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Regulation of TCRβ Allelic Exclusion by Gene Segment Proximity and Accessibility

Hrisavgi D. Kondilis-Mangum,* Han-Yu Shih,* Grace Mahowald,† Barry P. Sleckman,† and Michael S. Krangel*

Ag receptor loci are regulated to promote allelic exclusion, but the mechanisms are not well understood. Assembly of a functional TCR β-chain gene triggers feedback inhibition of Vβ-to-DJβ recombination in double-positive (DP) thymocytes, which correlates with reduced Vβ chromatin accessibility and a locus conformational change that separates Vβ from DJβ gene segments. We previously generated a Tcrb allele that maintained Vβ accessibility but was still subject to feedback inhibition in DP thymocytes. We have now further analyzed the contributions of chromatin accessibility and locus conformation to feedback inhibition using two novel TCR alleles. We show that reduced Vβ accessibility and increased distance between Vβ and DJβ gene segments both enforce feedback inhibition in DP thymocytes. The Journal of Immunology, 2011, 187: 6374–6381.

Abbreviations used in this article: 3D-FISH, three-dimensional fluorescence in situ hybridization; DN, double-negative; DP, double-positive; Eκ, Tcra enhancer; ES, embryonal stem; HKκ4me3, histone H3 lysine 4 trimethylation; LM-PCR, ligation-mediated PCR; neo, neomycin-resistance; PDκ1, promoter Dκ1; Rag2−/−, Rag2-deficient; RSS, recombination signal sequence; SE, signal end; TD-qPCR, touchdown quantitative PCR; ig, transgene.

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Vβ1 NT alleles only, but no data evaluated whether altered Vβ10 recombination reflected a loss of feedback inhibition in DP thymocytes as opposed to dysregulated rearrangement in DN thymocytes. Another study simply inserted a Vβ gene segment just upstream of DJβ gene segments (19). Although allelic exclusion was perturbed at the level of Vβ recombination, whether this reflected a loss of feedback inhibition in DP thymocytes was not evaluated in this study either. Bates et al. (20) generated a modified Igh allele in which a Vβ gene segment was introduced just upstream of DH genes. This allele clearly displayed a disruption of feedback inhibition in pre-B cells. However, as the genetic manipulation moved the Vβ into an accessible chromatin domain and also modulated distance, the individual effects of accessibility and distance could not be distinguished.

Jackson et al. (21) previously generated a Tcrb allele in which Vβ accessibility was maintained in DP thymocytes by introducing the Tcra enhancer (Eα) into the middle of the Vβ array (EoKl allele). Despite accessible Vβ chromatin, feedback inhibition of Vβ-to-DJβ recombination was maintained in DP thymocytes, indicating that parameters other than chromatin accessibility must be essential to enforce feedback inhibition in DP thymocytes. We have now further analyzed contributions of gene segment accessibility and proximity to feedback inhibition through the generation of two novel TCR alleles. Our results establish that reduced RSS accessibility and increased distance between RSSs both contribute to feedback inhibition of Vβ-to-DJβ recombination in DP thymocytes.

Materials and Methods

Mice and gene targeting

Wild-type 129, Rag2-deficient (Rag2+/-) (22), and Rag2+/- mice containing a rearranged Tcrb transgene (tg) (23) were purchased from Taconic Farms. EoKL, EoK1b, Tcrb+/-, and EoK1b Tcrb+/- mice were previously described (21). All mice were used in accordance with protocols approved by the Duke University and Washington University Animal Care and Use Committees.

ΔEoKI mice were generated as follows: DJβ1 and homology arms were PCR amplified using Pfu Turbo (Stratagene) and cloned using a TOPO Cloning kit (Invitrogen, Carlsbad, CA). DJβ1 was PCR amplified from a plasmid carrying promoter Dβ1 (PD1) and a Dβ1-Jβ1.1 rearrangement (5′ end at nt 154066 and 3′ end at nt 154066 of GenBank file MMAE000665), and H3K4me3 antibody (04-745, Millipore) and normal rabbit IgG (AB-105-C; R&D Systems). All samples were resuspended in 0.7% agarose gel electrophoresis, and transferred to a nylon membrane. Blots were hybridized with 32P-labeled probes listed in Supplemental Table I. Probe-to-probe distances were calculated as previously described (25).

Chromatin immunoprecipitation

Chromatin was prepared from primary thymocytes of 2- to 3-wk-old mice by small-scale micrococcal nuclease digestion as previously described (27). Immunoprecipitations were performed using anti-H3K4me3 (04-745, clone MC5315; Millipore) and normal rabbit IgG (AB-105-C; R&D Systems). All samples were resuspended in 0.7% agarose gel electrophoresis, and transferred to a nylon membrane, and detected by hybridization with γ32P-labeled oligonucleotide probes. CD4+ cells were sorted to at least 95% purity using a DiVa cell sorter (BD Biosciences); analysis was with CellQuest software (BD Biosciences).

Germline transcription

Approximately 2 × 107 primary thymocytes were resuspended in 1 ml TRIzol (Invitrogen), and RNA was isolated according to the manufacturer’s instructions. cDNA was synthesized using the Super Script III kit (Invitrogen) using up to 2 μg purified RNA. Transcripts were quantified by SYBR Green Real-Time PCR using a LightCycler 480 (Roche). All quantitative PCR amplifications used a touchdown strategy (touchdown quantitative PCR [TD-qPCR]) in which annealing temperature was reduced gradually from 65 to 58°C over 10 cycles followed by 40 cycles at 62°C. Primers are listed in Supplemental Table I.

Coding joints

Genomic DNA isolated from sorted DN3 and DP thymocytes was amplified by touchdown PCR as follows: 5 min at 94°C, 31–35 cycles of 30 s at 94°C, and 0.1 mM EDTA; input samples were further diluted 1:50. Bound and input samples were quantified using 2 μl sample by SYBR Green Real-Time PCR using a LightCycler 480 (Roche). All quantitative PCR amplifications used a touchdown strategy (touchdown quantitative PCR [TD-qPCR]) in which annealing temperature was reduced gradually from 65 to 58°C over 10 cycles followed by 40 cycles at 62°C. Primers are listed in Supplemental Table I.

RSS retention

Generation of Cre-KI locus conformation

Three-dimensional fluorescence in situ hybridization

Bacterial artificial chromosome clones 75P5 (5′ Tcrb probe) and 203HS (3′ Tcrb probe) were directly labeled and used for three-dimensional fluorescence in situ hybridization (3D-FISH) as previously described (25). Probe-to-probe distances were calculated as previously described (25). Only nuclei with two distinguishable signals for both alleles were analyzed. Statistical tests were performed using Prism 3.0 (GraphPad).

Results

EoKI locus conformation

Previous studies of mice carrying Tcrb alleles with an introduced Eα (EoKI; Fig. 1A) indicated that elevation of Vβ accessibility, by itself, could not subvert feedback inhibition of Vβ-to-DJβ recom
bination in DP thymocytes. We hypothesized that recombination might remain suppressed on accessible EαKI alleles if, like wild-type alleles, they were decontracted in DP thymocytes. To assess this, we used 3D-FISH to measure the distance between the 5′ and 3′ ends of wild-type and EαKI Tcrb alleles in recombinase-deficient DN and DP thymocytes (Fig. 2). Consistent with previous experiments (16), we found that, on average, wild-type alleles were contracted in DN thymocytes and decontracted in DP thymocytes. The behavior of EαKI alleles was indistinguishable from wild-type, indicating that they decontract in DP thymocytes despite the presence of Eα and an accessible chromatin configuration. Therefore, to formally test whether the distance between accessible Vβ and DJβ gene segments limited Vβ-to-DJβ recombination in DP thymocytes, we generated and characterized two novel TCR locus alleles that approximated accessible Vβ and DJβ gene segments in DP thymocytes.

Regulation of the DJEαKI allele

We used homologous recombination to introduce a cassette containing PDβ1 and a rearranged Dβ1Jβ1.1 (DJβ1KI) ∼1.0 kb 3′ of the Vβ13 RSS on the EαKI allele (DJEαKI allele; Fig. 1A). Mice homozygous for the DJEαKI allele displayed normal thymocyte development as assessed by cell number and expression of cell-surface markers CD4, CD8, CD25, and CD44 (data not shown).

We addressed chromatin accessibility on the DJEαKI allele by introducing it onto Rag2-deficient (Rag2−/−) and Rag2−/− × Tcrb tg backgrounds for analysis of steady-state germline transcripts and histone modifications in DN and DP thymocytes. Like DJβ1 transcripts on wild-type alleles, DJβ1KI transcripts on DJEαKI alleles were of comparable abundance in DN and DP thymocytes (Fig. 3A, right panel). Moreover, like nucleosomes at the Dβ1 RSS on wild-type alleles, those at the DJβ1KI RSS on DJEαKI alleles were H3K4me3 modified in DN thymocytes and displayed increased H3K4me3 in DP thymocytes (Fig. 3B, right panel). Hence, the DJβ1KI RSS appears to reside in accessible chromatin in both DN and DP thymocytes of DJ EαKI mice.
As documented previously (21), germline transcription of Vβ13 is downregulated on transition from DN to DP on wild-type alleles but is upregulated on EkoI alleles (Fig. 3A, center panel). Unexpectedly, we found that introduction of the D13KI blunt the effect of EkoI on Vβ13 in DjeEkI DP thymocytes, such that Vβ13 transcripts were upregulated as compared with wild-type DP thymocytes, but were no more abundant in DjeEkI DP than in DjeEkI DN thymocytes. This might reflect a suppression of transcription due to competition between PD1 and Actb promoters or an effect of the D13KI on Vβ13 transcript stability. The D13KI also unexpectedly suppressed H3K4me3 at the Vβ13 RSS in both DN and DP thymocytes (Fig. 3B, center panel). Given these results, we also analyzed accessibility at Vβ8.1, which lies 5 kb upstream of Vβ13. Unlike Vβ13, the upregulation of Vβ8.1 transcription in EkoI DP thymocytes was maintained in DjeEkI DP thymocytes (Fig. 3A, left panel). Moreover, the upregulation of Vβ8.1 H3K4me3 in EkoI DP thymocytes was only partly suppressed by the D13KI (Fig. 3B, left panel). Taken together, the transcription and chromatin data suggest that Vβ13 is moderately accessible and that Vβ8.1 is highly accessible in DjeEkI DN and DP thymocytes.

To analyze Vβ-to-DJβ recombination, we prepared genomic DNA from purified DN3 and DP thymocytes of EkoI and DjeEkI mice, amplified with Vβ13 and Jβ1.1 primers, and distinguished D13KI from endogenous DJβ1.1 rearrangement using a DJβ1-specific probe (Fig. 4A). Vβ13-to-DJβ recombination was readily detected in sorted DN3 and DP thymocytes of DjeEkI mice but not in EkoI controls. To measure the frequency of DJβ1 recombination, we quantified residual unrearranged Vβ13 and DJβ1 RSSs in DP thymocyte genomic DNA (Fig. 4B, center and right panels). We found that ~70% of the DJβ1KI and Vβ13 RSSs were lost in DjeEkI DP thymocytes. These losses likely reflect recombination to DJβ1 as well as recombination to the endogenous Dβ gene segments that would delete Vβ13 and DJβ1.

We also analyzed DJβ1 recombination by genomic Southern blot of EcoRI-digested wild thymus DNA (Fig. 4C). As compared with DjeEkI kidney (lane 2), a Vβ13 probe detected substantial loss of DNA carrying unrearranged Vβ13 and DJβ1KI and detected two major and several minor rearranged fragments (lane 3). However, the predicted 8.0 kb fragment representing Vβ13-DJβ1 rearrangement was not detected. The additional rearranged fragments may represent excision circles carrying signal joints generated by rearrangement of upstream Vβ segments to DJβ1KI (e.g., Vβ8.1 = 6.7 kb, Vβ8.2 = 5.5 kb, Vβ8.3 = 3.0 kb, Vβ5.1 = 4.2 kb, and Vβ5.2 = 4.6 kb), as well as DJβ1KI signal end (SE) recombination intermediates (2.5 kb), all of which would hybridize to the Vβ13 probe. Consistent with the former, we detected Vβ8.1-to-DJβ1KI recombination using a PCR strategy (Fig. 4A). Excision circles and SE intermediates generated in DN thymocytes should be undetectable by genomic Southern blot of whole thymus because they would be diluted by the proliferative burst that accompanies the DN to DP transition. The apparent abundance of Vβ13-containing excision circles and DJβ1KI SE intermediates in DjeEkI thymocytes suggested that they were generated by recombination events occurring in DP rather than in DN thymocytes.

To test directly for Vβ13 and DJβ1KI recombination in DP thymocytes, we used LM-PCR to detect SE recombination intermediates in sorted thymocyte subpopulations (Fig. 4D). Because this assay cannot distinguish DJβ1KI from endogenous Dβ1 SEs, we evaluated DJβ1KI SEs by comparison of DjeEkI to EkoI samples. As expected, 5′ D13, Vβ13 and Vβ8.1 SEs were readily detected in EkoI DN thymocytes but were barely detected in EkoI DP thymocytes. However, these SE intermediates were all readily detected in DjeEkI DP thymocytes (Fig. 4D). In contrast, control 5′ D13 SEs were undetectable in DjeEkI DP thymocytes, indicating a selective loss of feedback inhibition involving DJβ1KI and upstream Vβ gene segments.

To formally demonstrate that DJβ1KI rearrangements in DP thymocytes occurred chromosomally rather than on excision circles generated by Vβ-to-endogenous Dβ recombination in DN thymocytes, we analyzed Vβ-to-DJβ1KI recombination in thymocytes of DjeEkI mice that express a Tcrb tg. Feedback inhibition by such transgenes specifically suppresses Vβ-to-DJβ1 recombination and the excision circles generated by these recombination events (21). Indeed, increased retention of a DNA segment situated 5′ of DJβ1, normally lost during Vβ-to-endogenous DJβ recombination, was apparent in wild-type, EkoI, and DjeEkI Tcrb tg DP thymocytes (Fig. 4B, left panel). However, suppression of Vβ13 and DJβ1RSS loss was only partial in DjeEkI Tcrb tg DP thymocytes and for Vβ13 was much diminished as compared with the complete suppression in EkoI Tcrb tg DP thymocytes (Fig. 4B, center and right panels). This indicates continued chromosomal recombination of Vβ13 and upstream Vβ to DJβ1KI in DP thymocytes, despite feedback inhibition of endogenous Vβ-to-DJβ1 recombination by the Tcrb tg. Consistent with this interpretation,
recombination events detected by the V_{13} probe were more abundant on Southern blots of DJEoKI Tcrb tg thymus DNA (Fig. 4C, compare lanes 3 and 4), and V_{13} and DJEoKI SE intermediate were more abundant in DJEoKI Tcrb tg thymus DNA (Fig. 4D). We conclude that by reducing the distance between accessible gene segments, the DJEoKI promotes chromosomal V_{13} recombination in DP thymocytes and thereby subverts the process of feedback inhibition.

**Regulation of the \( \beta \)-in-\( \alpha \) allele**

To further assess constraints on Tcrb gene segment recombination in DP thymocytes, we introduced a Tcrb recombination substrate into the Tcra locus, because this locus normally undergoes recombination in DP thymocytes (Tcrb-in-Tcra; \( \beta \)-in-\( \alpha \) allele). The Tcrb substrate contained the same DJEoKI as in the DJEoKI allele, with the V_{13} promoter and gene segment (V_{13}KI) situated just upstream (Fig. 1B). A BamHI site introduced ∼90 bp 3’ of the V_{13} RSS was used in some experiments to distinguish the V_{13}KI from the endogenous V_{13}. We used homologous recombination to introduce this Tcrb recombination substrate into a previously generated Tcra allele [Tcra^{4}], which contains only the J_{\beta}61 and J_{\beta}56 gene segments (24), such that it replaces the TEA promoter and the entire J_{\beta} array of the wild-type Tcra locus. In this way, the Tcrb recombination substrate carries V_{13} and DJEoKI segments that are in close physical proximity and that will be accessible in DP thymocytes due to the activity of the endogenous E_{\alpha}. We generated heterozygous \( \beta \)-in-\( \alpha \) mice, which were then intercrossed to produce \( \beta \)-in-\( \alpha \) homozygous mice (Fig. 1B). These mice displayed normal DN thymocyte development and efficient differentiation to the DP stage, but were blocked in their development beyond the DP stage (data not shown). We presume that the chimeric TCR\( \alpha \) proteins encoded by \( \beta \)-in-\( \alpha \) alleles (which would include D_{\beta} and J_{\beta}, rather than J_{\alpha} sequences) are either unstable, cannot assemble with TCR\( \beta \) proteins, or cannot create a TCR\( \alpha \beta \) complex that can support positive selection.

We analyzed germline transcription and H3K4me3-modified nucleosomes on the \( \beta \)-in-\( \alpha \) allele after introducing it onto Rag2^{−/−} and Rag2^{−/−} × Tcrb tg backgrounds. As expected, transcription of the DJEoKI was low in DN thymocytes and substantially upregulated in DP thymocytes (Fig. 5A, left panel). Specific amplification of V_{13}KI revealed it to behave similarly (Fig. 5A, center panel). We also directly compared V_{13}KI transcription to endogenous V_{13} transcription using a PCR strategy that amplified both equally (Fig. 5B, right panel). V_{13}KI transcripts were more abundant in \( \beta \)-in-\( \alpha \) DP thymocytes than were endogenous V_{13}KI transcripts in wild-type DN thymocytes. Similar conclusions were drawn from analysis of H3K4me3 on \( \beta \)-in-\( \alpha \) alleles (Fig. 5B). These data suggest that the Tcrb substrate, like J_{\alpha} gene segments on a wild-type allele, is regulated by E_{\alpha} and is highly accessible in \( \beta \)-in-\( \alpha \) DP thymocytes.

We assayed V_{13}KI-to-DJEoKI recombination in wild-type and \( \beta \)-in-\( \alpha \) DN3 and DP thymocytes by amplification with V_{13} and J_{\beta}1.1 primers followed by hybridization with a DJEoKI-specific probe (Fig. 6A). This strategy detected the germline substrate in \( \beta \)-in-\( \alpha \) DN and DP thymocytes (Fig. 6A), but detected abundant V_{13}KI-to-DJEoKI rearranged alleles selectively in \( \beta \)-in-\( \alpha \) DP thymocytes (Fig. 6A). On the basis of germline RSS loss, fully 60% of the DJEoKI had undergone recombination in \( \beta \)-in-\( \alpha \) DP thymocytes (Fig. 6B). However, the same analysis indicated that only ∼10% of the V_{13}KI had undergone recombination (Fig. 6B), indicating that DJEoKI could rearrange to RSSs other than V_{13}KI. To further address this, we hybridized EcoRI-digested whole thymus genomic DNA to V_{13} and C_{p} probes. In addition to rearranged V_{13} (7.8 kb) and unrearranged V_{13}KI (5.9 kb),
Discussion

Numerous studies have correlated reduced Ag receptor locus accessibility and an extended Ag receptor locus conformation with the feedback inhibition of V(D)J recombination that mediates allelic exclusion (2, 13, 14). We previously forced V_{β} accessibility in DP thymocytes but could not overcome the inhibition of V_{β}-to-DJ_{β} recombination that normally characterizes this compartment (21). In this study, we found that, like wild-type alleles, those accessible EoKI alleles are extended in DP thymocytes. We therefore generated two new alleles (DJ_{EoKI} and β-in-α) to formally test whether gene segment proximity and accessibility are both critical effectors of feedback inhibition. By comparing the behavior of DJ_{EoKI} to EoKI alleles, we varied the proximity of accessible V_{β} and DJ_{β} segments; in DP thymocytes, these gene segments are accessible on both alleles, but they are in physical proximity on DJ_{EoKI} alleles only. We found that DJ_{EoKI} but not EoKI alleles supported V_{β}-to-DJ_{β} recombination in DP thymocytes. By comparing β-in-α alleles in DN and DP thymocytes, we varied the accessibility of proximal V_{β} and DJ_{β} segments; these gene segments are in physical proximity in both compartments, but become accessible due to developmental activation of EoKI in DP thymocytes only. We found that β-in-α alleles supported V_{β}-to-DJ_{β} recombination in DP but not in DN thymocytes. Based on...
the data from both models, we conclude that gene segment accessibility and gene segment proximity are both essential for chromosomal V(DJ) recombination and that feedback inhibition of \( V_{\mu} \)-to-DJ\( _{\beta} \) recombination on wild-type \( Tcrb \) alleles in DP thymocytes is normally enforced by both a loss of RSS accessibility to RAG1/2 and a decontracted locus conformation that inhibits RSS synopsis.

Pre-TCR signals initiate feedback inhibition and promote \( Tcrb \) epigenetic changes that enforce feedback inhibition, but the critical signaling pathways and downstream effector proteins are only partially understood (14). To the best of our knowledge, the only signaling pathway or downstream effector that has clearly been shown to impact \( Tcrb \) allelic exclusion through effects in DP thymocytes is the transcription factor E47. E47 supports \( Tcrb \) locus accessibility and recombination in DP thymocytes and is downregulated in response to pre-TCR signaling in DP thymocytes (31). Notably, its overexpression was shown to override feedback inhibition and promote \( V_{\mu} \)-to-DJ\( _{\beta} \) recombination in DP thymocytes (31). However, \( Tcrb \) locus accessibility and conformation were not evaluated in E47-overexpressing DP thymocytes, leaving the basis for this override of feedback inhibition undefined. We predict that E47 must support \( V_{\mu} \) accessibility and \( Tcrb \) locus contraction to account for the described effects on \( Tcrb \) recombination.

Although modulation of gene segment proximity appears to represent an important component of the feedback inhibition program, the mechanisms of locus contraction and decontraction are poorly understood. Recent studies have implicated architectural proteins cohesin and CTCF as regulators of long-distance interactions and V(DJ) recombination at the \( Tcra \) and \( Igh \) loci (32–34), but it is not known whether these proteins regulate overall locus conformation. Egr (35) and transcription factors Pax5 (11, 36), YY1 (37), and Ikaros (38) have all been implicated in \( Igh \) locus contraction, but whether and how they might trigger \( Igh \) locus decontraction is uncertain. Much less is known about the roles of architectural proteins and transcriptional regulators in \( Tcrb \) locus contraction and decontraction events. This will certainly be an important avenue for future studies.

Our data argue that gene segment proximity and accessibility are critical determinants of the \( Tcrb \) locus feedback inhibition program. Moreover, our results suggest that there are not likely any additional constraints imposed on the rearrangement of most \( V_{\mu} \) gene segments to DJ\( _{\beta} \) in DP thymocytes; for example, specific factors that regulate the usage of \( V_{\mu} \) and 5′ DJ\( _{\beta} \) RSSs. Were such constraints to exist, they should have been unperturbed by our genetic manipulations, and feedback would have remained intact on both the DJE\( _{\alpha} \)KI and \( \beta \)-in-\( \alpha \) alleles. We caution that we cannot formally eliminate the possibility that what we interpret to result from a change in physical distance could actually reflect the loss of an intervening regulatory element that is intrinsically inhibitory to \( V_{\mu} \)-to-DJ\( _{\beta} \) recombination. The identity of that element would be a matter of speculation. However, we imagine that it would function, like a change in physical proximity, to limit synopsis of \( V_{\mu} \) and DJ\( _{\beta} \) RSSs.

Despite the conclusions outlined above, additional layers of regulation may be required to explain the suppression of certain types of \( Tcrb \) locus recombination events in DP thymocytes. A particularly vexing issue is \( V_{\mu} \)-to-DJ\( _{\beta} \) recombination, because, unlike all other \( V_{\mu} \) gene segments, \( V_{\mu} \)-14 is located near DJ\( _{\beta} \) and J\( _{\beta} \) gene segments, and its accessibility is not downregulated by pre-TCR signaling and is apparently high in DP thymocytes (17, 39–41). Because DJ\( _{\beta} \) and J\( _{\beta} \) segments are also accessible and support RAG1/2 binding in DP thymocytes (42), the suppression of \( V_{\mu} \)-14 rearrangement may depend on unique features of inversional rearrangement (40) or of the \( V_{\mu} \)-14 RSS (41).

A second issue is the problem of secondary rearrangements. Reduced accessibility and locus decontraction can account for inhibition of \( V_{\mu} \)-to-DJ\( _{\beta} \) or -DJ2 rearrangement on a \( Tcrb \) allele that had not yet undergone \( V_{\gamma} \) rearrangement. However, because the \( V_{\mu} \) segments immediately upstream of a rearranged \( V_{\mu} \) are accessible in DP thymocytes (43, 44) and proximal to accessible downstream DJ\( _{\beta} \) and J\( _{\beta} \) segments (42), it is not clear what would suppress secondary \( V_{\mu} \)-to-DJ\( _{\beta} \) rearrangement on an allele that had already undergone primary \( V_{\mu} \)-to-DJ\( _{\beta} \) rearrangement. Recent work has demonstrated that secondary rearrangements can occur on these alleles and that they can replace even an in-frame VDJ\( _{\beta} \) rearrangement (18), but there was no indication that this occurred in DP as opposed to DN thymocytes. Indeed, analysis of DP thymocytes failed to detect SE intermediates at the accessible \( V_{\mu} \) segments upstream of a rearranged \( V_{\gamma} \) gene segment (43). Moreover, SEs at 5′ DJ2 RSS are strongly suppressed in DP thymocytes (21 and this study). Thus, DP thymocytes appear not to be permissive for secondary \( Tcrb \) recombination.

Because we found that accessible \( V_{\mu} \)s can rearrange to the DJ\( _{\beta} \)K1 in DJE\( _{\alpha} \)KI DP thymocytes, it seems unlikely that any additional mechanism that might be required to suppress secondary recombination would be directed at \( V_{\mu} \) RSSs. However, it remains possible that there is a specific regulatory mechanism directed at the 5′ DJ2 RSS. Indeed, DJ2 regulation appears to be unusually complex, with promoters both upstream and downstream of DJ2 (45, 46). The downstream promoter is preferentially active on unrearranged alleles and presumably directs DJ2-to-J\( _{\beta} \)2 rearrangement; the upstream promoter only becomes active once the downstream promoter is eliminated by DJ2-to-J\( _{\beta} \)2 rearrangement and is likely important to direct \( V_{\mu} \)-to-DJ\( _{\beta} \)2 rearrangement. Activity of the 5′ promoter suggests that the 5′ DJ2 RSS resides in accessible chromatin on DJ2-to-\( J_{\beta} \)2 rearranged alleles in DP thymocytes. However, it is unclear whether these alleles support RAG1/2 binding, because the only assays of RAG1/2 binding at DJ2 in DP thymocytes were conducted on alleles that were in germline configuration and in which only the downstream promoter should have been active (42). Therefore, it is not known whether RAG1/2 can bind to the 5′ DJ2 RSS in DP thymocytes, and it remains possible that secondary rearrangements could be suppressed in DP thymocytes by a specific mechanism that occludes RAG1/2 binding to the 5′ DJ2 RSS. Our results demonstrate conclusively that, for most \( V_{\mu} \) gene segments, accessibility and conformational constraints alone can fully account for the suppression of \( V_{\mu} \)-to-DJ\( _{\beta} \)1 recombination in DP thymocytes. However, additional work will be required to clarify the mechanisms, beyond accessibility and conformational constraints, that impart feedback inhibition to \( V_{\mu} \)-14 recombination and secondary recombination events involving DJ2.

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