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Long-distance regulation of fetal V_δ gene segment TRDV4 by the *Tcrd* enhancer1,,2

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Abstract

Murine *Tcra* and *Tcrd* gene segments are organized into single genetic locus (*Tcra/Tcrd* locus) which undergoes V(D)J recombination in $CD4^-CD8^-$ double negative (DN) thymocytes to assemble *Tcrd* genes and in $CD4^+CD8^+$ double positive (DP) thymocytes to assemble *Tcra* genes. Recombination events are regulated by two developmental stage-specific enhancers, E_δ and E_α. Effects of E_α on *Trca/Tcrd* locus chromatin have been well documented, but effects of E_δ have not. In this regard, E_α acts over long distances to activate many V_α and J_α segments for recombination in DP thymocytes. However, in DN thymocytes it is unclear whether E_δ functions over long distances to regulate V_δ gene segments, or only functions locally to regulate D_δ and J_δ gene segments. Here we analyzed germline transcription, histone modifications and recombination on wild-type and E_δ-deficient alleles in adult and fetal thymocytes. We found that E_δ functions as a local enhancer whose influence is limited to no more than about 10 kb in either direction (including D_δ, J_δ and TRDV5 gene segments) in adult DN thymocytes. However we identified a unique long-distance role for E_δ promoting accessibility and recombination of fetal V_δ 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.

Introduction

The development of αβ and γδ T lymphocytes depends on the somatic assembly of T cell receptor (TCR) genes by V(D)J recombination (1). Among the four TCR genes, *Tcrd* and *Tcra* are uniquely organized into a single, complex genetic locus (the *Tcra/Tcrd* locus) that spans 1.6 megabases of murine chromosome 14 (2). *Tcra/Tcrd* locus recombination events occur according to a strict developmental program during thymocyte maturation, with *Tcrd* genes assembled in $CD4^-CD8^-$ double negative (DN) thymocytes and *Tcra* genes assembled in those thymocytes that progress to the $CD4^+CD8^+$ 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 (RAG) complex and by changes in locus organization that allow specific pairs of RSSs to undergo synapsis for recombination (3). How such changes are effected is only partially understood.

Two developmental stage-specific enhancers, E_δ and E_α, regulate the switch from *Tcrd* to *Tcra* rearrangement (4–7). E_δ is active in DN thymocytes; it promotes high level germline transcription from nearby D_δ and J_δ promoters and supports normal levels of fully V_δ-D_δ-J_δ

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rearranged genes (6, 8). However inefficient and incomplete (V_δ-D_δ, D_δ-D_δ, D_δ-J_δ) *Tcrd* gene assembly occurs even on E_δ-deleted alleles, implying that *Tcrd* recombination may be partially supported by additional *cis*-elements. E_α becomes active subsequently, in DP thymocytes; it provokes transcription from the T early α promoter upstream of the J_α segments, at a distance of 70 kb (9, 10). E_α- and T early α-dependent germline transcription across the J_α segments is essential for J_α accessibility and for V_α-to-J_α recombination at this stage (11, 12).

A pool of about 100 V gene segments is distributed across 1.5 megabases of DNA upstream of D_δ and J_α gene segments and supplies V genes for both the TCRδ and TCRα repertoires. A small subset of these V gene segments undergoes recombination to D_δ gene segments; sixteen have been classified as V_δ but a smaller number (TRDV2-2, TRDV5 and the four-membered TRAV15/TRDV6 family) tend to dominate the adult TCR δ repertoire (2, 13–15). In addition, one V_δ (TRDV4) rearranges specifically in fetal thymocytes. In contrast, the vast majority of the V gene segments may undergo recombination to J_α gene segments during subsequent *Tcra* gene assembly (14, 16).

Although the regulation of *Tcra/Tcrd* V gene segment usage is crucial for the development of distinct TCRδ and TCRα repertoires, the molecular basis for this regulation is only partly understood (1). Several V_δ gene segments are positioned relatively proximal to D_δ, J_δ and C_δ gene segments. However, prominent adult V_δ 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_δ gene segment accessibility depends solely on intrinsic features of V_δ gene promoters, or whether there are contributions from distant *cis*-regulatory elements as well. Regardless, it is clear that many additional *Tcra/Tcrd* locus V gene segments become transcriptionally active and accessible in DP thymocytes, particularly within the proximal 500kb of the V segment array (15). These changes do not occur on E_α-deficient alleles, demonstrating that E_α can influence V gene segment accessibility over very long distances.

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

To investigate a role for E_δ in regulating V_δ gene segments in DN thymocytes, we analyzed germline transcription, histone modifications, and recombination events on wild-type and E_δ-deleted alleles in adult and fetal thymocytes. These studies led us to identify a specific, long-distance role for E_δ 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 D_δ and J_δ gene segments.

Materials and Methods

Mice

The following mouse strains were used: 129 (Jackson Laboratory), *Rag2*$^{-/-}$ (20) (Jackson Laboratory) on a 129 background, $E_\delta^{-/-}$ (6) and $E_\delta^{-/-}$*Rag2*$^{-/-}$. $E_\delta^{-/-}$ mice were kindly provided by Barry Sleckman (Washington University, St. Louis MO). Strains carrying E_δ-deleted alleles were on a mixed background but carried the 129 *Tcra/Tcrd* locus. Adult thymi were harvested from three week old recombinase-deficient mice or four to six week old recombinase-sufficient mice. 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 (ChIP)

Chromatin was prepared as described (21, 22). Thymocytes ($0.5–10.0 \times 10^6$) were washed with Wash Buffer I (10 mM sodium butyrate, 5mM Na_3EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.1 mM PMSF, 0.1 mM benzamidine, pH7.0, in PBS without Mg^{2+} and Ca^{2+}) followed by Wash Buffer II (Wash Buffer I without Na_3EDTA). Washed cells were then lysed in 200–400 μl of 80 mM NaCl, 10 mM Tris-HCl pH8.0, 10 mM sodium butyrate, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 250 mM sucrose, 0.15 mM spermine, 0.5mM spermidine, 0.02% (vol/vol) NP40, 0.1 mM PMSF and 0.1 mM benzamidine for 5 min on ice. Nuclei were pelleted and washed once with 10 mM NaCl, 10 mM Tris-HCl pH8.0, 10 mM sodium butyrate, 3 mM $MgCl_2$, 1 mM $CaCl_2$ 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 pH8.0, 5 mM EDTAand 10 mM sodium butyrate. Sonication was then conducted on ice for eight cycles of 15 seconds on and 20 seconds 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% (vol/vol). Chromatin was then precleared with Protein A-Sepharose/salmon sperm DNA slurry (Millipore) and was subsequently incubated overnight at 4°C with anti-acetylated H3 (Millipore 06-599), anti-acetylated H4 (Millipore 06-598), anti-dimethylated H3K4 (Millipore 07-030), anti-trimethylated H3K4 (Millipore 04-745), anti-dimethylated H3K9 (Abcam ab1220), anti-trimethylated H3K27 (Millipore, 07-449) or control rabbit IgG (R&D Systems, ab-105-c). Protein A-Sepharose/salmon sperm DNA slurry was added for an additional 1 hr 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 β_2-microglobulin (*B2m*) was used to normalize ratios of bound/input in different samples. Analysis of *MageA2* was used to normalize immunoprecipitations using anti-dimethylated H3K9. Primers sequences are provided in Supplementary Table 1. 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 unfractionated thymocytes using TRIzol (Invitrogen) according to the manufacturer's instructions. Contaminating genomic DNA was removed by incubation with 1U 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 ^{32}P-labeled oligonucleotide probe. Primer and probe sequences are listed in Supplementary Table 1. Real-time PCR was carried out as described above; primers are listed in Supplementary Table 1.

PCR and Southern blot analysis of rearrangements

Total or sorted DN3 thymocytes were lysed by incubation in 10 mM Tris-HCl pH8.0, 150 mM NaCl, 10 mM EDTA, 0.4% (wt/vol) 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 ^{32}P-labeled oligonucleotide probes. Primer and probe sequences are provided in Supplementary Table 1.

PCR analysis of RSS cleavage

Thymocyte genomic DNA obtained from recombinase-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 recombinase-deficient mice. Loss of the RSS amplicon in recombinase-sufficient thymocytes can result from its rearrangement or its deletion due to rearrangement of upstream RSSs; loss of the neighboring amplicon reflects its deletion only. Unrearranged RSS was calculated as residual RSS amplicon/residual neighbor amplicon to specifically reflect RSS loss due to RSS rearrangement. Primer sequences are provided in Supplementary Table 1. PCR conditions were as described for ChIP analysis.

OP9-DL1 culture

Monolayers of OP9-DL1 cells (23) were cultured in MEM Alpha (GIBCO) supplemented with 20% fetal bovine serum (Atlanta Biologicals), 1×Penicillin-Streptomycin (GIBCO) and 1mM sodium pyruvate (GIBCO). Antibodies PE-Cy 5 anti-CD4 (553654), PE-Cy 5 anti-CD8a (553034), PE-Cy 5 anti-CD3e (553065), FITC anti-CD25 (553072) and PE anti-CD44 (553134) were purchased from BD-Pharmingen. Anti-CD117 magnetic beads (18757) were purchased from Stemcell Technologies. CD117$^+$ thymocytes were enriched by AutoMACS in Possels mode. Sorted DN1 (CD4$^-$CD8$^-$CD3$^-$CD117$^+$CD44$^+$CD25$^-$) thymocytes from adult 129 or E$\delta^{-/-}$ mice were then cultured on an OP9-DL1 monolayer in IMDM (GIBCO) supplemented with 5% fetal bovine serum, 1×penicillin/streptomycin, 1 mM sodium pyruvate, 55 μM 2-mercaptoethanol (GIBCO), 5 ng/ml rmFlt-3 Ligand (R&D Systems, 427-FL) and 5 ng/ml IL-7 (R&D Systems, 407-ML). After 14 days of culture in the presence or absence of 3 ng/ml trichostatin A (TSA, Sigma), DN3 cells (CD25$^+$CD44$^-$) were sorted for analysis.

Results

E_δ behaves as a local enhancer in adult DN thymocytes

In our previous studies we observed that the V_δ gene segments that dominate the adult TCRδ repertoire (TRDV2-2, TRDV5, TRAV15D-1, TRAV15D-2, 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*$^{-/-}$ mice (15). To investigate whether V_δ chromatin structure depends on E_δ, we used ChIP to monitor four histone modifications in thymocytes from *Rag2*$^{-/-}$ and $E_\delta{}^{-/-}$ *Rag2*$^{-/-}$ mice. On wild-type alleles, we found H3ac and H4ac to be high (>50% of *B2m*) from TRDD2 (D2) to TRDJ2 (J2) (Fig. 1B, C). As noted previously (15), H3ac and H4ac were present at moderate

(5-50% of *B2m*) to high levels at six dominant V_δ gene segments (TRDV2-2, TRDV5 and TRAV15 family) and at certain other V segments (TRAV2 and TRAV14 family). By the criteria noted above, we also detected moderate acetylation at TRAV17, TRDV4 and TRDD1. On E_δ-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, C).

H3 lysine 4 dimethylation (H3K4me2) is a chromatin mark that is distributed across active genes; 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 RAG2 protein during V(D)J recombination (25–27). Profiles of these histone modifications followed the trend of E_δ-dependence near D_δ and J_δ gene segments and E_δ-independence at more distal locations (Fig. 1D, E). However, unlike H3ac, H4ac and H3K4me3, we found that H3K4me2 was E_δ-independent at D2 and J1. Interpretation of this finding is difficult since 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_δ, D_δ and J_δ gene segments by semiquantitative RT-PCR (Fig. 2A). The results matched the chromatin data quite closely, as germline transcription at D2, TRDJ1 (J1) and J2 was reduced substantially on E_δ-deficient as compared to wild-type alleles, 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 approximately 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, as 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 E_δ-deficient alleles. Nevertheless, although H3K4me3 at TRDV5 was very low on wild-type alleles, it was still 5–10-fold over the IgG control in different experiments (data not shown), and, consistent with the transcription data, averaged 8.5-fold over $E_\delta^{-/-}$ alleles (Fig. 1E). Taken together, the chromatin and transcription data support the notion that E_δ functions as a local enhancer whose influence is limited to no more than about 10 kb in either direction in adult DN thymocytes.

E_δ regulates TRDV4 and TRDV5 chromatin in fetal thymocytes

The nearest V_δ gene segment upstream of E_δ 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 adult as has been described for $V_\gamma3$ and $V_\gamma4$ (28–31). To ask whether E_δ regulates TRDV4 chromatin we analyzed H3ac at the TRDV4 promoter using chromatin prepared from fetal day 17.5 (F17.5) thymocytes of *Rag2*$^{-/-}$ and $E_\delta^{-/-}$*Rag2*$^{-/-}$ mice. Consistent with the unique developmental profile of TRDV4 usage, TRDV4 displayed an 85% increase in H3ac in fetal as compared to adult thymocytes, whereas none of the other V_δ gene segments tested displayed similar increases (Fig. 3A). Moreover, elevated H3ac at TRDV4 in fetal thymocytes was clearly E_δ-dependent (Fig. 3B). H3ac at TRDV5 was E_δ-dependent at this stage as well (Fig. 3B).

To confirm the conclusions from chromatin analysis, we measured germline transcription of V_δ gene segments in fetal thymocytes of *Rag2*$^{-/-}$ and $E_\delta^{-/-}$*Rag2*$^{-/-}$ mice (Fig. 3C). Consistent with the chromatin data, TRDV4 and TRDV5 both displayed substantial reductions in germline transcripts on E_δ-deficient alleles, whereas there were no significant changes in germline transcription of TRDV2-2 and TRAV15. These results demonstrate that

E_δ supports TRDV4 and TRDV5 promoter activity and chromatin structure in fetal thymocytes.

E_δ regulates TRDV4 and TRDV5 recombination fetal thymocytes

Based on the above results we predicted that E_δ 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 wild-type alleles in F15.5, F17.5 and adult thymocytes (Fig. 4A). The *Tcrd* gene is unique among antigen 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) (Fig. 4A). 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, as amplicon sizes would be virtually indistinguishable regardless of its inclusion. Using this strategy, we detected complete (VDJ1) and partial (VD2) rearrangements of TRDV4 in wild-type 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 moreso in adult thymocytes (Fig. 4A). Thus, TRDV4 rearrangement was restricted to the fetal period.

Quantitative real-time PCR across V_δ RSSs was then used to assess RSS loss and thus the extent of V_δ rearrangement in fetal thymocytes. The results indicated that TRDV4 dominates V_δ rearrangement events in the fetal period, as it was rearranged on 52% of wild-type alleles (Fig. 4B). In contrast, TRDV2-2 was rearranged on 14% and TRDV5 on only 9% of wild-type alleles (Fig. 4B). TRAV15 rearrangement was not detectable using this approach, indicating that this V_δ family makes at best a very minor contribution to the fetal V_δ repertoire. Analysis of E_δ-deficient fetal thymocytes revealed TRDV4 rearrangement to be reduced by 60% (to 21% of alleles) (Fig. 4B). In contrast, reductions in TRDV2-2 and TRDV5 rearrangement were notapparent. Thus, E_δ is critical for high frequency TRVD4 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 E_δ-deficiency on the production of complete (VDJ1) and partial (VD2) fetal *Tcrd* rearrangement events (Fig. 4C). Complete rearrangements involving TRDV4 were abolished on E_δ-deficient alleles. Rather, the residual TRDV4 rearrangements on E_δ-deficient alleles were exclusively in the form of TRDV4-D2 rearrangement intermediates. A similar recombination defect was observed for TRDV5 (Fig. 4C). Since there was no apparent quantitative defect in TRDV5 recombination as assessed by RSS loss (Fig. 4B), this result may indicate a selective defect in TRDV5-D2-to-J1 rearrangement in E_δ-deficient fetal thymocytes. However, because the frequency of TRDV5 recombination is low even in wild-type fetal thymocytes, the assay for RSS loss may not have had sufficient accuracy to distinguish TRDV5 usage on wild-type and E_δ-deficient alleles (Fig. 4B).

In contrast to 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 wild-type and E_δ-deficient alleles (Fig. 4B). Given that D2-to-J1 rearrangement is dramatically impaired on E_δ-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 E_δ can stimulate H3ac at TRDV4 in fetal thymocytes but cannot do so in adult thymocytes (Figs. 1B, 3A). We wondered whether potential effects of E_δ at TRDV4 in adult thymocytes were counteracted by suppressive histone modifications specifically targeted to TRDV4. In this regard, the suppressive histone modification H3 lysine 27 trimethylation (H3K27me3) was shown to be elevated at proximal V_H gene segments in adult but not fetal pro-B cells (34) and to promote distal V_H gene recombination in adults (35). However, we detected only low levels of H3K27me3 at TRDV4 in adult DN thymocytes (data not shown). We did detect the suppressive histone modification 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 E_δ-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 trichostatin A (TSA) increased H3ac and H4ac at TRDV2-2, TRDV4 and TRDV5 (Fig. 6A, B), 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 day cultures of DN thymocytes with OP9-DL1 stromal cells. TSA treatment stimulated more than a ten-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 V_δ 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.

Discussion

Although more than ten years has passed since E_δ was first shown to regulate *Tcrd* locus recombination events (6), a detailed characterization of the regulation of *Tcrd* locus chromatin accessibility has been lacking. In this study we provide new insights into the influences of E_δ on *Tcrd* locus chromatin, and how these influences support the recombination of *Tcrd* gene segments. We found that E_δ acts relatively locally to regulate chromatin structure and transcription in the 20 kb region extending from TRDD2 to TRDV5 in adult DN thymocytes. A relatively local influence of E_δ on D_δ and J_δ chromatin can readily explain the previously described recombination defect in E_δ-deficient mice, with reductions in complete VDJ-rearrangements and increases in VD and DD rearrangement intermediates (6). However, although E_δ regulates the nearby TRDV5, it appears to play no role in activating more distant V_δ gene segments in adult DN thymocytes. Thus, with the exception of TRDV5, E_δ does not function to define *Tcra/Tcrd* locus V gene segments as V_δ 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_δ plays a distinct and unanticipated role in fetal thymocytes as a regulator of chromatin structure, transcription and

recombination of fetal V_δ gene segment TRDV4. As a consequence, complete rearrangements of TRDV4 and TRDV5 were absolutely dependent on E_δ. The data for TRDV4 indicate that, at least in fetal thymocytes, E_δ 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_δ, D_δ and J_δ gene segments (8). In this context, the distinct recombination defects displayed by TRDV4, TRDV5 and TRDV2-2 in $E_\delta^{-/-}$ 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_δ-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_δ-independent TRDV2-2, the frequency of V-to-D2 rearrangement on E_δ-deficient alleles is near that on wild-type alleles, whereas for the E_δ-dependent TRDV4, the frequency of V-to-D2 rearrangement is substantially reduced. Further, following initial TRDV2-2-to-D2 rearrangement, we suspect that potent E_δ-independent TRDV2-2 promoter activity can create substantial accessibility at both D2 and J1, providing the driving force for complete TRDV2-2-D2-J1 rearrangements on E_δ-deficient alleles. In contrast, for the E_δ-dependent TRDV4, reduced promoter activity on E_δ-deficient alleles is unable to support TRDV4-D2-to-J1 rearrangement following 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 $E_\delta^{-/-}$ alleles is fully consistent with the unusually constellation of recombination intermediates previously detected on these alleles in adult thymocytes (6).

Although E_δ can activate TRDV4 in fetal thymocytes, it cannot do so in adult thymocytes. Previous work has demonstrated that the inability of fetal $V_\gamma3$ and $V_\gamma4$ gene segments to rearrange in adult thymocytes is due to suppression mediated by local promoter sequences (28, 30). TRDV4 usage may be regulated similarly. Notably, mice deficient in basic-helix-loop-helix transcription factor E2A display dysregulated rearrangement of $V_\gamma3$ and TRDV4 in adult thymocytes (29) implicating E2A as a suppressor of fetal V_γ and V_δ gene segments in adults. However it is not known whether this represents a direct effect of E2A on V_γ and V_δ 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 $V_\gamma3$ 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_γ segment promoters are subject to a developmentally regulated suppressive mechanism in adult thymocytes (28–30). Our current data suggests that for TRDV4 at least 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

ChIP	chromatin immunoprecipitation
D2	TRDD2
DN	double negative
DP	double positive
E_{δ}	*Tcrd* enhancer
E_{α}	*Tcra* enhancer
H3ac	histone H3 acetylation
F15.5	fetal day 15.5
F17.5	fetal day 17.5
H4ac	histone H4 acetylation
H3K4me2	histone H3 lysine 4 dimethylation
H3K4me3	histone H3 lysine 4 trimethylation
H3K27me3	histone H3 lysine 27 trimethylation
H3K9me2	histone H3 lysine 9 dimethylation
HDAC	histone deacetylase
J1	TRDJ1
J2	TRDJ2
RSS	recombination signal sequence
TSA	trichostatin A

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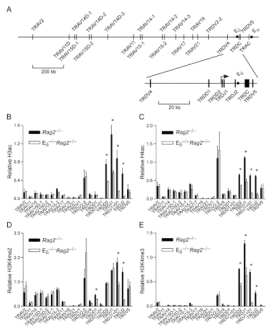

Figure 1.

Influence of Eδ 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_δ and E_α (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 $Rag2^{-/-}$ and $E_\delta{}^{-/-}Rag2^{-/-}$ thymocytes. TRDD1+01 and TRDJ1+01 are sites situated 1kb 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 one) 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,-1,-2,-3). The significance of differences between $E_\delta{}^{-/-}$ and wild-type were evaluated by two-tailed Student's t-test: *, P<.01.

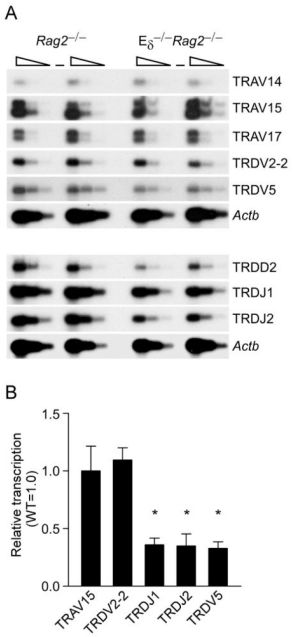

Figure 2.

Influence of E_δ on *Tcra/Tcrd* locus germline transcription in adult DN thymocytes. A. Germline transcription was measured by RT-PCR using serial three-fold dilutions of cDNA (wedges) prepared from four independent cDNA preparations from *Rag2*$^{-/-}$ and $E_\delta^{-/-}$*Rag2*$^{-/-}$ thymocytes. Two preparations for each genotype are analyzed in the top set of panels; two different preparations for each genotype are analyzed in the bottom set of panels. (−) no reverse transcriptase. 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 $E_\delta^{-/-}$*Rag2*$^{-/-}$ thymocytes. The data represent the mean ± SEM of five independent cDNA preparations for each genotype, all normalized to values for β-actin (*Actb*). The values for $E_\delta^{-/-}$*Rag2*$^{-/-}$ for each site were then expressed relative to those for *Rag2*$^{-/-}$, which were normalized to one. The significance of differences between $E_\delta^{-/-}$ and wild-type were evaluated by two-tailed Student's t-test: *, P<.05.

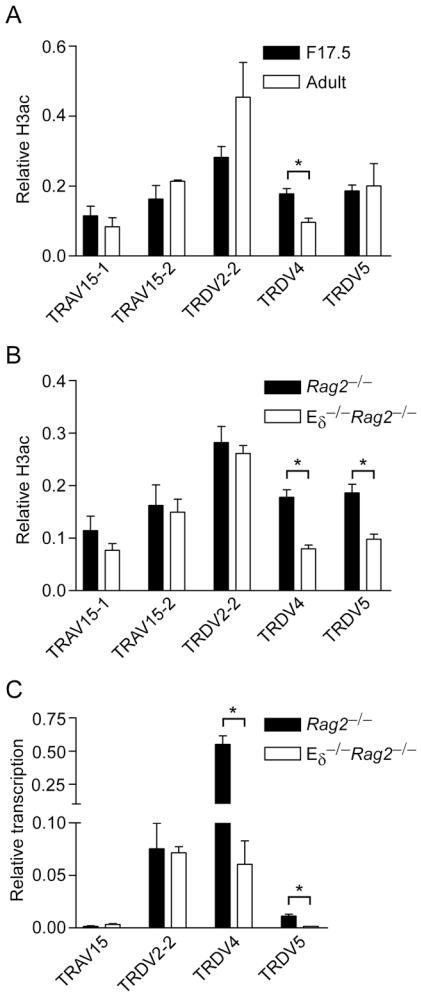

Figure 3.

Influence of E_δ 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 $E_\delta{}^{-/-}$*Rag2*$^{-/-}$ thymocytes. The 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 one) in each sample. C. Germline transcription was measured by quantitative real-time PCR using cDNA prepared from F17.5 *Rag2*$^{-/-}$ and $E_\delta{}^{-/-}$*Rag2*$^{-/-}$ thymocytes. The data represent the mean ± SEM of two independent cDNA preparations for each genotype, all normalized to values for *Actb*. *, P<.05 by two-tailed Student's *t*-test.

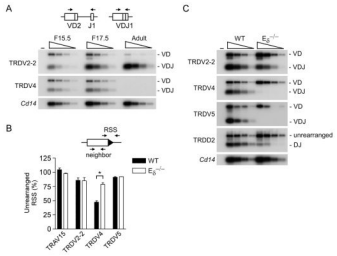

Figure 4.

Influence of E_δ 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 ^{32}P-labeled internal J1 oligonucleotide was used as a probe. *Cd14* PCR insured use of similar amounts of DNA. (−), no DNA. The data are representative of three independent experiments. B. RSS cleavage in genomic DNA of wild-type (WT) 129 and $E_\delta^{-/-}$ 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. The data represent the mean ± SEM of four and two independent genomic DNA preparations, respectively, from WT and $E_\delta^{-/-}$ F17.5 thymocytes. *, P<.05 by two-tailed Student's *t*-test. C. *Tcrd* rearrangement in fetal thymocytes of wild-type (WT) 129 and $E_\delta^{-/-}$ 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 ^{32}P-labeled internal J1 oligonucleotide probe. The data are representative of two to three independent experiments.

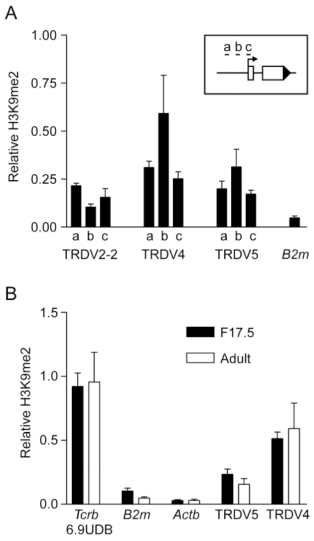

Figure 5.

H3K9 dimethylation of V_δ 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 V gene segment was analyzed at three sites (a, b, c, as diagrammed. Values of bound/input were expressed relative to *MageA2* (normalized to one). The data represent the mean ± SEM of three independent chromatin preparations. B. H3K9me2 was compared in chromatin prepared from F17.5 and adult *Rag2*$^{-/-}$ thymocytes. The 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 *Tcrb* locus (39); *B2m* and *Actb* served as negative control sites.

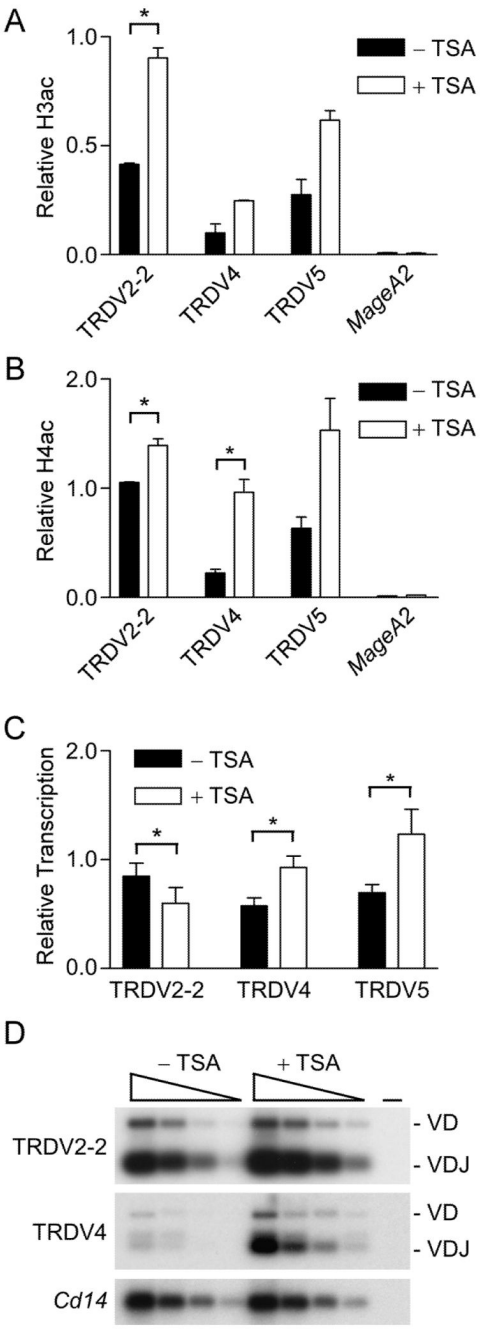

Figure 6.

Activation of V_δ gene segment chromatin and rearrangement using a HDAC inhibitor. H3ac (A) and H4ac (B) were measured by ChIP using chromatin prepared from adult *Rag2*$^{-/-}$ thymocytes that were cultured for 8 hrs with or without 3 ng/ml TSA. Values of bound/input were expressed relative to *B2m* (normalized to one) in each sample. The data represent the mean ± SEM of two to three independent chromatin preparations for each treatment. *, P<. 05 by two-tailed Student's *t*-test. C. Germline transcription was measured by quantitative real-time PCR using cDNA prepared from adult *Rag2*$^{-/-}$ thymocytes that were cultured for 8 hrs with or without 3 ng/ml TSA. The data represent the mean ± SEM of four independent cDNA preparations for each genotype, all normalized to values for *Actb*. *, P<.05 by two-

tailed paired Student's *t*-test. D. Adult 129 DN1 thymocytes were placed in culture for 14 days 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 ^{32}P-labeled internal J1 oligonucleotide was used as a probe. *Cd14* PCR insured use of similar amounts of DNA. (−), no DNA. The data is representative of three independent experiments.