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*J Immunol* 2011; 187:2484-2491; Prepublished online 22 July 2011;
doi: 10.4049/jimmunol.1100468
http://www.jimmunol.org/content/187/5/2484

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/07/22/jimmunol.1100468.DC1

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Long-Distance Regulation of Fetal V\textsubscript{\textalpha} Gene Segment TRDV4 by the Tcrd Enhancer

Bingtao Hao and Michael S. Krangel

Murine T\textalpha and Tcrd gene segments are organized into a single genetic locus (T\textalpha/Tcrd locus) that undergoes V(D)J recombination in CD4\textsuperscript{+}CD8\textsuperscript{−} double-negative (DN) thymocytes to assemble Tcrd genes and in CD4\textsuperscript{+}CD8\textsuperscript{+} double-positive thymocytes to assemble T\textalpha genes. Recombination events are regulated by two developmental stage-specific enhancers, E\textalpha and Ecrd. Effects of Ecrd on T\textalpha/Tcrd locus chromatin have been well documented, but effects of E\textalpha have not. In this regard, E\textalpha acts over long distances to activate many V\textalpha and J\textalpha segments for recombination in double-positive thymocytes. However, in DN thymocytes, it is unclear whether E\textalpha functions over long distances to regulate V\textalpha gene segments or functions only locally to regulate D\textalpha and J\textalpha gene segments. In this study, we analyzed germline transcription, histone modifications, and recombination on wild-type and Ecrd-deficient alleles in adult and fetal thymocytes. We found that Ecrd functions as a local enhancer whose influence is limited to no more than \textsim10 kb in either direction (including D\textalpha, J\textalpha, and TRDV5 gene segments) in adult DN thymocytes. However, we identified a unique long-distance role for Ecrd promoting accessibility and recombination of fetal V\textalpha gene segment TRDV4, over a distance of 55 kb, in fetal thymocytes. TRDV4 recombination is specifically repressed in adult thymocytes. We found that this repression is enforced by a developmentally regulated loss of histone acetylation. Constitutively high levels of a suppressive modification, histone H3 lysine 9 dimethylation, may contribute to repression as well. *The Journal of Immunology*, 2011, 187: 2484–2491.

The development of \alpha\beta and \gamma\delta T lymphocytes depends on the somatic assembly of TCR genes by V(D)J recombination (1). Among the four TCR genes, Tcrd and T\textalpha are uniquely organized into a single, complex genetic locus (the T\textalpha/Tcrd locus) that spans 1.6 megabases of murine chromosome 14 (2). T\textalpha/Tcrd locus recombination events occur according to a strict developmental program during thymocyte maturation, with Tcrd genes assembled in CD4\textsuperscript{+}CD8\textsuperscript{−} double-negative (DN) thymocytes and T\textalpha genes assembled in those thymocytes that progress to the CD4\textsuperscript{+}CD8\textsuperscript{+} double-positive (DP) stage. Developmental programming is thought to be mediated by changes in chromatin structure that make certain recombination signal sequences (RSSs) accessible to the recombination activating gene complex and by changes in locus organization that allow specific pairs of RSSs to undergo synthesis for recombination (3). How such changes are effected is only partially understood.

Two developmental stage-specific enhancers, E\textalpha and Ecrd, regulate the switch from Tcrd to T\textalpha rearrangement (4–7). E\textalpha is active in DN thymocytes; it promotes high-level germline transcription from nearby D\textalpha and J\textalpha promoters and supports normal levels of fully V\textgamma(D0)J\textalpha rearranged genes (6, 8). However, inefficient and incomplete (V\textgamma(D0)D\textalpha,J\textalpha) Tcrd gene assembly occurs even on E\textalpha-deleted alleles, implying that Tcrd recombination may be partially supported by additional cis-elements. Ecrd becomes active subsequently in DP thymocytes; it provokes transcription from the T early \alpha promoter upstream of the J\textalpha segments, at a distance of 70 kb (9, 10). E\textalpha and T early \alpha-dependent germline transcription across the J\textalpha segments is essential for J\textalpha accessibility and for V\textgamma to-J\textalpha recombination at this stage (11, 12).

A pool of \textsim100 V基因 segments is distributed across 1.5 megabases of DNA upstream of D\textalpha and J\textalpha gene segments and supplies V genes for both the TCR\textgamma and TCR\textalpha repertoires. A small subset of these V gene segments undergoes recombination to D\textalpha gene segments; 16 have been classified as V\textgamma, but a smaller number (TRDV2-2, TRDV5, and the four-membered TRAV15/TRDV6 family) tend to dominate the adult TCR\textgamma repertoire (2, 13–15). In addition, one V\textdelta (TRDV4) rearranges specifically in fetal thymocytes. In contrast, most of the V gene segments may undergo recombination to J\textalpha gene segments during subsequent T\textalpha gene assembly (14, 16).

Although the regulation of T\textalpha/Tcrd V gene segment usage is crucial for the development of distinct TCR\textgamma and TCR\textalpha repertoires, the molecular basis for this regulation is only partly understood (1). Several V\textgamma gene segments are positioned relatively proximal to D\textalpha, J\textalpha, and C\textalpha gene segments. However, prominent adult V\textdelta gene segments are characterized by active germline transcription and an accessible chromatin configuration in adult DN thymocytes, independent of their position in the locus (15). Moreover, recombination biases imposed by RSSs themselves cannot explain gene segment usage (17). Thus, there must be regulation at the level of RSS accessibility (15, 17). However, it is not clear whether V\textdelta gene segment accessibility depends solely on intrinsic features of V\textdelta gene promoters, or whether there are contributions from distant cis-regulatory elements as well. Regardless, it is clear that many additional T\textalpha/Tcrd locus V gene segments become transcriptionally active and accessible in DP
lymphocytes, particularly within the proximal 500 kb of the V segment array (15). These changes do not occur on Eα-deficient alleles, demonstrating that Eα can influence V gene segment accessibility over very long distances.

Although the studies outlined earlier suggest that Eα can shape the Vα repertoire through long-distance effects on Vα promoters, surprisingly little is known regarding the role of Eα in shaping the Vα repertoire. Although germline transcription of TRDV5, located 10 kb downstream of Eα, was found to be reduced on Eα-deficient alleles (18), information about more distant Vα gene segments has been lacking. Furthermore, no information is available regarding the chromatin modification profile of Eα-deficient alleles. Thus, it is unknown whether support of Tcrd gene assembly by Eα reflects a broad role for Eα in promoting accessibility of Vα, Dα, and Jα gene segments in DN thymocytes or a selective and localized role in Dα or Jα accessibility. Indeed, it has been generally assumed that Eα functions over relatively short distances, based, in part, on the observation that Eα cannot support Tcrd gene recombination when repositioned 100 kb distant from Dα and Jα gene segments in the normal location of Eα (19).

To investigate a role for Eα in regulating Vα gene segments in DN thymocytes, we analyzed germline transcription, histone modifications, and recombination events on wild-type (WT) and Eα-deleted alleles in adult and fetal thymocytes. These studies led us to identify a specific, long-distance role for Eα in promoting accessibility and recombination of the TRDV4 gene segment in fetal thymocytes. They have also provided some insights into the mechanisms by which accessibility is generated at Dα and Jα gene segments.

Materials and Methods

Mice

The following mouse strains were used: 129 (The Jackson Laboratory), Rag2<sup>−/−</sup> (20) (The Jackson Laboratory) on a 129 background, Eα<sup>−/−</sup> (6), and Eα<sup>−/−</sup>Rag2<sup>−/−</sup>; Eα<sup>−/−</sup> mice were kindly provided by Barry Sleckman (Washington University, St. Louis, MO). Strains carrying Eα-deleted alleles were on a mixed background but carried the 129 Tcrd/Tcrd locus. Adult thymus were harvested from 3-wk-old recombination-deficient mice or 4- to 6-wk-old recombination-sufficient fetuses. Fetuses were harvested from timed pregnancies, with the day of detection of a vaginal plug designated F0.5. All mice were used in accordance with protocols approved by the Duke University Animal Care and Use Committee.

Chromatin immunoprecipitation

Chromatin was prepared as described previously (21, 22). Thymocytes (0.5–10.0 × 10<sup>6</sup>) were washed with Wash Buffer I (10 mM sodium butyrate, 5 mM Na2EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM PMSE, 0.1 mM benzamidine, pH 7.0, in PBS without Mg<sup>2+</sup> and Ca<sup>2+</sup>) followed by Wash Buffer II (Wash Buffer I without Na2EDTA). Washed cells were then lysed in 200–400 μl of 80 mM NaCl, 10 mM Tris-HCl pH 8.0, 10 mM sodium butyrate, 6 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 250 mM sucrose, 0.15 mM spermine, 0.5 mM spermidine, 0.02% (v/v) NP40, 0.1 mM PMSE, and 0.1 mM benzamidine for 5 min on ice. Nuclei were pelleted and washed once with 10 mM NaCl, 10 mM Tris-HCl pH 8.0, 10 mM sodium butyrate, 3 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 250 mM sucrose. Digestion was then performed to generate mainly mononucleosomes with a minor fraction of dinucleosomes, by incubation for 5 min at 37°C in 200 μl of the same buffer containing 5 U micrococcal nuclease (Worthington). The reaction was stopped by addition of 300 μl 10 mM Tris-HCl pH 8.0, 5 mM EDTA, and 10 mM sodium butyrate. Sonication was then conducted on ice for eight cycles of 15 s on and 20 s off using a Sonicator 3000 (Misonix) with the output set to 3.0. After centrifugation for 10 min at 18,000 × g, the supernatant was transferred to fresh tube and Triton X-100 was added to a final concentration of 1% (v/v). Chromatin was then precleared with protein A-Sepharose/salmon sperm DNA slurry (Millipore) and was subsequently incubated overnight at 4°C with anti-acetylated H4 (6E9-M98; Millipore), anti-acetylated H1 (6E6-M98; Millipore), anti-dimethylated H3K4 (07-030; Millipore), anti-trimethylated H3K4 (04-745; Millipore), anti-dimethylated H3K9 (ab1220; Abcam), anti-tri-methylated H3K27 (07-449; Millipore), or control rabbit IgG (ab-105-c; R&D Systems). Protein A-Sepharose/salmon sperm DNA slurry was added for an additional 1-h incubation, after which immunoprecipitates were washed vigorously and DNAs were purified. Immunoprecipitated and input DNAs were quantified by real-time PCR using a Roche LightCycler and a FastStart DNA Master SYBR Green I kit (Roche). For immunoprecipitations using anti-acetylated H3, anti-acetylated H4, anti-dimethylated H3K4, and anti-trimethylated H3K4, analysis of β2-microglobulin (B2m) was used to normalize ratios of bound/input in different samples. Analysis of MouseEα2 was used to normalize immunoprecipitations using anti-dimethylated H3K9. Primers sequences are provided in Supplemental Table I. PCR conditions were as follows: 5 min at 95°C followed by 45 cycles of 1 s at 95°C, 5 s at 62°C, 7 s at 72°C.

Germline transcription

RNA was extracted from unfractinated thymocytes using TRIzol (Invitrogen) according to the manufacturer’s instructions. Contaminating genomic DNA was removed by incubation with 1 U DNase I (New England Biolabs) for 10 min at 37°C. SuperScript reverse transcriptase (Invitrogen) and random hexamer primers were used to synthesize cDNA according to the manufacturer’s instructions. PCR conditions were described previously (15). After agarose gel electrophoresis and transfer to nylon membranes, PCR products were detected by hybridization with a [32P]-labeled oligonucleotide probe. Primer and probe sequences are listed in Supplemental Table I. Real-time PCR was carried out as described earlier; primers are listed in Supplemental Table I.

PCR and Southern blot analysis of rearrangements

Total or sorted DN3 thymocytes were lysed by incubation in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.4% (v/v) SDS, and 0.1 mg/ml proteinase K overnight at 37°C. Genomic DNA was prepared by phenol/chloroform extraction and ethanol precipitation. PCR conditions were as follows: 3 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 60°C, 1 min at 72°C; 5 min at 72°C. After agarose gel electrophoresis and transfer to nylon membranes, PCR products were detected by hybridization with [32P]-labeled oligonucleotide probes. Primer and probe sequences are provided in Supplemental Table I.

PCR analysis of RSS cleavage

Thymocyte genomic DNA obtained from recombination-sufficient mice was analyzed by real-time PCR using primer pairs that define an RSS-spanning and a neighboring amplicon. Quantification was performed using standard curves constructed from amplification of thymocyte genomic DNA obtained from recombination-deficient mice. Loss of the RSS amplicon in recombination-sufficient thymocytes can result from its rearrangement or its deletion because of rearrangement of upstream RSSs; loss of the neighboring amplicon reflects its deletion only. Unarranged RSS was calculated as residual RSS amplicon/residual neighbor amplicon to specifically reflect RSS loss caused by RSS rearrangement. Primer sequences are provided in Supplemental Table I. PCR conditions were as described above for chromatin immunoprecipitation (ChIP) analysis.

OP9-DL1 culture

Monolayers of OP9-DL1 cells (23) were cultured in MEM Alpha (Life Technologies) supplemented with 20% FBS (Atlanta Biologicals), 1% penicillin-streptomycin (Life Technologies), and 1 mM sodium pyruvate (Life Technologies). Abs PE-Cy5 anti-CD5 (553654), PE-Cy5 anti-CD8α (553034), PE-Cy5 anti-CD3ε (553065), FITC anti-CD25 (553072), and PE-CD44 (553134) were purchased from BD Pharmingen. Anti-CD117 magnetic beads (18757) were purchased from Stemcell Technologies. CD117+ thymocytes were enriched by AutoMACS in Pansomes mode. Sorted DN1 (CD4+ CD8<sup>−</sup> CD3<sup>+</sup>CD117<sup>+</sup>CD44<sup>+</sup>CD25<sup>−</sup>) thymocytes from adult 129 or Eα<sup>−/−</sup> mice were then cultured on an OP9-DL1 monolayer in IMDM (Life Technologies) supplemented with 5% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, 55 μM 2-ME (Life Technologies), 5 ng/ml rmFlt-3 ligand (427-FL; R&D Systems), and 5 ng/ml IL-7 (407-ML; R&D Systems). After 14 d of culture in the presence or absence of 3 ng/ml trichostatin A (TSA; Sigma), DN3 cells (CD25<sup>−</sup>CD44<sup>+</sup>) were sorted for analysis.

Results

Eα behaves as a local enhancer in adult DN thymocytes

In our previous studies, we observed that the Vα gene segments that dominate the adult TCRβ repertoire (TRDV2-2, TRDV5, TRAV15D1, TRAV15D2, TRAV15-1, and TRAV15-2; Fig. 1A)
displayed elevated histone H3 acetylation (H3ac) and H4 acetylation (H4ac) and germline transcription in DN thymocytes of adult Rag2<sup>-/-</sup> mice (15). To investigate whether V<sub>d</sub> chromatin structure depends on E<sub>δ</sub>, we used ChIP to monitor four histone modifications in thymocytes from Rag2<sup>-/-</sup> and E<sub>δ</sub>-<sup>-/-</sup> Rag2<sup>-/-</sup> mice. On WT alleles, we found H3ac and H4ac to be high (>50% of B2m) from TRDD2 (D2) to TRDJ2 (J2) (Fig. 1B, 1C). As noted previously (15), H3ac and H4ac were present at moderate (5–50% of B2m) to high levels at six dominant V<sub>d</sub> gene segments (TRDV2-2, TRDV5, and TRAV15 family) and at certain other V segments (TRAV2 and TRAV14 family). By the criteria noted earlier, we also detected moderate acetylation at TRAV17, TRDV4, and TRDD1 (D1). On E<sub>δ</sub>-deficient alleles, H3ac and H4ac were reduced in the D2-to-J2 region, but we observed no substantial changes at V gene segments (Fig. 1B, 1C).

Histone H3 lysine 4 dimethylation (H3K4me2) is a chromatin mark that is distributed across active genes; histone H3 lysine 4 trimethylation (H3K4me3) better correlates with transcriptional activity, is generally associated with the 5′ portion of active transcription units (24), and notably serves as a docking site for recombination activating gene 2 protein during V(D)J recombination (25–27). Profiles of these histone modifications followed the trend of E<sub>δ</sub> dependence near D<sub>d</sub> and J<sub>d</sub> gene segments and E<sub>δ</sub> independence at more distal locations (Fig. 1D, 1E). However, unlike H3ac, H4ac, and H3K4me3, we found that H3K4me2 was E<sub>δ</sub> independent at D2 and TRDJ1 (J1). Interpretation of this finding is difficult because H3K4me2 abundance varies in a complex way that is determined, in part, by its conversion to H3K4me3 at the 5′ end of active transcription units (24).

To extend these observations, we analyzed germline transcription of V<sub>d</sub>, D<sub>d</sub>, and J<sub>d</sub> gene segments by semiquantitative RT-PCR (Fig. 2A). The results matched the chromatin data quite closely, because germline transcription at D2, J1, and J2 was reduced substantially on E<sub>δ</sub>-deficient as compared with WT alleles,

![Figure 1](http://www.jimmunol.org/Downloadedfrom/http://www.jimmunol.org/)

**FIGURE 1.** Influence of E<sub>δ</sub> on Tcra/Tcrd locus histone modifications in adult DN thymocytes. A, Map of the Tcra/Tcrd locus depicting the relative positioning of gene segments analyzed in this study. Enhancers E<sub>δ</sub> and E<sub>a</sub> (circles), and the D2 promoter (bent arrow) are also depicted. Not all Tcra/Tcrd gene segments are shown. H3ac (B), H4ac (C), H3K4me2 (D), and H3K4me3 (E) were measured by ChIP using chromatin prepared from Rag2<sup>-/-</sup> and E<sub>δ</sub>-<sup>-/-</sup> Rag2<sup>-/-</sup> thymocytes. TRDD1+01 and J1+01 are sites situated 1 kb downstream of D1 and J1, respectively. The data represent the mean ± SEM of three independent chromatin preparations for each genotype. Values of bound/input were expressed relative to those for B2m (normalized to 1) in each sample. Note that PCR for TRAV11 detects both TRAV11 and TRAV11D; PCR for TRAV14 detects six members of the TRAV14 family (TRAV14D-1, D-2, D-3, TRAV14-1, -2, -3). The significance of differences between E<sub>δ</sub>-<sup>-/-</sup> and WT were evaluated by two-tailed Student t test: *p < 0.01.
whereas (with the exception of TRDV5) germline transcription of V gene segments was unaffected. These conclusions were confirmed by quantitative real-time PCR, which detected ∼70% reductions in transcription at J1, J2, and TRDV5, but not at the more distant TRAV15 and TRDV2-2 gene segments (Fig. 2B). We note that among the histone modifications tested, transcription correlated least well with H3K4me2, because reduced transcription at D2 and J1 occurred without concomitant reductions in this modification (Fig. 1D). However, as noted previously, the abundance of H3K4me2 can be difficult to interpret. Reduced transcription at TRDV5 was consistent with previous data (18); however, we did not detect differences in H3ac, H4ac, or H3K4me2 at this site on Eδ-deficient alleles. Nevertheless, although H3K4me3 at TRDV5 was very low on WT alleles, it was still 5- to 10-fold over the IgG control in different experiments (data not shown), and, consistent with the transcription data, averaged 8.5-fold over Eδ-/- alleles (Fig. 1E). Taken together, the chromatin and transcription data support the notion that Eδ functions as a local enhancer whose influence is limited to no more than ∼10 kb in either direction in adult DN thymocytes.

Eδ regulates TRDV4 and TRDV5 chromatin in fetal thymocytes

The nearest Vδ gene segment upstream of Eδ is TRDV4, at a distance of 55 kb. TRDV4 recombination is restricted to the fetal thymus, likely the result of a specific suppression mechanism in adults as has been described for Vδ3 and Vδ4 (28–31). To ask whether Eδ regulates TRDV4 chromatin, we analyzed H3ac at the TRDV4 promoter using chromatin prepared from fetal day 17.5 (F17.5) thymocytes of Rag2−/− and Eδ−/−Rag2−/− mice. Consistent with the unique developmental profile of TRDV4 usage, TRDV4 displayed an 85% increase in H3ac in fetal as compared with adult thymocytes, whereas none of the other Vδ gene segments tested displayed similar increases (Fig. 3A). Moreover, elevated H3ac at TRDV4 in fetal thymocytes was clearly Eδ dependent (Fig. 3B). H3ac at TRDV5 was Eδ independent at this stage as well (Fig. 3B).

To confirm the conclusions from chromatin analysis, we measured germline transcription of Vδ gene segments in fetal thymocytes of Rag2−/− and Eδ−/−Rag2−/− mice (Fig. 3C). Consistent with the chromatin data, TRDV4 and TRDV5 both displayed substantial reductions in germline transcripts on Eδ-deficient alleles, whereas there were no significant changes in germline transcription of TRDV2-2 and TRAV15. These results demonstrate that Eδ supports TRDV4 and TRDV5 promoter activity and chromatin structure in fetal thymocytes.

**FIGURE 2.** Influence of Eδ on Tcra/Tcrd locus germline transcription in adult DN thymocytes. A, Germline transcription was measured by RT-PCR using serial 3-fold dilutions of cDNA (wedges) prepared from four independent cDNA preparations from Rag2−/− and Eδ−/−Rag2−/− thymocytes. Two preparations for each genotype are analyzed in the top panels; two different preparations for each genotype are analyzed in the bottom panels. The TRAV14 and TRAV15 primers detect all members of the TRAV14 and TRAV15 families. B, Real-time PCR of germline transcription using cDNA preparations from Rag2−/− and Eδ−/−Rag2−/− thymocytes. Data represent the mean ± SEM of five independent cDNA preparations for each genotype, all normalized to values for β-actin (Actb). The values for Eδ−/−Rag2−/− for each site were then expressed relative to those for Rag2−/−, which were normalized to 1. The significance of differences between Eδ−/− and WT were evaluated by two-tailed Student t test; *p < 0.05. (−), no reverse transcriptase.

**FIGURE 3.** Influence of Eδ on Tcra/Tcrd locus histone acetylation and germline transcription in fetal thymocytes. H3ac was measured by ChIP using chromatin prepared from (A) F17.5 and adult Rag2−/− DN thymocytes, and (B) F17.5 Rag2−/− and Eδ−/−Rag2−/− thymocytes. Data represent the mean ± SEM of three to five independent chromatin preparations for each genotype and developmental stage. Values of bound/input were expressed relative to B2m (normalized to 1) in each sample. C, Germline transcription was measured by quantitative real-time PCR using cDNA prepared from F17.5 Rag2−/− and Eδ−/−Rag2−/− thymocytes. Data represent the mean ± SEM of two independent cDNA preparations for each genotype, all normalized to values for Actb. *p < 0.05 by two-tailed Student t test.
E6 regulates TRDV4 and TRDV5 recombination fetal thymocytes

Based on the earlier results, we predicted that E6 would play a critical role in TRDV4 recombination in fetal thymocytes. To begin to test this, we first compared TRDV4 and TRDV2-2 recombination on WT alleles in F15.5, F17.5, and adult thymocytes (Fig. 4A). The Tcrd gene is unique among Ag receptor genes in that its recombination is not strictly ordered. Thus, VD, DD, and DJ recombination products are all detectable, and fully rearranged VDJ alleles can presumably be assembled through multiple pathways (32). PCR using a V-specific primer and a J1 primer can amplify two species indicative of Tcrd recombination events, a smaller product arising from fully rearranged templates (VDJ1) and a larger product arising from partially rearranged templates (VD2). Prior work has shown that D1 and D2 are both incorporated into Tcrd rearrangements in adult thymocytes, whereas D2 is selectively incorporated in fetal thymocytes (13, 33). Our PCR strategy does not report on D1 usage, because amplicon sizes would be virtually indistinguishable regardless of its inclusion. Using this strategy, we detected complete (VDJ1) and partial (VD2) rearrangements of TRDV4 in WT F15.5 thymocytes. These rearrangements increased slightly in F17.5 thymocytes but were undetectable in adult thymocytes (Fig. 4A). In contrast, we detected low levels of complete and partial TRDV2-2 rearrangements in F15.5 thymocytes, and complete rearrangements increased in F17.5 and even more so in adult thymocytes (Fig. 4A). Thus, TRDV4 rearrangement was restricted to the fetal period.

Quantitative real-time PCR across V6 RSSs was then used to assess RSS loss, and thus the extent of V6 rearrangement in fetal thymocytes. The results indicated that TRDV4 dominates V6 rearrangement events in the fetal period, as it was rearranged on 82% of WT alleles (Fig. 4B). In contrast, TRDV2-2 was rearranged on 14% and TRDV5 on only 9% of WT alleles (Fig. 4B). TRAV15 rearrangement was not detectable using this approach, indicating that this V6 family makes at best a very minor contribution to the fetal V6 repertoire. Analysis of E6-deficient fetal thymocytes revealed TRDV4 rearrangement to be reduced by 40% (to 21% of alleles; Fig. 4B). In contrast, reductions in TRDV2-2 and TRDV5 rearrangement were not apparent. Thus, E6 is critical for high-frequency TRDV4 rearrangement in the fetal thymus.

To confirm and extend these findings, we performed PCR with V-specific and J1 primers to assess the effects of E6 deficiency on the production of complete (VDJ1) and partial (VD2) fetal Tcrd rearrangement events (Fig. 4C). Complete rearrangements involving TRDV4 were abolished on E6-deficient alleles. Rather, the residual TRDV4 rearrangements on E6-deficient alleles were exclusively in the form of TRDV4-D2 rearrangement intermediates. A similar recombination defect was observed for TRDV5 (Fig. 4C). Because there was no apparent quantitative defect in TRDV5 rearrangement as assessed by RSS loss (Fig. 4B), this result may indicate a selective defect in TRDV5-D2-to-J1 rearrangement in E6-deficient fetal thymocytes. However, because the frequency of TRDV5 recombination is low even in WT fetal thymocytes, the assay for RSS loss may not have had sufficient accuracy to distinguish TRDV5 usage on WT and E6-deficient alleles (Fig. 4B).

In contrast with TRDV4 and TRDV5, we detected a very mild defect in TRDV2-2 rearrangement, with complete rearrangements slightly reduced and partial rearrangements slightly increased (Fig. 4C). This result is consistent with the fact that TRDV2-2 RSS loss is similar on WT and E6-deficient alleles (Fig. 4B). Given that D2-to-J1 rearrangement is dramatically impaired on E6-deficient alleles (Fig. 4C), we conclude that TRDV2-2 rearrangement occurs primarily by way of a VD intermediate on these alleles, and that the VD-to-J step may be mildly impaired. In contrast, TRDV4 rearrangement is substantially impaired at the V-to-D step, and TRDV4 and TRDV5 rearrangements are both blocked at the VD-to-J step.

Developmental regulation of TRDV4 recombination by histone acetylation

Our data indicated that E6 can stimulate H3ac at TRDV4 in fetal thymocytes but cannot do so in adult thymocytes (Figs. 1B, 3A). We wondered whether potential effects of E6 in adult thymocytes were counteracted by suppressive histone modifications specifically targeted to TRDV4. In this regard, the suppressive histone modification H3 lysine 27 trimethylation was shown to be elevated at proximal VH gene segments in adult but not fetal pro-B cells (34) and to promote distal VH gene recombination in adults (35). However, we detected only low levels of histone H3 lysine 27 trimethylation at TRDV4 in adult DN thymocytes (data not shown). We did detect the suppressive

**FIGURE 4.** Influence of E6 on Tcrd gene rearrangement in fetal thymocytes. **A,** J1 rearrangement in 129 mice. Three-fold serial dilutions (wedges) of genomic DNA from thymocytes of F15.5, F17.5, and adult 129 mice were analyzed by PCR followed by Southern blot. PCR was performed using TRDV2-2 or TRDV4 primers in combination with a J1 primer, a [32P]-labeled internal J1 oligonucleotide was used as a probe. Ct4 PCR ensured use of similar amounts of DNA. Data are representative of three independent experiments. **B,** RSS cleavage in genomic DNA of WT 129 and E6−/− fetal thymocytes. Real-time PCR was used to quantify percent unrearranged RSS relative to a neighboring amplicon. TRAV15 primers detect all members of the TRAV15 family. Data represent the mean ± SEM of four and two independent genomic DNA preparations, respectively, from WT and E6−/− F17.5 thymocytes. *p < 0.05, two-tailed Student t test. **C,** Tcrd rearrangement in fetal thymocytes of WT 129 and E6−/− mice. Genomic DNA samples from F17.5 thymocytes were analyzed by PCR using the indicated primers in conjunction with a J1 primer, followed by Southern blot using a [32P]-labeled internal J1 oligonucleotide probe. Data are representative of two to three independent experiments. (−), no DNA.
modification histone H3 lysine 9 dimethylation (H3K9me2) at levels that were substantially higher at TRDV4 than at TRDV2-2 or TRDV5 (Fig. 5A). Surprisingly, however, H3K9me2 at TRDV4 was similar in fetal and adult thymocytes (Fig. 5B), indicating that TRDV4 repression in adults is not a consequence of developmentally regulated H3K9me2.

To assess whether TRDV4 recombination was suppressed in adult thymocytes by the observed loss of Eδ-dependent histone acetylation, we first asked whether TRDV4 histone acetylation could be modulated by inhibition of histone deacetylases (HDACs). Indeed, incubation of adult Rag2−/− thymocytes with HDAC inhibitor TSA increased H3ac and H4ac at TRDV2-2, TRDV4, and TRDV5 (Fig. 6A, 6B), although some of the differences noted fell just short of reaching statistical significance. In conjunction with these changes, we detected increases in TRDV4 and TRDV5 germline transcription (Fig. 6C). We note that these increases could be more substantial, and the apparent decrease in TRDV2-2 transcription could be illusory, if TSA treatment were found to upregulate transcription of the control Actb gene that was used for normalization. We then tested the effects of TSA on TRDV4 rearrangement in 14-d cultures of DN thymocytes with OP9-DL1 stromal cells. TSA treatment stimulated a >10-fold increase in fully rearranged TRDV4 (Fig. 6D). In contrast, TSA had a relatively modest effect on rearrangement of TRDV2-2, likely because untreated adult thymocytes are already permissive for rearrangement of this Vδ gene segment. We conclude that the balance between histone acetyltransferase and HDAC activity differs at TRDV4 in fetal and adult thymocytes, and that this difference is causal in restricting TRDV4 usage to fetal thymocytes.

**FIGURE 6.** Activation of Vδ gene segment chromatin and rearrangement using an HDAC inhibitor. H3ac (A) and H4ac (B) were measured by ChIP using chromatin prepared from adult Rag2−/− thymocytes that were cultured for 8 h with or without 3 ng/ml TSA. Values of bound/input were expressed relative to B2m (normalized to 1) in each sample. Data represent the mean ± SEM of two to three independent chromatin preparations for each treatment. *p < 0.05, two-tailed Student t test. C, Germline transcription was measured by quantitative real-time PCR using cDNA prepared from adult Rag2−/− thymocytes that were cultured for 8 h with or without 3 ng/ml TSA. Data represent the mean ± SEM of four independent cDNA preparations for each genotype, all normalized to values for Actb. *p < 0.05, two-tailed paired Student t test. D, Adult 129 DN1 thymocytes were placed in culture for 14 d with or without 3 ng/ml TSA on OP9-DL1 stromal cells; DN3 thymocytes were sorted from cultured cells for preparation of genomic DNA. Three-fold serial dilutions (wedges) of genomic DNA were analyzed by PCR, followed by Southern blot. PCR was performed using TRDV2-2 or TRDV4 primers in combination with a J1 primer; a [32P]-labeled internal J1 oligonucleotide was used as a probe. Cd14 PCR ensured use of similar amounts of DNA. Data are representative of three independent experiments. (—), no DNA.

**Discussion**

Although >10 y have passed since Eδ was first shown to regulate Tcrδ locus recombination events (6), a detailed characterization of the regulation of Tcrδ locus chromatin accessibility has been lacking. In this study, we provide new insights into the influences of Eδ on Tcrδ locus chromatin and how these influences support the recombination of Tcrδ gene segments. We found that Eδ acts relatively locally to regulate chromatin structure and transcription in the 20-kb region extending from D2 to TRDV5 in adult DN thymocytes. A relatively local influence of Eδ on Dδ and Jδ chromatin can readily explain the previously described recombination defect in Eδ-deficient mice, with reductions in

![Graph](http://www.jimmunol.org/Downloadedfrom/0035f482d50732f34e4e03.png)

**FIGURE 5.** H3K9 dimethylation of Vδ gene segments in adult and fetal thymocytes. A, H3K9me2 of TRDV2-2, TRDV4, and TRDV5 was measured by ChIP using chromatin prepared from adult Rag2−/− thymocytes. Each Vδ gene segment was analyzed at three sites (a, b, c, as diagrammed). Values of bound/input were expressed relative to MageA2 (normalized to 1). Data represent the mean ± SEM of three independent chromatin preparations. B, H3K9me2 was compared in chromatin prepared from F17.5 and adult Rag2−/− thymocytes. Data represent the mean ± SEM of three to four independent chromatin preparations. TRDV4 and TRDV5 were analyzed at sites b. 6.9UDB is a positive control site within the Tcrδ locus (39); B2m and Actb served as negative control sites.
REGULATION OF FETAL V\textsubscript{\alpha} GENE REARRANGEMENT BY E\textsubscript{\alpha}

complete VDJ-rearrangements and increases in VD and DD rearrangement intermediates (6). However, although E\textsubscript{\alpha} regulates the nearby TRDV5, it appears to play no role in activating more distant V\textsubscript{\alpha} gene segments in adult DN thymocytes. Thus, with the exception of TRDV5, E\textsubscript{\alpha} does not function to define Tcrd/Tcrd locus V gene segments as V\textsubscript{\alpha} in these cells. Rather, this may be determined by unique features of individual V segment promoters. Notably, we found that in addition to regulating TRDV5, E\textsubscript{\alpha} plays a distinct and unanticipated role in fetal thymocytes as a regulator of chromatin structure, transcription, and recombination of fetal V\textsubscript{\alpha} gene segment TRDV4. As a consequence, complete rearrangements of TRDV4 and TRDV5 were absolutely dependent on E\textsubscript{\alpha}. The data for TRDV4 indicate that, at least in fetal thymocytes, E\textsubscript{\alpha} can function over a distance of 55 kb.

A primary mechanism by which enhancers exert control over recombination is by activating transcription from germline promoters (11, 12). Germline transcription can then disrupt chromatin structure (36) and distribute histone modifications such as H3K4me3 that are crucial for recruitment and activation of the recombinase (25–27, 37). Tcrd gene recombination likely depends on the activities of distinct promoters that control transcription and accessibility of V\textsubscript{\alpha}, D\textsubscript{\alpha}, and J\textsubscript{\alpha} gene segments (8). In this context, the distinct recombination defects displayed by TRDV4, TRDV5, and TRDV2-2 in E\textsubscript{\alpha}–/– fetal thymocytes are most easily explained by a hierarchy of promoter and accessibility defects on these alleles. We suggest that defective D2-to-J1 rearrangement predominantly reflects a major defect in J1 accessibility on E\textsubscript{\alpha}-deficient alleles. We suspect, however, that there is only a modest defect in D2 accessibility; in this way, residual D2 accessibility can support levels of V-to-D2 rearrangement that vary largely as a function of V segment promoter activity and accessibility. Thus, for the E\textsubscript{\alpha}-independent TRDV2-2, the frequency of V-to-D2 rearrangement on E\textsubscript{\alpha}-deficient alleles is near that on WT alleles, whereas for the E\textsubscript{\alpha}-dependent TRDV4, the frequency of V-to-D2 rearrangement is substantially reduced. Furthermore, after initial TRDV2-2-to-D2 rearrangement, we suspect that potent E\textsubscript{\alpha}-independent TRDV2-2 promoter activity can create substantial accessibility at both D2 and J1, providing the driving force for complete TRDV2-2-to-D2-J1 rearrangements on E\textsubscript{\alpha}-deficient alleles. In contrast, for the E\textsubscript{\alpha}-dependent TRDV4, reduced promoter activity on E\textsubscript{\alpha}-deficient alleles is unable to support TRDV4-D2-to-J1 rearrangement after initial TRDV4-to-D2 rearrangement. Promoter and accessibility defects for TRDV5 are similar to those for TRDV4, thereby leading to a similar recombination profile. We note that our model for accessibility defects on E\textsubscript{\alpha}–/– alleles is fully consistent with the unusual constellation of recombination intermediates previously detected on these alleles in adult thymocytes (6).

Although E\textsubscript{\alpha} can activate TRDV4 in fetal thymocytes, it cannot do so in adult thymocytes. Previous work has demonstrated that the inability of fetal V\textsubscript{\alpha}3 and V\textsubscript{\alpha}4 gene segments to rearrange in adult thymocytes is due to suppression mediated by local promoter sequences (28, 30). TRDV4 usage may be regulated similarly, mice deficient in basic-helix-loop-helix transcription factor E2A display dysregulated rearrangement of V\textsubscript{\alpha}3 and TRDV4 in adult thymocytes (29), implicating E2A as a suppressor of fetal V\textsubscript{\alpha} and V\textsubscript{\alpha} gene segments in adults. However, it is not known whether this represents a direct effect of E2A on V\textsubscript{\alpha} and V\textsubscript{\alpha} promoters. Deletion of a TRDV4 promoter E box resulted in elevated TRDV4 promoter activity (38), but this result was obtained in transient transfection experiments and has not been further studied or confirmed in vivo. As in previous studies examining fetal V\textsubscript{\alpha}3 gene rearrangement (30), we found that fetal TRDV4 rearrangement can be stimulated in adult DN thymocytes by artificially elevating histone acetylation through the use of HDAC inhibitor TSA. This suggests that the balance between histone acetyltransferase and HDAC activity at TRDV4 may regulate its developmental activation. Of particular interest, we found that the TRDV4 promoter displays unusually high levels of suppressive H3K9me2 in both adult and fetal thymocytes. Thus, in adult thymocytes, the TRDV4 histone modification profile is typical of a repressed gene, whereas in fetal thymocytes, it appears to be a mosaic of histone modifications typical of both activation and repression. Prior data may be interpreted to indicate that fetal V\textsubscript{\alpha} segment promoters are subject to a developmentally regulated suppressive mechanism in adult thymocytes (28–30). Our current data suggest that for TRDV4, a component of this suppression may be constitutive, and that even in fetal thymocytes, it may be primed for suppression in adults. Further studies will be required to better understand how the balance of additional suppressive and activating influences is modulated during development.

Acknowledgments

We thank Dr. Keji Zhao (National Institutes of Health) for ChIP protocol, Dr. Barry Sleckman (Washington University) for E\textsubscript{\alpha}–/– mice, Dr. Motonari Kondo (Duke University) for OP9-DL1 cells and for use of the AutoMACS, Dr. Mary Elizabeth Jones (Duke University) for advice with MACS, Dr. Keji Zhao (National Institutes of Health) for ChIP protocol, Dr. Yuan Zhuang for guidance on microdissection of OP9-DL1 cultures, Dr. M. S. Krangel for TCR-J1 accessibility. Of particular interest, we found that the TRDV4 promoter displays unusually high levels of suppressive H3K9me2 in both adult and fetal thymocytes. Thus, in adult thymocytes, the TRDV4 histone modification profile is typical of a repressed gene, whereas in fetal thymocytes, it appears to be a mosaic of histone modifications typical of both activation and repression. Prior data may be interpreted to indicate that fetal V\textsubscript{\alpha} segment promoters are subject to a developmentally regulated suppressive mechanism in adult thymocytes (28–30). Our current data suggest that for TRDV4, a component of this suppression may be constitutive, and that even in fetal thymocytes, it may be primed for suppression in adults. Further studies will be required to better understand how the balance of additional suppressive and activating influences is modulated during development.

Disclosures

The authors have no financial interests of interest.

References


