weak protections were observed in genomic footprinting analysis of Eβ in γδ T cell hybridomas (C. H. M., unpublished data). Alternatively, the degree of cooperativity among DNA-binding proteins could determine the strength of binding and level of protection from DMS modification. Strong footprints detected over Eα could reflect highly cooperative occupancy (Hernández-Munain et al., 1998). In contrast, cooperativity among Eβ binding factors is clearly much weaker (Hernández-Munain and Krangel, 1995; C. H. M., unpublished data).

The molecular basis for a loss of Eα occupancy in DP thymocytes is unclear. This phenomenon could be intrinsic to Eα and its binding factors, for example, as a consequence of the differential expression or modification of such factors in DN III and DP thymocytes. Alternatively, Eα occupancy might be determined by other regulatory elements within the TCRα/β locus that act at a distance.

Local versus Long-Distance Regulation of Accessibility by Enhancers and Promoters

Finally, our results have implications for the mechanism of accessibility control at antigen receptor loci. Because our analysis of chromatin structure using DNase I indicates the presence of local nucleosomal distortions at Eα and Eα and its binding factors, for example, as a consequence of the differential expression or modification of such factors in DN III and DP thymocytes. Alternatively, Eα occupancy might be determined by other regulatory elements within the TCRα/β locus that act at a distance.

Experimental Procedures

Mice

Rag-2Δ mice (Shinkai et al., 1992) were purchased from Taconic. Rα mice (Shinkai et al., 1993), transgenic mice containing a TCRα minilocus driven by Eα or Eα (Lauzurica and Krangel, 1994a, 1994b), and Eα mice (Sleckman et al., 1997) were previously described. All mice were analyzed at 4–6 weeks of age.

DNase I Hypersensitivity

DNase I treatments were performed as described (Boyes and Felsenfeld, 1996), with minor modifications. In brief, 1–5 × 10⁷ unfraccionated thymocytes were permeabilized in 2–5 ml of 0.15 M
Genomic Footprinting

Genomic footprinting was performed as described (Mueller et al., 1992). Oligonucleotides for E1 top strand analysis were GGGGACTT CTTGGAACTGGA, GGTTGTACCAAAAGGACATCGCA, and CCAC CAAGACGTCCAGGCGCACCC. Those for E1 bottom strand analysis were GCCCAGAATAAGAAGAAAGAT, GGTCCCTACTCCCTCC AGGTTTGG, and CCACCCTCTCCCTAGGTTGGTC. Oligonucleotides for TEA bottom strand analysis were AGATCGCCCTGC GGCTGTTT, GGAAAGGACATAGAGAGAAAGG, and GAAAAGG ACCGATAAGGAGCGAACG. Oligonucleotides for E1 top strand analysis were TCAAGTTTTTTCCCGAGAAGCTC, GAAAGATT GGGAAGCTTCTTGGCAC, and GGGAGGTCCCTGGCCAGCT CAGG. Those for E1 bottom strand analysis were CCTGAGTAAC CATAAGGTTAGG, GGGGCTTTTTGGTTGCTGTTTAG, and GGG GTTTTGGATGCTTTGAGTTTGTG.

Northern Blot

Total RNA samples isolated from unfractionated thymocytes (Chomezynski and Saachi, 1987) were electrophoresed through formaldehyde-agarose gels, transferred to nitrocellulose membranes, and hybridized with $^{32}$P-labeled probes generated by random priming (Amersham). Genomic footprinting was performed as described (Mueller et al., 1992). Oligonucleotides for E1 top strand analysis were GGGGACTT CTTGGAACTGGA, GGTTGTACCAAAAGGACATCGCA, and CCAC CAAGACGTCCAGGCGCACCC. Those for E1 bottom strand analysis were GCCCAGAATAAGAAGAAAGAT, GGTCCCTACTCCCTCC AGGTTTGG, and CCACCCTCTCCCTAGGTTGGTC. Oligonucleotides for E1 top strand analysis were TCAAGTTTTTTCCCGAGAAGCTC, GAAAGATT GGGAAGCTTCTTGGCAC, and GGGAGGTCCCTGGCCAGCT CAGG. Those for E1 bottom strand analysis were CCTGAGTAAC CATAAGGTTAGG, GGGGCTTTTTGGTTGCTGTTTAG, and GGG GTTTTGGATGCTTTGAGTTTGTG.

Analysis of V(D)J Recombination in Sorted T Cell Populations

For cell sorting, total thymocytes or DN-enriched thymocytes obtained as described (Di Santo et al., 1997) were stained with relevant antibodies and reagents (all purchased from PharMingen). To sort DN subpopulations, DN-enriched thymocytes were incubated with anti-CD4-Cy5, anti-CD8-Cy5, anti-CD3-Cy5, anti-CD24-biotin, anti-TCR-FITC, and their wings. Trends Genet.

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References


