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Specification of V$\delta$ and V$\alpha$ Usage by Tcra/Tcrd Locus V Gene Segment Promoters

Abani Kanta Naik,* Abbas Hawwari,†,1 and Michael S. Krangel*1

The Tcra/Tcrd locus undergoes V-D$\delta$-J$\delta$ rearrangement in CD4$^+$CD8$^-$ thymocytes to form the TCR$\delta$ chain of the $\gamma$+$\delta$ TCR and V-J$\alpha$ rearrangement in CD4$^+$CD8$^+$ thymocytes to form the TCR$\alpha$-chain of the $\alpha$$\beta$ TCR. Most V segments in the locus participate in V-J$\alpha$ rearrangement, but only a small and partially overlapping subset participates in V-D$\delta$-J$\delta$ rearrangement. What specifies any particular Tcra/Tcrd locus V gene segment as a V$\delta$ a V$\alpha$, or both is currently unknown. We tested the hypothesis that V segment usage is specified by V segment promoter-dependent chromatin accessibility in developing thymocytes. TRAV15/DV6 family V gene segments contribute to both the Tcrd and the Tcra repertoires, whereas TRAV12 family V gene segments contribute almost exclusively to the Tcra repertoire. To understand whether the TRAV15/DV6 promoter region specifies TRAV15/DV6 as a V$\delta$, we used gene targeting to replace the promoter region of a TRAV12 family member with one from a TRAV15/DV6 family member. The TRAV15/DV6 promoter region conferred increased germline transcription and histone modifications to TRAV12 in double-negative thymocytes and caused a substantial increase in usage of TRAV15/DV6 in double-negative thymocytes and caused a substantial increase in usage of TRAV15/DV6 in double-negative thymocytes. Our results demonstrate that usage of TRAV15/DV6 family V gene segments for Tcrd rearrangement in double-negative thymocytes is regulated, at least in part, by intrinsic features of TRAV15/DV6 promoters, and argue that Tcra/Tcrd locus V$\delta$ gene segments are defined by their local chromatin accessibility in CD4$^+$CD8$^-$ thymocytes. The Journal of Immunology, 2015, 194: 000–000.

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Abbreviations used in this article: DN, double negative; DP, double positive; E<sub>\alpha</sub>, Tcrd enhancer; ES, embryonal stem; H3ac, acetylated histone H3; H3K4me3, histone H3 lysine 4 trimethylation; RSS, recombination signal sequence.

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cis-regulatory elements, the Tcra enhancer (E5) and the Tcra enhancer, are known to control Tcra and Tcra recombination events, respectively (10, 11). E6 is activated in DN thymocytes and contributes to both V6-D6 and D6-J6 recombination (11). However, E6 cannot function over long distances (12) and in adult thymocytes its effects appear to be limited to D6, J6, and C6 gene segments, as well as TRDV4 (13). Although its influence extends as far as 55 kb to TRDV4 in fetal thymocytes, it does not provide accessibility to more distal V6 gene segments in fetal or adult thymocytes (13). Therefore, if V6 gene segments are specified by their chromatin accessibility in DN thymocytes, that accessibility must be directed by cis-elements other than E6.

The TRAV15/DV6 family is efficiently used in Tcra recombination and also participates in Tcra recombination. In strain 129 mice, this family is composed of four members: TRAV15D-1/DV6D-1, TRAV15D-2/DV6D-2, TRAV15-1/DV6-1, and TRAV15-2/DV6-2. The TRAV12 family, which is used almost exclusively in Tcra recombination, is composed of TRAV12D-1, TRAV12D-2, TRAV12D-3, TRAV12-1, TRAV12-2, and TRAV12-3 (14–17). TRAV15/DV6 but not TRAV12 family V gene segments show evidence of accessibility in DN thymocytes (6), suggesting a correlation between V segment accessibility and usage. However, a causal relation between accessibility and usage has not been established. In this study, we formally tested this hypothesis, hypothesizing that TRAV15/DV6 gene segment promoters provide local chromatin accessibility in DN thymocytes that enables efficient V segment usage in Tcra recombination. Specifically, we asked whether a TRAV15 promoter could confer on a TRAV12 gene segment the ability to rearrange to D6 gene segments in DN thymocytes. Our results show that the presence of a knock-in 1.74-kb promoter region of TRAV15-1/DV6-1 caused increases in transcription and chromatin accessibility of the TRAV12-2 gene segment in DN thymocytes, and significantly enhanced the usage of TRAV12-2 in Tcra recombination events.

Materials and Methods

Mice

The targeting construct, embryonal stem (ES) cell clones, and mice were generated at TaconicArtemis GmbH (Germany). A 1742-bp genomic region including upstream sequences, exon 1, intron 1, and 5 bp of exon 2 of TRAV15-1/DV6-1 (nt 945193-946934 of GenBank accession number NT_039614, http://www.ncbi.nlm.nih.gov/genbank) was used to replace the analogous portion of TRAV12-2 (nt 1007954–1009697) by homologous recombination. The targeting vector was generated by PCR amplification of the insert region, as well as 3.0- and 6.7-kb homology arms from 129/SvEvTac ES cell genomic DNA. The fragments were cloned into a proprietary vector backbone. The targeting vector was electroporated into a proprietary 129SvEvTac ES cell line (ART129S626), and targeted clones were isolated using positive (neomycin resistance) and negative (thymidine kinase) selection. The targeting frequency was 14.7%. The flippase recognition site flanked neomycin resistance gene was excised by transfection with pCAG-Flpe-pA. Successfully deleted clones were used to generate chimeric mice using blastocysts of superovulated BALB/c females. Chimeric mice were bred to 129 for several generations and were then intercrossed to generate homozygous 15-12 Rag2−/− mice, or were further bred to Rag2−/− mice on a 129 background to generate homozygous Rag2−/− mice. All mice were used in accordance with protocols approved by the Duke University Animal Care and Use Committee and the German Animal Welfare Act.

Germline transcription

RNA was prepared from total thymocytes of 3- to 4-wk-old Rag2−/− or homozygous 15-12 Rag2−/− mice and was immunoprecipitated using Abs specific for acetylated histone H3 (H3ac; Millipore, 06-599), trimethylated histone H3 lysine 4 (H3K4me3; ab1012; Abcam) or control rabbit IgG (ab105-c; R&D Systems) following a protocol described previously (13). Immunoprecipitated and input samples were quantified by SYBR green real-time PCR as described earlier. Experimental values were expressed as bound/input and were normalized to values for β2-microglobulin in each sample.

Cell sorting

DN and DP thymocytes were collected from 3- to 4-wk-old mice by cell sorting. To isolate the DN2/DN3 fraction, we stained total thymocytes with PE-conjugated Abs against CD4 (100410; BioLegend) and CD8 (100710; BioLegend), followed by 20 min on ice. Cells were washed twice with RPMI 1640 containing 10% FBS, after which CD4+ and CD8+ cells were removed using Dynabeads (Life Technologies) according to the manufacturer’s instructions. Remaining thymocytes were stained with FITC-conjugated Ab against CD25 (102006; BioLegend), allophycocyanin-conjugated Ab against CD44 (103012; BioLegend), PECy5-conjugated TAAD (A1310; Invitrogen), and PECy5-conjugated Abs against CD4, CD8, CD3e (103010; BioLegend), B220 (103210; BioLegend), Mac1 (15-0112-830; BioLegend), Gr1 (108410; BioLegend), and Ter119 (106210; BioLegend) for 20 min on ice. The stained thymocytes were washed and CD25+ PECy5− cells were obtained by cell sorting. DP thymocytes were collected by sorting as previously described (18).

Recombination assays

Genomic DNA was prepared according to standard procedures. RNA isolation and cDNA synthesis were performed as described earlier. To create plasmid standards for quantification of rearrangements, we amplified TRAV15-TRDC and TRA V12-DC products by PCR from 15-12 cDNA using primers listed in Supplemental Table I under the following conditions: 95°C for 5 min followed by 25 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 5 min. TRAV15-TRDC PCR products were then cloned into a TOPO PCR Cloning Vector PCR 2.1 (Life Technologies). The resulting plasmids were quantified by PicoGreen (Life Technologies), were serially diluted into Rag2−/− Tcrb transgenic genomic DNA to be used as recombination standards, and were further normalized to each other by SYBR green real-time PCR using primers that anneal to the PCR 2.1 vector backbone. TRAV12-D1 and TRAV15-D11 recombination was then measured in DN thymocyte genomic DNA samples and in the plasmid standards by TagMan real-time PCR using primers and probes listed in Supplemental Table I under the following conditions: 95°C for 10 min followed by 48 cycles of 95°C for 10 s, 62°C for 30 s. Values for genomic DNA amplification in wild-type and 15-12 were normalized to each other by SYBR green real-time PCR amplification of Cd14. Absolute numbers of rearranged products were then determined based on the calculated molar amount of input genomic DNA and plasmid standards.

TRAV12-D1 and TRAV15-D11 recombination was measured in cDNA samples by TaqMan PCR under conditions outlined earlier. Experimental values were normalized to those for β-actin (Actb) in different samples.

Results

To understand whether the TRAV15/DV6 promoter region enables TRAV15/DV6 participation in Tcra recombination in DN thymocytes, we used gene targeting to replace the promoter of a TRAV12 family member with one from a TRAV15/DV6 family member. Previous studies had shown that TRAV15/DV6 family members (then called ADV7) participate in Tcra rearrangement, whereas TRAV12 family members (then called ADV8 or Vo8) participate in Tcra rearrangement almost exclusively (14–17). TRAV15-1/DV6-1 and TRAV12-2 are separated by only 62 kb at a distance of >500 kb from D6 (Fig. 1A). We replaced a 1.74-kb region of TRAV12-2 with the equivalent portion of TRAV15-1/DV6-1, so that the hybrid gene contained the following TRAV15-1/DV6-1 sequences: 1460 bp of intron 1, and 5 bp of exon 2 (Fig. 1B–D). The intron and splice sites of TRAV15-1/DV6-1 were kept intact to minimize the likelihood

Chromatin immunoprecipitation

Chromatin was prepared from total thymocytes of 3- to 4-wk-old Rag2−/− or homozygous 15-12 Rag2−/− mice and was immunoprecipitated using Abs specific for acetylated histone H3 (H3ac; Millipore, 06-599), trimethylated histone H3 lysine 4 (H3K4me3; ab1012; Abcam) or control rabbit IgG (ab105-c; R&D Systems) following a protocol described previously (13). Immunoprecipitated and input samples were quantified by SYBR green real-time PCR as described earlier. Experimental values were expressed as bound/input and were normalized to values for β2-microglobulin in each sample.
that splicing of the hybrid gene would be disrupted. The knock-in mouse line generated (called 15-12) has a wild-type version of TRAV15-1/DV6-1 and the TRAV15-1/DV6-1:TRA V12-2 hybrid gene located 62 kb downstream.

We asked whether the hybrid TRAV12-2 gene segment was regulated differently than the wild-type TRAV12-2 gene segment by analyzing germline transcription and chromatin modifications of both gene segments on a \( \text{Rag2}^{2/-} \) background. TRAV12-2 germline transcription in \( \text{Rag2}^{2/-} \) DN thymocytes was significantly higher on 15-12 as compared with wild-type alleles, whereas TRAV15 family and total C\( _d \) transcripts were comparable (Fig. 2A). Small increases in germline transcription of TRAV4-3, 29 kb upstream of TRAV12-2, and of TRAV9-3, 5 kb downstream of TRAV12-2, were not statistically significant (Fig. 2A).

We assessed H3ac and histone H3 lysine 4 trimethylation (H3K4me3) as measures of active chromatin that are thought to reflect transcription and accessibility of Ag receptor gene segments to the RAG recombinase (19, 20). Transcription-dependent H3K4me3 deposition has been tied directly to RAG-mediated recombination because this mark has been shown to facilitate RAG2 protein binding to chromatin via its plant homeodomain finger and to facilitate RAG2 catalytic activity (21–23). We prepared chromatin from DN thymocytes of \( \text{Rag2}^{2/-} \) mice carrying wild-type or 15-12 alleles and immunoprecipitated H3ac or H3K4me3 chromatin followed by PCR using TRAV12-2 primers. In comparison with TRAV12-2 on wild-type alleles, TRAV12-2 on 15-12 alleles showed significantly greater levels of H3ac (Fig. 2B) and H3K4me3 (Fig. 2C). Thus, the TRAV15-1/DV6-1 promoter region conferred to the hybrid gene segment elevated germline transcription and histone modifications that are typical of TRAV15-1/DV6-1 in DN thymocytes (6).

We determined whether these promoter-dependent changes in germline transcription and histone modifications translated to changes in TRAV12-2 recombination on 15-12 alleles. To accomplish this, we first amplified and cloned PCR products representing TRAV12-TRDC and TRAV15/DV6-TRDC recombination events from 15-12 thymus cDNA. We then used the resulting plasmids as standards to design a highly quantitative PCR assay to measure TRAV12 and TRAV15/DV6 recombination to TRDJ1 in genomic DNA of wild-type and 15-12 DN thymocytes. We confirmed a dramatic difference between TRAV15/DV6 and TRAV12 rearrangement to TRDJ1 in DN thymocytes of 129 mice, because the former displayed 387 rearrangements per 10,000 genomes, whereas the latter displayed only 6 rearrangements per 10,000 genomes (Fig. 3A). As expected, TRAV15/DV6-TRDJ1 products were not significantly different in 15-12 as compared with wild-type DN thymocytes. However, TRAV12-TRDJ1 products were ∼24-fold more abundant in 15-12 as compared with wild-type (Fig. 3A). Although the frequency of TRAV12-TRDJ1 rearrangement in 15-12 thymocytes was only ∼1/3 that of TRAV15/DV6-TRDJ1, we note that four TRAV15/DV6-TRDJ1 family members may contribute to the TRAV15/DV6-TRDJ1 PCR signal. By contrast, only the hybrid TRAV12-2 gene should contribute to the TRAV12-TRDJ1 PCR signal. Thus, the activation of TRAV12-TRDJ1 rearrangement in 15-12 thymocytes is substantial.

We confirmed the upregulation of TRAV12-2 usage in 15-12 DN thymocytes by analysis of cDNA prepared from wild-type and 15-12 mice (Fig. 3B). The results showed that TRAV15/DV6-TRDJ1 transcripts were comparable in wild-type and 15-12 DN thymocytes, whereas TRAV12-TRDJ1 transcripts were ∼4.5-fold higher in 15-12 than in wild-type samples. The smaller increase in TRAV12 usage in cDNA as compared with genomic DNA could reflect perturbations in hybrid gene transcription, splicing, or transcript stability. Nevertheless, the overall trend was consistent with the more direct and more quantitative analysis of rearrangement in genomic DNA (Fig. 3A). To determine whether the TRAV12-TRDJ1 recombination products in 15-12 cDNA samples originated from the hybrid gene, we used a nested PCR approach. We first amplified TRAV15-1/DV6-1-TRDC products from wild-type and 15-12 mice.

![FIGURE 1. Gene targeting to create TRAV12-2 with a TRAV15-1/DV6-1 promoter region.](http://www.jimmunol.org/)

(A) Schematic of the Tcra/Tcrd locus, with TRAV15-1/DV6-1 in red and TRAV12-2 in blue. N, NcoI; neo, neomycin resistance; S, Sbf1; TK, thymidine kinase. Small triangles denote flipase recognition target sites. (B) Targeting strategy, with the introduced portion of TRAV15-1/DV6-1 in red and the TRAV12-2 in blue. (C) Southern blot analysis of genomic DNA from control (C) and correctly targeted (T) ES cells. (D) PCR typing of 129 and 15-12 alleles in tail DNA.
FIGURE 2. TRAV12-2 accessibility on wild-type and 15-12 alleles in DN thymocytes. (A) TRAV12-2 germline transcription compared with that for TRAV4-3, TRAV9-3, the TRAV15/DV6 family, and C8, in cDNA prepared from Rag2<sup>-/-</sup> and 15-12 Rag2<sup>-/-</sup> thymocytes. Results were normalized to Actb and for each amplicon the results for 15-12 alleles were expressed relative to those for wild-type alleles (WT = 1). Results represent the mean ± SEM of four to five WT and four 15-12 samples. (B) TRAV12-2 H3 acetylation (H3Ac) in chromatin prepared from Rag2<sup>-/-</sup> and 15-12 Rag2<sup>-/-</sup> thymocytes. Results were normalized to those for the β2-microglobulin promoter and represent the mean ± SEM of three WT and three 15-12 samples. (C) TRAV12-2 H3 K4 trimethylation (H3K4me3) measured as in (B). Results represent the mean ± SEM of three WT and four 15-12 samples. *p < 0.05 two-tailed Student t test.

cDNA samples using a TRAV15-1/DV6-1 exon 1 primer that recognizes both the wild-type TRAV15-1/DV6-1 and the hybrid gene. Gel-purified PCR products were then used as templates for second-round amplification with TRAV12 and TRDJ1 primers. The results showed that TRAV12-TRDJ1 recombination products were only detected in 15-12 samples (Fig. 3C), implying that TRAV12 usage was driven by the hybrid gene.

Discussion

Our findings demonstrate that usage of TRAV15/DV6 family V gene segments for Tcra rearrangement in DN thymocytes is regulated, at least in part, by intrinsic features of TRAV15/DV6 promoters. Because the TRAV15-1/DV6-1 promoter region conferred increased germline transcription and histone modifications to TRAV12-2 in DN thymocytes, our data offer the best evidence yet that Tcra/Tcrl locus V6 gene segments are defined by the accessibility of their local chromatin environment to the RAG recombinase in DN thymocytes. At least in the case of TRAV15/DV6 family V gene segments, this accessibility is independent of E6 (13). Thus, accessibility must be determined solely by local promoter sequences, or by local promoter sequences together with undiscovered long-distance regulatory elements. Our experiments also rule out any specificity contributed by RSSs or chromosomal position, at least in the case of TRAV15/DV6.

Although the developmental regulation of gene segment usage at Ag receptor loci has been the subject of intensive investigation for many years, mechanistic studies of V gene segment recombination have been rather limited. Deletion of the Vβ13 promoter substantially impaired VB13 rearrangement, implicating the V gene segment promoter as an important determinant of V gene rearrangement in vivo (24). A recent study assessed the molecular correlates of V8 usage and supported major roles for RSS quality and an accessible chromatin environment, as defined by RNA polymerase II and active histone marks such as H3Ac and H3K4me3 (25). Similar conclusions were drawn regarding VH usage, although for a subset of VH gene segments, proximity to CTCF and cohesin binding sites seemed critical (26). Nevertheless, we are aware of only one set of studies that directly examined the parameters accounting for developmentally programmed usage of V gene segments. That work showed that within the Tcrg Cγ1 locus, usage of Vγ7 genes in early fetal thymocytes is determined by their relative positioning, whereas their usage in adults is determined by their promoter sequences (27–29). Although our data rule out a role for chromosomal position in specifying TRAV15/DV6 usage, chromosomal positioning near D6 could contribute to exclusive use of some V6 gene segments in Tcrl rearrangements.

TRAV15/DV6 promoters could enable efficient recombination to D6 gene segments by hosting the binding of specific transcription factors that mediate their activation at the DN stage. In addition, these promoters could efficiently mediate looping that positions TRAV15/DV6 family members near D6 gene segments in three-dimensional space in DN thymocytes; such positioning should be facilitated by the relatively contracted conformation of the Tcra/Tcrl locus in DN thymocytes (30). In either case, we note that TRAV15/DV6 family promoter regions display remarkably high levels of DNA sequence conservation as compared with some

FIGURE 3. TRAV15/DV6 and TRAV12 rearrangement in DN thymocytes. (A) TRAV15/DV6-TRDJ1 and TRAV12-TRDJ1 rearrangements in genomic DNA isolated from wild-type (WT) and 15-12 DN thymocytes were quantified by PCR. Experimentally determined values were normalized to those for Cdi4 and then to those for quantified plasmid standards carrying cloned rearrangements. Results are expressed as the mean ± SEM of four WT and five 15-12 samples. (B) TRAV15/DV6-TRDJ1 and TRAV12-TRDJ1 rearrangements in cDNA samples prepared from DN thymocytes of WT and 15-12 mice were quantified by PCR. Experimentally determined values were normalized to those for Actb, and the results for 15-12 were then expressed relative to those for WT (WT = 1). Results are expressed as the mean ± SEM of four WT and five 15-12 samples. (C) TRAV12-TRDJ1 rearrangements in TRAV15/DV6-TRDC PCR products of WT and 15-12 mice. TRAV15/DV6-TRDC PCR products were amplified from cDNA samples prepared from WT and 15-12 DN thymocytes. Gel-purified PCR products were then used as templates for TRAV12-TRDJ1 PCR. Results are expressed as the mean ± SEM of four WT and four 15-12 samples. *p < 0.05 two-tailed Student t test.

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other V gene segment families. The 2.7-kb regions upstream of TRAV15 family exons 1 display 62% homology with each other, whereas the equivalent regions of TRAV4, TRAV12, TRAV13, and TRAV14 family members show 19, 46, 18, and 15% conservation, respectively. These conserved TRAV15/DV6 sequences are likely to recruit a common array of factors that specify their distinct developmental program.

Given that CTCF and cohesin bind to many Tcra/Tcra locus V segment promoters and are important regulators of Vα-δα recombination (31, 32), the disposition of CTCF and cohesin binding in the vicinity of TRAV15-1/DV6-1 and TRAV12-2 is of some interest. Notably, in both DN and DP thymocytes, CTCF and cohesin bind strongly to a promoter site ~100 bp upstream of the first TRAV12-2 exon and to a second site located 1.8 kb further upstream (32). In contrast, the TRAV15-1/DV6-1 is devoid of nearby CTCF and cohesin binding. Our promoter replacement eliminated the binding site proximal to the first TRAV12-2 exon, but left the distal site intact and introduced no new binding sites for CTCF and cohesin. Thus, CTCF and cohesin binding cannot readily explain the ability of the TRAV15-1/DV6-1 promoter to confer Vδ status to TRAV12-2. Consistent with this, Dδ6 and Jδ segments and Eδ also lack substantial binding of CTCF and cohesin (32). These considerations do not eliminate the possibility that the TRAV15-1/DV6-1 promoter could regulate recombination in DN thymocytes in part by facilitating long-distance looping. However, this looping would likely be independent of CTCF and cohesin, and would likely occur at quite low frequency, because we have been unable to detect contact between Eδ and distant Vδ gene segments by chromosome conformation capture approaches. Further work will be required to more fully understand the unique features of TRAV15/DV6 promoters that confer Vδ status to linked V gene segments.

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Disclosures

The authors have no financial conflicts of interest.

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