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Orchestrating T cell receptor α gene assembly through changes in chromatin structure and organization

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Abstract

V(D)J recombination is regulated through changes in chromatin structure that allow recombinase proteins access to recombination signal sequences and through changes in three-dimensional chromatin organization that bring pairs of distant recombination signal sequences into proximity. The *Tcra/Tcrd* locus is complex and undergoes distinct recombination programs in double negative and double positive thymocytes that lead to the assembly of *Tcrd* and *Tcra* genes, respectively. Our studies provide insights into how locus chromatin structure is regulated and how changes in locus chromatin structure can target and then retarget the recombinase to create developmental progressions of recombination events. Our studies also reveal distinct locus conformations in double negative and double positive thymocytes and suggest how these conformations may support the distinct recombination programs in the two compartments.

Keywords

T cell receptor; chromatin; V(D)J recombination; T lymphocyte

Introduction

Antigen-specific immunity depends on the display of diverse and clonally distributed antigen receptors on the surfaces of T and B lymphocytes. These receptors, T cell receptors (TCRs) and surface immunoglobulins (Ig), are acquired as T and B lymphocytes develop in the thymus and bone marrow, respectively. Antigen receptor diversity depends, in large measure, on V(D)J recombination, the somatic DNA recombination process that is used to assemble mature TCR and Ig genes (1, 2). V(D)J recombination selects individual variable (V), diversity (D) and joining (J) gene segments from large arrays of these gene segments, and joins them to create the variable region exon of the mature, rearranged gene.

V(D)J recombination is directed by the lymphoid-specific recombinase activating gene (RAG)1 and RAG2 protein complex (referred to as RAG) (1–3). RAG can recognize recombination signal sequences (RSSs) that flank each TCR or Ig V, D, or J gene segment, and with two RSSs in a synaptic complex, can generate double-strand breaks at the borders of the RSSs and the coding segments. DNA repair proteins then join the four ends to create novel coding joints (D-to-J, V-to-D or V-to-J, which remain chromosomal) and signal joints (which are often extrachromosomal). V(D)J recombination creates diverse antigen receptor repertoires through the combinatorial nature of the recombination process and nucleotide loss and addition at the site of the coding joint. T lymphocyte development generates T cells that bear either an $\alpha\beta$ or a $\gamma\delta$ TCR (4). Notably, the four TCR genes that encode these receptors are organized into three genetic loci: *Tcrb*, encoding TCR β chain; *Tcrg*, encoding

the TCR γ chain; and *Tcra/Tcrd*, encoding both TCR α and TCR δ chains. During T lymphocyte development, *Tcrg*, *Tcrd* and *Tcrb* all undergo recombination in CD4⁻CD8⁻ double negative (DN) thymocytes, when RAG is first expressed. Successful recombination of *Tcrg* and *Tcrd* promotes assembly of a $\gamma\delta$ TCR and commitment to the $\gamma\delta$ T cell lineage. Successful *Tcrb* recombination, on the other hand, promotes assembly of a pre-TCR and commitment to the $\alpha\beta$ lineage. Such committed cells downregulate recombinase expression, enter the cell cycle and differentiate to the CD4⁺CD8⁺ double positive (DP) stage. Newly generated DP thymocytes then re-express RAG, initiate *Tcra* recombination, and ultimately assemble a cell surface $\alpha\beta$ TCR. DP thymocyte fate then depends on TCR specificity: those with useful TCRs are saved (positive selection) whereas those with autoreactive TCRs are eliminated (negative selection). Therefore, TCR gene assembly not only creates a T cell repertoire, but also instructs multiple cell fate decisions during T cell development.

In both the B and T cell lineages, V(D)J recombination events are thought to be developmentally programmed through changes in chromatin structure and chromatin organization that provide RAG proteins access to pairs of RSSs (5–7). Such developmental programming takes multiple forms, but nowhere is the task more complex than at the *Tcra/Tcrd* locus (4, 8). This locus contains more than 160 gene segments that undergo recombination at two different stages of T cell development to form two different TCR proteins (Fig. 1). The major portion of the locus (about 1.5 Mb) is devoted to an array of about 100 V gene segments. Downstream of this, a 0.1 Mb region includes two D δ and J δ gene segments, followed by C δ , and an array of 61 J α gene segments, followed by C α . *Tcrd* gene assembly in DN thymocytes occurs in two steps, V δ -to-D δ and D δ -to-J δ , whereas *Tcra* gene assembly in DP thymocytes occurs in a single step, V α -to-J α . Both TCRs draw from the same array of V gene segments, but in fact only a small number of V gene segments typically rearrange to D δ in DN thymocytes, and are therefore considered V δ gene segments, whereas the vast majority of V gene segments (including some V δ) can rearrange to J α in DP thymocytes, and are therefore considered V α gene segments.

The dynamics of *Tcra/Tcrd* locus recombination events are fascinating: *Tcrd* alleles typically undergo complete (V δ -to-D δ -to-J δ) or partial (V δ -to-D δ or D δ -to-J δ) recombination in DN thymocytes; in those thymocytes that commit to the $\alpha\beta$ lineage, *Tcrd* rearrangements can then be excised by V α -to-J α recombination (4, 8). Strikingly, there can be multiple cycles of V α -to-J α recombination. Early DP thymocytes initially join an available 3' V α gene segment to a J α segments at the 5' end of the J α array, and these initial, or primary, V α -to-J α recombination events can then be replaced by secondary events that join progressively more 5' V α gene segments to progressively more 3' J α gene segments. The recombination process is ultimately terminated by downregulation of recombinase expression in positively selected DP thymocytes (or by cell death in those DP thymocytes that are not selected). Multiple cycles of V α -to-J α recombination increase the efficiency of thymocyte development by providing individual DP thymocytes with several opportunities to be positively selected.

The unique behavior of the *Tcra/Tcrd* locus raises many questions about mechanisms of developmental control. What targets initial recombination events in DN thymocytes to D δ and J δ gene segments? What features of V δ gene segments distinguish them from V α gene segments and promote their rearrangement in DN thymocytes? What retargets recombination events to the J α array in DP thymocytes, and what directs the 5'-to-3' progression of J α usage? What, if anything, promotes a reciprocal progression across the V α array? These questions have been the subject of intensive investigation in our laboratory and in other laboratories for many years; we will discuss each of them in turn. Central to the discussion are two concepts: *accessibility*, which has guided the field of V(D)J

recombination for many years, and *locus conformation*, which has been recognized more recently.

The “accessibility hypothesis” was developed from the observation that V(D)J recombination events at the *Igh* locus correlated developmentally with the activation of transcription of the unarranged gene segments (germline transcription) (9). It was proposed that chromatin structure forms an intrinsic barrier that prevents RAG proteins from accessing RSSs, and that an accessible state (characterized by active transcription and recombination) could be reached through developmentally regulated changes to chromatin structure. In the ensuing years, it has been shown that TCR and Ig locus transcriptional enhancers and promoters are essential for and impart developmental control to V(D)J recombination in chromatin (10–17), and that they do so by recruiting chromatin remodeling enzymes that covalently modify histones and that can move or remove nucleosomes (18–21). Recent studies have also revealed that a passive view of “accessibility” is likely an oversimplification since RAG2 has been found to interact directly with a modified form of histone H3 (H3K4me3) and appears to be recruited to H3K4me3 modified nucleosomes genome-wide (22–24). Remarkably, RAG1 appears to distribute independent of RAG2, and to localize selectively to regions of antigen receptor loci with accessible RSSs, presumably through direct RAG1-RSS interactions (23).

Because V(D)J recombination represents a transaction between two RSSs that may be widely separated in antigen receptor loci, the three dimensional organization or conformation of antigen receptor loci is also thought to be a critical regulator of the recombination process (25). Using pairs of DNA probes in three dimensional fluorescence in situ hybridization (3D-FISH) experiments, it has been found that Ig and TCR loci adopt relatively extended configurations in non-lymphoid cells and undergo developmental stage-specific contraction events in lymphoid cells to set the stage for V(D)J recombination (26–28). Thus, the *Igh* locus contracts in pro-B cells to promote V_H-to-DJ_H recombination, and does so in a fashion that brings all V_H gene segments into roughly equal proximity to DJ_H gene segments (29). It then decontracts in pre-B cells, presumably to limit further V_H-to-DJ_H recombination and promote allelic exclusion (27).

Accessibility control of *Tcra/Tcrd* gene recombination

Efficient *Tcrd* gene recombination in DN thymocytes depends on the *Tcrd* enhancer (E δ). Elimination of E δ by gene targeting inhibits, but does not completely block, both V δ -to-D δ and D δ -to-J δ recombination events (11). This likely reflects reduced accessibility of D δ -J δ chromatin: germline *Tcrd* transcripts, initiating predominantly at D δ 2 and J δ 1 (30), are reduced, but not eliminated, by E δ -deletion, and histone modifications such as H3 and H4 acetylation and H3K4 di- and tri-methylation are diminished across the D δ and J δ segments as well (BH and MSK, unpublished). Thus, E δ appears to collaborate with local promoters to target the D δ and J δ segments for recombination. However, we have found no appreciable effect of E δ deletion on chromatin structure at the common V δ gene segments (BH and dMSK, unpublished), arguing that V δ gene segments are made accessible in an E δ -independent fashion. In previous experiments we found that the commonly used V δ gene segments (in particular TRDV5, TRDV2-2 and members of the TRAV15/TRDV6 family) are actively transcribed and display histone modifications characteristic of accessible chromatin in DN thymocytes (31). Thus we assume that these V gene segments are defined as V δ on the basis of E δ -independent promoter activity and chromatin remodeling in DN thymocytes. It appears unlikely that physical location in the *Tcra/Tcrd* locus defines them as V δ , since some (TRDV5, TRDV2-2) are D δ -proximal, whereas others (TRAV15/TRDV6 family) are hundreds of kb away. It also appears unlikely that unique properties of their RSSs define them as V δ , since the D δ 5'RSS rearranges effectively with both V α and V δ RSSs (32).

The transition to the DP stage is marked by a downregulation of E δ activity, an upregulation of E α activity, and a retargeting of recombination events from *Tcrd* gene segments to *Tcra* gene segments(33). Gene targeting experiments revealed E α to be required for essentially all V α -to-J α recombination events (10). However, as noted previously, the earliest V α -to-J α recombination events (so-called primary rearrangements) are targeted preferentially to the most 5' J α gene segments (34, 35). This 5' targeting depends on two E α -dependent promoters, T early- α (TEA) and J α 49 (15, 36). Primary rearrangements are directed almost exclusively to two clusters of J α segments, one immediately downstream of TEA, and one in proximity of J α 49. Elimination of these promoters by gene-targeting suppressed recombination events involving these J α segments, and caused residual primary recombination events to be scattered across the central portion of the J α array (Fig. 2) (36). This retargeting was associated with upregulated transcription from a set of otherwise cryptic germline promoters associated with central J α gene segments.

These studies established germline J α promoters as local regulators of recombination, but did not establish a mechanism. Several years ago, we asked whether transcription across 5' J α RSSs was critical for their recombination by introducing a transcription terminator into the J α array (37, 38). Termination of transcription downstream of J α 56 impaired rearrangement of J α 53 and J α 52 (37), whereas termination of transcription downstream of TEA impaired rearrangement of the entire J α 61-J α 52 interval (Fig. 2) (38). Importantly, blockade of TEA transcription mimicked exactly the recombination defect in TEA promoter-deleted mice, indicating that the TEA promoter targets 5' J α recombination events by driving transcription across this region. Notably, like TEA promoter deletion, termination of TEA transcription caused the derepression of cryptic promoters associated with central J α gene segments, indicating that these promoters are normally suppressed by transcriptional interference. Thus, TEA transcription focuses primary V α to J α recombination through a combination of positive effects across 5' J α gene segments and negative effects across central J α gene segments.

We have also addressed the effects of TEA transcription on 5' J α chromatin. Wild-type alleles are characterized by very high levels of several histone modifications, including H3 acetylation, H3K4 di- and tri-methylation, and H3K36 tri-methylation in the region from J α 61 to J α 56. All of these modifications are potently suppressed by transcriptional blockade (37, 38). Transcriptional elongation also influences nucleosome organization. We recently identified three well positioned nucleosomes spanning the J α 61 region on inaccessible E α -deficient alleles and demonstrated nucleosome loss and repositioning on accessible wild-type alleles (19). Transcriptional blockade reversed several of these changes, indication that in addition to covalently modifying nucleosomes, transcriptional elongation can move and remove nucleosomes. We suspect that accessible alleles are actually mosaics with nucleosomes missing at some sites and covalently modified at others, in this manner simultaneously promoting RAG1 interaction with RSS DNA and RAG2 interaction with a nearby H3K4 tri-methylated nucleosome.

These studies provide a reasonably satisfying view of how J α segments are targeted for primary recombination events. To understand the targeting of secondary recombination, we analyze an allele engineered to mimic a primary rearrangement: *Tcrd* gene segments, the TEA promoter and 5' J α gene segments were eliminated, and a V α -J α 57 rearrangement replaced the germline J α 57 gene segment (39). We found that this configuration targeted histone modifications preferentially to the J α segments immediately downstream of the introduced V α -J α 57, and that V α -to-J α recombination events that replaced the V α -J α 57 rearrangement in early DP thymocytes were targeted to this region as well (40). As such, we think that the promoter of the rearranged V α effectively replaces the TEA promoter and locally targets recombination to downstream J α gene segments through similar mechanisms.

Moreover, since at each round of recombination the promoter of the rearranged V α would be repositioned further 3' along the J α array, the net effect would be a stepwise progression of chromatin remodeling and recombinase targeting events. Of note, all of our inferences about recombinase targeting on the different *Tcra* alleles discussed above have recently been confirmed in studies analyzing RAG1 binding by chromatin immunoprecipitation (Y.Ji, B.Hao, E.M.Oltz, M.S.Krangel and D.G.Schatz, unpublished observations).

Can regulated changes in accessibility account for preferential use of 3' V α gene segments in primary V α to J α recombination and a progression from 3' to 5' across the V α array? We suspect that accessibility is indeed part of the story, since the 3' one-third of the V α array displays dramatic, E α -dependent increases in histone modifications in DP thymocytes (31). The ability of E α to activate these V segment promoters over a distance of 500 kb may provide these proximal V gene segments with an advantage over central and distal V gene segments as competitors for RAG proteins. However, we suspect that the real key to this behavior is a unique conformation of the *Tcra/Tcrd* locus that is adopted in DP thymocytes.

Conformational control of *Tcra/Tcrd* gene recombination

Recent 3D-FISH analysis using 12 genomic markers spanning the 3Mb *Igh* locus provided a detailed characterization of the conformational change that occurs between pre-pro B and pro B cells (29). In pre-pro B cells, the locus is relatively extended, with most V_H gene segments relatively distant from D_H and J_H gene segments; in contrast, the locus is much more compact in pro-B cells, with both distal and proximal V_H gene segments moved into close proximity of D_H and J_H gene segments. This conformation presumably provides all V_H gene segments a similar opportunity for V_H-to-D_HJ_H rearrangement, which is likely of particular importance since an individual allele can undergo only a single round of V_H-to-D_HJ_H rearrangement. We reasoned that the *Tcra/Tcrd* locus might display more complex conformational properties since the pattern of recombination events switches between the DN and DP stages of thymocyte development. Like the *Igh* locus in pro-B cells, the *Tcra/Tcrd* locus undergoes a single round of V δ -to-D δ -to-J δ rearrangement, and this rearrangement must access both proximal and more distant V δ gene segments. However, as noted above, V α -to-J α recombination in DP thymocytes can occur multiple times on an allele and tends to be targeted to the more 3' of the available V α gene segments at each round of recombination. We recently addressed this issue by comparing *Tcra/Tcrd* locus conformation in B cells, DN thymocytes and DP thymocytes using four DNA probes in 3D-FISH experiments (41). We found that, as compared to splenic B cells, the locus was contracted in both DN and DP thymocytes. Remarkably, however, we detected two distinct modes of contraction in the two thymocyte compartments: in DN thymocytes the locus is contracted along its entire length, whereas in DP thymocytes, the locus displays a 3' contracted and 5' decontracted conformation (Fig. 3). Hence, the 3' end of the locus (including proximal but not central and distal V gene segments) is contracted in DN thymocytes and remains contracted upon transition to the DP stage, whereas the 5' end of the locus (including distal and central V gene segments) is contracted in DN thymocytes and then decontracts upon transition to the DP stage (41). We interpret this 5' decontraction to be a mechanism that would force initial V α -to-J α recombination events to use the relatively proximal V α gene segments and that would similarly bias V α usage during subsequent rounds of recombination. This idea is supported by proximal V_H-biased rearrangements documented in mice carrying a decontracted *Igh* locus: *Igh* transgenic (39), *Pax5*^{-/-} (42), *Ikaros*^{-/-} (43) and *YY1*^{-/-} mice (44). However, the *Tcra/Tcrd* locus would be the only example in which a decontracted state would promote a bias to V(D)J recombination events that is of physiological significance.

The molecular mechanisms of antigen receptor locus contraction and decontraction are not well understood. Although deficiencies in transcription factors Pax5, Ikaros and YY1 disrupt the contracted *Igh* locus configuration, it is unclear how they do so. Since enhancers are thought to activate promoters by looping interactions, and since E α in particular is known to activate promoters at a distance of 500 kb, we imagined that E δ and E α might function to establish locus conformational states. However, examination of the conformation of E α - and E δ -deficient alleles in DN and DP thymocytes provided no evidence that locus contraction depended on these elements (41). On the contrary, we think it likely that the ability of E α to activate promoters over the proximal 1/3 of the locus in DP thymocytes may be a consequence of the contracted configuration of this portion of the locus.

The regulation of antigen receptor locus conformation thus remains a major unanswered question. However, there are several molecules previously implicated as mediators of chromatin architecture, including SATB1, CTCF, and CTCF-associated cohesin, that are potential candidates (45–47). As a transcription factor and a chromatin organizer, SATB1 is thought to bridge specific AT-rich DNA sequences to the nuclear matrix and to function as an anchor for tethering chromatin into loops. It is directly implicated in the formation of a complex looped structure at the Th2 cytokine locus (48). CTCF and cohesin have been shown to colocalize at many sites genome-wide (49–51), and have been directly implicated as mediators of long-distance interactions at the *Ifng* (52), *H19/Igf2* (53), and beta-globin loci (54). Moreover, colocalized CTCF and cohesin binding sites are abundant at both the *Igh* and *Igk* loci in developing B cells (55). However, binding profiles across the *Tcra/Tcrd* locus in DN and DP thymocytes have not yet been reported, and any roles in locus conformation remain purely speculative.

Conclusions

The *Tcra/Tcrd* locus provides an intriguing system in which to explore the rules governing gene segment usage during V(D)J recombination. Our studies reveal how regulated changes in local chromatin accessibility are established and how developmentally regulated changes in accessibility are likely critical to establish the dynamics of V(D)J recombination events at the *Tcra/Tcrd* locus. Our studies also suggest how changes in locus conformation may impart a critical second level of control. Finally, although we have discussed accessibility and conformation as two independent parameters that direct V(D)J recombination events at the locus, we suspect that this view is substantially oversimplified, since looping of DNA over long distances has the potential to inhibit (eg., enhancer-blocking) or potentiate enhancer-promoter interactions. These considerations suggest that it will be essential to establish an integrated view of conformational and accessibility effects to bring our understanding of *Tcra/Tcrd* locus regulation to the next level.

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Figure 1. Organization of the *Tcrα/Tcrδ* locus

Gene segments are depicted by filled rectangles, enhancers are depicted by filled ovals, and prominent promoters in the $J\alpha$ array are depicted by bent arrows. The commonly used V δ gene segments (TRAV15/DV6 family, TRDV2-2, TRDV4, TRDV5) are identified. Note that TRDV4 rearranges preferentially in fetal thymocytes.

Figure 2. Regulation of J α chromatin accessibility

Wild-type and genetically manipulated versions of the J α array are depicted. Δ TEA and Δ TEA Δ J49 carry deletions of the TEA and J49 promoters. TEA-T and 56R carry insertions of a transcription terminator downstream of TEA or J α 56, respectively. HY α carries an insertion of a TRAV17-J α 57 rearrangement. Large arrows identify major promoters (TEA, J49). Small arrows identify minor or cryptic promoter activity. Shaded portions of the Δ TEA, Δ TEA Δ J49, TEA-T and 56R alleles identify areas of diminished chromatin modifications and disrupted V α -to-J α recombination.

Figure 3. *Tcrα/Tcrδ* locus conformational states

The diagram depicts the relatively extended configuration of the locus in B cells, the fully contracted configuration in DN thymocytes, and the unique 3' contracted and 5' decontracted configuration in DP thymocytes. The V array is segregated into distal (5'), central, and proximal (3') regions.