Published in final edited form as:

Curr Opin Immunol. 2009 April ; 21(2): 133–139. doi:10.1016/j.coi.2009.03.009.

Mechanics of T cell receptor gene rearrangement

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Summary

The four T cell receptor genes (*Tcra*, *Tcrb*, *Tcrg*, *Tcrd*) are assembled by V(D)J recombination according to distinct programs during intrathymic T cell development. These programs depend on genetic factors, including gene segment order and recombination signal sequences. They also depend on epigenetic factors. Regulated changes in chromatin structure, directed by enhancers and promoter, can modify the availability of recombination signal sequences to the RAG recombinase. Regulated changes in locus conformation may control the synapsis of distant recombination signal sequences, and regulated changes in subnuclear positioning may influence locus recombination events by unknown mechanisms. Together these influences may explain the ordered activation and inactivation of T cell receptor locus recombination events, and the phenomenon of *Tcrb* allelic exclusion.

Introduction

The somatic assembly of T cell receptor (TCR) genes (*Tcra*, *Tcrb*, *Tcrg*, *Tcrd*) generates a diverse T cell repertoire and is an essential component of thymocyte development that instructs numerous cell lineage and cell fate decisions. TCR genes are assembled through V(D)J recombination, a site specific recombination process directed by the lymphoid-specific recombinase (RAG, composed of RAG1 and RAG2) and ubiquitously expressed DNA repair proteins [1]. RAG proteins create double-strand breaks at recombination signal sequences (RSSs) that flank TCR variable (V), diversity (D) and joining (J) gene segments, and these breaks are subsequently resolved by nonhomologous end joining. The four TCR genes are assembled according to distinct developmental programs. Recombination of *Tcrd*, *Tcrg* and *Tcrb* all occur during the CD4⁺CD8⁺ double-negative (DN) 2 (CD44⁺CD25⁺) and DN3 (CD44⁺CD25⁺) stages of thymocyte development, during an initial period of recombinase expression. Thymocytes commit to the $\alpha\beta$ or $\gamma\delta$ lineages concurrent with, and likely as an outcome of, these TCR gene recombination events [2]. Successful recombination of *Tcrd* and *Tcrg* promotes assembly of a $\gamma\delta$ TCR, whereas successful recombination of *Tcrb* promotes assembly of TCR β with pre-T α to form a pre-TCR. Pre-TCR signals then provoke downregulation of recombinase expression, several rounds of proliferation, and differentiation of thymocytes to the CD4⁺CD8⁺ double positive (DP) compartment, within which *Rag* genes are re-expressed and *Tcra* recombination initiated. The specificity of assembled $\alpha\beta$ TCR for intrathymic ligands then dictates cell survival and differentiation into the CD4, CD8 or other $\alpha\beta$ T cell lineages.

TCR gene assembly is regulated through controls exerted at the level of chromatin structure, at the level of locus conformation, and at the level of locus positioning in thymocyte nuclei

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[3;4]. Recombination biases are also dictated by information encrypted in RSSs themselves. This review outlines current thinking on how these regulatory influences are integrated to orchestrate the unique developmental programs at murine TCR loci.

***Tcr*g: Programmed V segment rearrangements**

The *Tcr*g locus has two properties that have provoked substantial interest. First, certain V_γ -to- J_γ recombination events are developmentally programmed, with V_γ usage defining $\gamma\delta$ T cell subsets that populate distinct anatomical locations [2]. Second, V_γ -to- J_γ recombination events are directed by γ c family cytokines. The murine *Tcr*g locus is composed of three functional V_γ - J_γ - C_γ clusters, $C_\gamma1$, $C_\gamma2$ and $C_\gamma4$ (Figure 1). The $C_\gamma1$ cluster is the best studied, because its four V_γ gene segments are used in a highly regulated way: the $J_\gamma1$ -proximal $V_\gamma3$ and $V_\gamma4$ gene segments undergo recombination in early fetal thymocytes and encode invariant $\gamma\delta$ TCRs of intraepithelial lymphocytes in the epidermis and in vaginal and tongue epithelia, respectively. The more distal $V_\gamma2$ and $V_\gamma5$ gene segments rearrange subsequently and contribute, along with $V_\gamma1.1$ ($C_\gamma4$ cluster) and $V_\gamma1.2$ ($C_\gamma2$ cluster), to the diverse $\gamma\delta$ repertoires in secondary lymphoid organs. $V_\gamma5$ usage predominates in the intestinal epithelium.

RSS accessibility at antigen receptor loci is mediated by regulatory elements such as enhancers and promoters that disrupt nucleosome structure through effects on transcription, on histone modifications, and by recruitment of chromatin remodeling complexes [3;4]. Two elements in the $C_\gamma1$ cluster, transcriptional enhancer 3'EC $\gamma1$ and DNase hypersensitive site HsA, function together to regulate transcription of rearranged *Tcr*g genes, but have only a modest impact on *Tcr*g recombination [5]. In contrast, V_γ promoters have been implicated as critical developmental regulators of V_γ to $J_\gamma1$ recombination. As measured by germline transcription and histone acetylation, the fetal $V_\gamma3$ gene segment is active in fetal but not adult thymocytes, whereas the adult $V_\gamma2$ gene segment is active at both stages [6]. Exchange of the $V_\gamma3$ and $V_\gamma2$ promoters in a transgenic recombination substrate reprogrammed $V_\gamma3$ and $V_\gamma2$ rearrangement in adults, favoring $V_\gamma3$ rearrangement over $V_\gamma2$ [7]. However, promoter exchange did not impact the bias towards $V_\gamma3$ rearrangement in fetal thymocytes. Rather, the fetal bias depends on gene order, because exchange of $V_\gamma3$ and $V_\gamma2$ in a transgenic recombination substrate caused $V_\gamma2$ rather than $V_\gamma3$ rearrangement to predominate in fetal thymocytes, even though both displayed accessible chromatin [8]. Moreover, excision of $V_\gamma3$ and $V_\gamma4$ from the endogenous *Tcr*g locus increased $V_\gamma2$ rearrangement in fetal thymocytes without modifying $V_\gamma2$ chromatin structure [9]. Thus, all V_γ gene segments in the $C_\gamma1$ cluster are accessible in fetal thymocytes, with proximity to $J_\gamma1$ biasing rearrangement to $V_\gamma3$ and $V_\gamma4$; this bias is then overcome in adults by specific repression enforced by the $V_\gamma3$ and $V_\gamma4$ promoters. This suppression depends on transcription factor E2A [10]. Interestingly, an analogous proximal to distal switch occurs for V_H segments between fetal and adult B cell development. The suppression of proximal V_H segments in the adult occurs, in part, through Ezh2-dependent trimethylation of histone H3K27 [11;12]. Thus in both loci, proximal V segments outcompete distal V segments unless the proximal V segments are specifically suppressed. However, in comparison to *Igh*, the $C_\gamma1$ cluster is extremely compact with V_γ segments tightly clustered. Therefore the bias towards proximal V_γ usage may depend on a specific locus conformation rather than overall distance, or perhaps by recombinase tracking along the DNA [13].

Il7^{-/-} and *Il7ra*^{-/-} mice lack $\gamma\delta$ T cells because IL-7/IL-7R signaling in DN thymocytes is essential for accessibility and recombination of *Tcr*g [14–16]. IL-7R signaling activates STAT5, and indeed there are STAT5 binding sites in J_γ promoters and 3'E γ . Moreover, an activated STAT5 could induce $\gamma\delta$ T cell development by activating J_γ chromatin and V_γ -to- J_γ recombination [17;18]. A role for STAT5 in *Tcr*g recombination was questioned based on the analysis of *Stat5a*^{-/-} *Stat5b*^{-/-} mice with hypomorphic *Stat5* alleles [19], but mice carrying

null alleles indeed mimic the dramatic phenotype of *Il7*^{-/-} and *Il7ra*^{-/-} mice [20]. A distinct γ c cytokine, IL-15, specifically induces V γ 5-to-J γ 1 recombination through effects on V γ 5 and J γ 1 chromatin [21]. Like IL-7, IL-15 activates STAT5, and the selective activation of V γ 5 is STAT5-dependent. The basis for distinct responses to IL-7 and IL-15 remains an open question.

***Tcrb*: Order and allelic exclusion**

A hallmark of *Tcrb* is developmentally ordered recombination, with D β -to-J β preceding V β -to-D β J β recombination. All *Tcrb* recombination events depend strictly on E β , which is essential for chromatin modifications throughout a 25 kb domain encompassing the two D β -J β -C β clusters [22] (Figure 1). E β cooperates with the promoter PD β 1, which is tightly associated with D β 1, to stimulate D β 1-to-J β 1 recombination [23] and likely cooperates with a second promoter, PD β 2 [24], to stimulate D β 2-to-J β 2 recombination. D β 1 appears to be the critical control point for D β 1-to-J β 1 recombination, because although E β can independently influence chromatin structure across a broad region, a specific interaction between E β and PD β 1 is required to make the D β 1 gene segment accessible to the recombinase [25;26]. This interaction recruits the SWI-SNF chromatin remodeling complex to D β 1; recruitment of SWI/SNF is necessary for D β -to-J β recombination and, in the context of a minilocus V(D)J recombination reporter substrate, can replace PD β 1 function [27].

These experiments provide insight into accessibility control of D β -to-J β recombination, but do not directly address the basis for two-step, developmentally ordered rearrangement. What prevents direct V β -to-J β recombination? Control is exerted by RSSs themselves. RSSs have either 12- or 23-bp spacers separating conserved heptamer and nonamer elements, and recombination is restricted to gene segments with dissimilar RSSs (the 12/23 rule). D β -to-J β recombination is directed by a 3' D β 23 RSS and a J β 12 RSS; the V β -to-D β step is directed by a V β 23 RSS and a 5' D β 12 RSS. Although direct V β 23 RSS-to-J β 12 RSS recombination is permissible according to the 12/23 rule, it does not occur (a restriction known as “beyond 12/23” or B12/23) [28]. However, direct V β -to-J β recombination can occur when a J β RSS is replaced by the 5' D β 1 RSS or when a V β RSS is replaced by the 3' D β 1 RSS [28;29]. These restrictions are apparent even on nonchromatinized substrates and appear to depend on both RSS and flanking sequences [30]. In fact, B12/23 restrictions can be replicated using RAG and high mobility group proteins *in vitro*, with the reaction blocked at early steps of RSS nicking and synaptic complex formation [31].

Although B12/23 restrictions enforce a two step recombination process, they do not impose order. Why D β -to-J β recombination invariably precedes V β -to-D β recombination has been uncertain. Notably, the 3' D β 1 and 3' D β 2 23 RSSs were shown to include a highly conserved binding site for the transcription factor AP-1, and the AP-1 component c-Fos was found to recruit RAG to the 3' D β 1 23 RSS [32]. Although stimulatory for plasmid D β 1-to-J β 1 recombination, this recruitment inhibited V β -to-D β 1 recombination, presumably because it sterically interferes with RAG access to the nearby 5' D β 1 12 RSS. Notably, deletion of the D β 1 23 RSS [33] or gene-targeted loss of c-Fos [32] made *Tcrb* permissive for V β -to-D β 1 recombination. This suggests that during T cell development, removal of the 3' D β 1 23 RSS through D β 1-to-J β 1 recombination may signal to initiate V β -to-D β J β recombination.

The V β -to-D β J β step of *Tcrb* recombination is subject to allelic exclusion, thus insuring that developing T lymphocytes assemble a functional *Tcrb* gene on only a single allele. Allelic exclusion can be thought of as occurring in two phases [34;35]. First, in the initiation phase, it must be highly unlikely that both alleles attempt V β -to-D β J β recombination within the same time frame. Second, in the maintenance phase, in-frame recombination on one allele must provoke a feedback signal that suppresses further V β -to-D β J β recombination on the other allele.

From a mechanistic perspective, we have a much better understanding of the maintenance phase of allelic exclusion. Feedback inhibition is established by pre-TCR signaling, which provokes downregulation of *Rag-1* and *Rag-2* expression, entry of DN3 thymocytes into the cell cycle, and differentiation to the DP stage [36]. V_{β} -to- $D_{\beta}J_{\beta}$ recombination is then suppressed in DP thymocytes despite re-expression of *Rag-1* and *Rag-2*. Almost all V_{β} gene segments are several hundred kb distant from D_{β} - J_{β} - C_{β} and are regulated independently; these V_{β} segments transition to a less accessible chromatin structure in DP thymocytes [37]. However, chromatin accessibility cannot fully account for feedback inhibition because a genetic manipulation that maintained accessible V_{β} chromatin in DP thymocytes failed to override feedback inhibition [38]. Locus conformation is thought to provide an additional level of regulation. Use of three dimensional fluorescence *in-situ* hybridization (3D-FISH) and the chromosome conformation capture (3C) crosslinking technique have shown that the two ends of the *Tcrb* locus are farther apart in DP than in DN thymocytes [39]. This “decontraction” may limit synapsis of V_{β} and $D_{\beta}J_{\beta}$ segments in DP thymocytes. Similar decontraction of the *Igh* locus has been linked to *Igh* allelic exclusion [40]. The molecular mechanisms mediating large-scale locus conformational changes are unknown. However an intriguing candidate would be the transcription factor CTCF, which functions to recruit cohesins [41;42] and can tether distal portions of the β -globin locus [43].

Notably, it was recently shown that overexpression of transcription factor E2A, whose activity is normally downregulated in response to pre-TCR signals, can override feedback inhibition in DP thymocytes [44]. E2A was shown to be a dosage-dependent regulator of V_{β} accessibility [44;45], but one might predict that it must also regulate locus conformation. It should also be noted that there are examples in which V_{β} segments are close to D_{β} and apparently accessible in DP thymocytes, yet are still subject to feedback inhibition: (1) $V_{\beta}14$, which is situated downstream of $C_{\beta}2$ in an inverted orientation [46]; (2) unrearranged V_{β} gene segments upstream of a V_{β} - D_{β} - $J_{\beta}1$ rearrangement [47;48]; (3) $V_{\beta}10$ on an allele with a 475 kb deletion [49]. These examples suggest that feedback inhibition could involve constraints beyond reduced accessibility and locus decontraction.

Effective feedback inhibition requires that in the initiation phase of allelic exclusion in DN thymocytes, the two *Tcrb* alleles must attempt V_{β} -to- $D_{\beta}J_{\beta}$ recombination one allele at a time. Asynchronous recombination could occur through two types of mechanisms: (1) a deterministic mechanism, in which each DN thymocyte carries one *Tcrb* allele that provides an initial substrate for V_{β} -to- $D_{\beta}J_{\beta}$ recombination; or (2) a stochastic mechanism, in which inefficient recombination on both alleles makes simultaneous V_{β} -to- $D_{\beta}J_{\beta}$ recombination highly unlikely. Recent data appears consistent with the latter. *Tcrb* alleles in DN thymocytes were shown by 3D Immuno-FISH to have the unusual property of associating at high frequency with the nuclear lamina and with foci of pericentromeric heterochromatin, two nuclear compartments often thought to be suppressive for gene expression [39;50]. Notably, associated alleles were less likely to have undergone V_{β} -to- $D_{\beta}J_{\beta}$ recombination in DN thymocytes [50]; however, these associations were not strictly monoallelic. Rather, associated and free alleles distributed stochastically in DN thymocyte nuclei, with most nuclei having either one or two associated alleles. Thus, it was proposed that frequent and stochastically distributed associations with inhibitory subnuclear compartments reduce the likelihood that two *Tcrb* alleles attempt V_{β} -to- $D_{\beta}J_{\beta}$ recombination simultaneously [50]. Nevertheless, formal proof that these interactions inhibit V_{β} -to- $D_{\beta}J_{\beta}$ recombination, and the mechanism by which they do so, are lacking. In this regard, single cell analysis showed $V_{\beta}8.2$ transcription to be biallelic in all DN3 thymocytes [48]. Thus, inhibition may not involve modulation of V_{β} accessibility.

***Tcra/Tcrd*: Recombination from the inside out**

The *Tcra/Tcrd* locus is characterized by an even more complex developmental progression of recombination events [51]. Approximately 100 V gene segments are arrayed across 1.5 mb of the locus, with D $_{\delta}$, J $_{\delta}$ and C $_{\delta}$ gene segments, 61 J $_{\alpha}$ gene segments and C $_{\alpha}$, spanning 100 kb at the 3' end of the locus (Figure 1). V $_{\delta}$ -to-D $_{\delta}$ -to-J $_{\delta}$ recombination occurs in DN thymocytes, whereas V $_{\alpha}$ -to-J $_{\alpha}$ recombination (which deletes partially or fully rearranged *Tcrd* genes [52–54]) occurs in DP thymocytes. This developmental switch in recombinase targeting depends on the differential activation of developmental stage-specific enhancers, E $_{\delta}$ and E $_{\alpha}$ [55–57]. The locus displays regulated V segment usage, as only a small fraction of the V gene segments contribute substantially to the *Tcrd* repertoire (reviewed in [58]). Moreover, there are multiple rounds of V $_{\alpha}$ -to-J $_{\alpha}$ recombination in DP thymocytes, with primary rearrangements using 3' V $_{\alpha}$ and 5' J $_{\alpha}$ segments, and secondary rearrangements using more 5' V $_{\alpha}$ and more 3' J $_{\alpha}$ gene segments [51].

Only a handful of V gene segments dominate the adult V $_{\delta}$ repertoire (reviewed in [58]). Some (TRDV2-2, TRDV5) are situated proximal to D $_{\delta}$ gene segments, whereas others (TRAV15 family) are hundreds of kb distant. All members of this select group are characterized by active transcription and an accessible chromatin configuration in DN thymocytes [58], suggesting that promoter activity in DN thymocytes may define these as V $_{\delta}$ segments. Upon transition to DP, many additional V gene segments become active, particularly within the proximal 500 kb of the V segment array; this activation depends on the long-distance influence of E $_{\alpha}$ [58]. A concurrent locus contraction event brings proximal V gene segments much closer to J $_{\alpha}$ and C $_{\alpha}$ segments [39]. This conformational change may facilitate, or may be facilitated by, enhancer-promoter interactions. Regardless, both contraction and proximal V promoter activation may bias towards usage of 3' V $_{\alpha}$ segments in primary V $_{\alpha}$ -to-J $_{\alpha}$ recombination. Notably, TRDV4, the dominant fetal V $_{\delta}$, appears inactive in adult DN and DP thymocytes [58]; like V $_{\gamma}3$ and V $_{\gamma}4$, it may be subject to specific suppression in adults [10].

The targeting of primary V $_{\alpha}$ -to-J $_{\alpha}$ recombination events depends on the activity of two E $_{\alpha}$ -dependent promoters, T-early α (TEA) and J $_{\alpha}49$, associated with 5' J $_{\alpha}$ gene segments [59;60]. Transcriptional elongation from TEA was shown to be critical for chromatin remodeling and accessibility to the recombinase at J $_{\alpha}$ segments extending 12 kb downstream from the promoter [61;62]. Transcription-coupled trimethylation of histone H3K4, which is high across this region, may promote RAG protein binding and activity [63;64]. Nevertheless, transcription appears to suppress cryptic promoter activity and chromatin accessibility at J $_{\alpha}$ segments further downstream [60–62]. Primary V $_{\alpha}$ -to-J $_{\alpha}$ recombination deletes the TEA (and often the J $_{\alpha}49$) promoter, but places the promoter of the introduced V $_{\alpha}$ gene segment at the 5' end of the residual J $_{\alpha}$ array. Secondary V $_{\alpha}$ -to-J $_{\alpha}$ recombination is then targeted to J $_{\alpha}$ segments immediately downstream of the primary V $_{\alpha}$ -J $_{\alpha}$, presumably a consequence of chromatin remodeling events directed by transcription from the introduced V $_{\alpha}$ promoter [65]. These regulatory influences continually retarget recombination events to the most 5' of the available J $_{\alpha}$ gene segments, thereby promoting efficient use of the J $_{\alpha}$ array. Estimates suggest, on average, five rounds of V $_{\alpha}$ -to-J $_{\alpha}$ recombination on each allele [66], with the progression of recombination events terminated by either positive selection [67;68], which downregulates recombinase expression, or cell death [69]. Multiple rounds of V $_{\alpha}$ -to-J $_{\alpha}$ recombination on both alleles, in the absence of allelic exclusion, provides thymocytes multiple opportunities to generate a TCR α chain that can support positive selection [70–72].

Conclusions

Although we are developing a reasonable understanding of the factors that dictate programs of V(D)J recombination at the different TCR loci, in many cases we have only scratched the

surface when it comes to underlying mechanisms. Accessibility aside, it is still unclear how RAG proteins find RSSs and assemble synaptic complexes *in vivo*. How and to what sites is RAG recruited, or are RSSs recruited to RAG? We can only describe locus conformation in crude terms, and we can only guess at the molecular events directing conformational changes. We also lack mechanistic insight regarding changes in subnuclear positioning and their influence on recombination. Finally, it would seem that precise temporal regulation of recombination events should be critical for thymocyte development, since this would allow time to test TCR proteins for functionality. Without appropriate pacing, a productive V_{β} - D_{β} 1- J_{β} 1 rearrangement could be excised by V_{β} -to- D_{β} 2- J_{β} 2 rearrangement before it could induce β -selection, and a productive V_{α} -to- J_{α} rearrangement could be excised by further V_{α} -to- J_{α} rearrangement before it could induce positive selection. Might chromatin structure provide the “pacemaker”? Only time will tell.

Acknowledgements

I would like to thank Eugene Oltz, Hrisavgi Kondilis and Hanyu Shih for critical reading of the manuscript. Work in the author's laboratory was supported by the National Institutes of Health (R37 GM41052 and RO1 AI49934).

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Figure 1.

Structure and organization of TCR loci. Regulatory elements discussed in the text are identified above each diagram, and gene segments are identified below each diagram. Promoters are depicted by bent arrows and enhancers by ovals. Not shown in the diagram of *Tcrγ* is the nonfunctional C γ 3 cluster. The diagram of *Tcra/Tcrδ* identifies several of the most commonly used V δ gene segments (TRDV2-2, TRDV4, TRDV5, TRAV15). Diagrams of the loci are not drawn to scale and for *Tcra/Tcrδ* the diagram does not accurately reflect the number of V or J gene segments.