

The Center of Accessibility: D β Control of V(D)J Recombination

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Abstract Developmental patterning of antigen receptor gene assembly in lymphocyte precursors correlates with decondensation of the chromatin surrounding individual gene segments. Ongoing V(D)J recombination is associated with hyperacetylation of histones H3 and H4 and the expression of sterile germline transcripts across the region of recombinational accessibility. Likewise, histone acetyltransferase and SWI/SNF chromatin remodeling complexes each appear to be required for recombination, and the PHD-finger of RAG-2 preferentially associates with recombination signal sequence (RSS) chromatin that contains H3 trimethylated on lysine 4. However, the regulatory mechanisms that direct chromatin alteration and rearrangement have proven elusive, due in large part to the interdependency of individual stages in gene activation, our limited understanding of functional significance of changes to the histone code, and the difficulty of modeling recombinational accessibility in existing experimental systems. Examining *Tcrb* assembly in developing thymocytes, we review the central roles of RSS elements and germline promoters as foci for epigenetic reorganization of recombinationally accessible gene segments in light of recent findings and persistent questions.

Keywords T cell receptor · *Tcrb* · Transcription · V(D)J recombination · Thymocytes · Double negative

Abbreviations

RSS Recombination signal sequence
Tcrb T cell receptor β locus

Tcra T cell receptor α locus
DN Double negative
DP Double positive
ACEs Accessibility control elements
E β *Tcrb* enhancer, PD β 1, D β 1 promoter
Rag Recombination activating gene

Introduction

The genes that encode our antigen receptors undergo an elaborate and carefully orchestrated series of somatic rearrangements during lymphocyte development (Arstila et al. 1999; Rajewsky 1996). This process of antigen receptor gene assembly, dubbed V(D)J recombination after the Variable, Diversity and Joining gene segments that are recombined, provides for the enormous combinatorial and junctional diversity of antigen binding domains. Seven antigen receptor genes are each recombined by a conserved enzymatic complex centered around the lymphocyte-specific RAG1/2 proteins (Murre 2009; Oettinger 1990), which in turn recognize a conserved recombination signal sequence (RSS) that flanks each V, D, and J gene segment. Yet proper lymphocyte development and function requires that V(D)J recombination follow a precise program of ordered gene assembly, imposed in part by genetic variations in the functionality of individual RSS elements and in part by epigenetic regulation of the transcriptional control elements that populate each antigen receptor gene locus. Here, we review recent findings regarding the regulatory architecture surrounding the T cell receptor β locus (*Tcrb*) D and J gene segments (Fig. 1), focusing on RSS and promoter elements and their role in controlling the order of V(D)J recombination.

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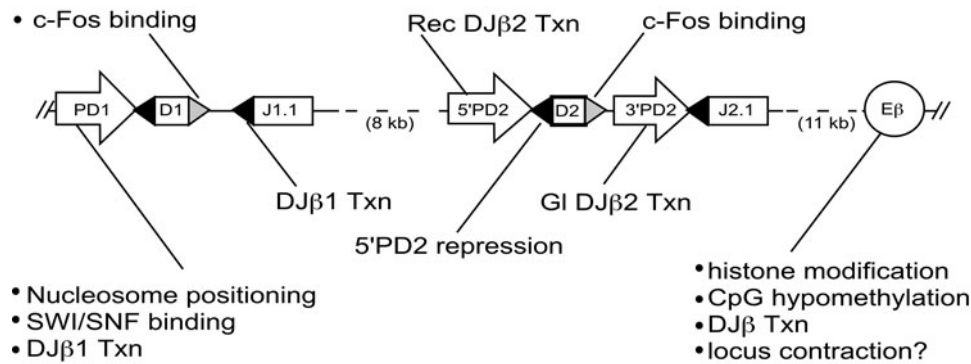


Fig. 1 Accessibility control elements across DJ β . In DN thymocytes, germline *Tcrb* transcription (abbreviated “Txn”) and recombination are controlled by D β promoters (black triangles) that establish physical contact with the downstream E β enhancer (circle). Recruitment of transcription factors, histone modifiers, and chromatin remodelers to the promoters and enhancer have each been linked to

DJ β recombinational accessibility. Additionally, both D β 23-RSSs (grey triangles) and at least two 12-RSSs (black triangles) have been implicated as accessibility control elements either directly through potential to guide nucleosome positioning in vitro, or indirectly by interacting with the transcriptional machinery

Assembly of a functional antigen receptor gene requires that one of a pool of V segments be ligated to one of several Js, or in loci that contain D elements, one V to one D to one J. During rearrangement, RSSs that flank the targeted gene segments are bound by complexes of RAG1 and RAG2 (Oettinger 1990; Schatz et al. 1989), which introduce double-stranded DNA breaks precisely at the boundaries between the RSSs and coding sequences (Gellert 2002). Cleavage products are then ligated by ubiquitous DNA repair enzymes to form extrachromosomal signal joints and chromosomal coding joints (Gellert 2002).

B and T cells each develop from a common lymphoid progenitor (Murre 2009). T cell development begins when early thymocyte progenitors (ETPs) emigrate from the bone marrow to the subcapsular region of the thymus cortex. Progression of early thymocytes from ETP through pro-T or CD4[−]CD8[−] double negative (DN) development is coincident with rearrangement of *Tcrb*, *Tcrg* and *Tcrd* (Krange 2009). Assembly of *Tcrb* and *Tcrd* is a two-step process, involving initial D-to-J assembly, and then V recombination exclusively to DJ joints. Lacking D elements, *Tcrg* and T cell receptor α locus (*Tcra*) are assembled in one V-to-J joint. If cells assemble functional *Tcrg* and *Tcrd* joints before completing *Tcrb* assembly, they commit to the $\gamma\delta$ lineage (von Boehmer 2004). Conversely, expression of a rearranged *Tcrb* gene drives development to the pre-T or CD4⁺CD8⁺ double positive (DP) stage where *Tcra* rearrangement occurs. Importantly, *Tcra* recombination leads to deletion of the *Tcrd* locus embedded between the V α and J α gene segments, regulating the ratio of $\alpha\beta$ to $\gamma\delta$ T cells (Krange 2009).

Analogous rearrangement and development patterns are observed for the immunoglobulin (*Ig*) heavy and then light chain loci during B cell development (Alt et al. 1984). As with *Tcrb* in DN thymocytes, *IgH* genes are assembled in

bone marrow CD117⁺CD25[−] pro-B cells via a two-step process in which D-to-J recombination is followed by V-to-DJ recombination. Assembly of an in-frame V(D)J_H joint leads to a burst of proliferation and developmental progression to CD117[−]CD25⁺ pre-B cells where the *Igk* and *Igl* light chain genes are assembled.

RSS Regulation of Recombination

Early experiments demonstrated that the order of V(D)J recombination is not imposed at the level of recombinase expression. While ectopic expression of the RAG proteins in murine fibroblasts directed efficient recombination of transiently transfected substrates, endogenous antigen receptor genes remained refractory to recombinase until transcriptionally activated (Goebel et al. 2001; Schatz and Baltimore 1988). Similarly, chromatinizing double-stranded RSS templates was sufficient to impair in vitro RAG-mediated cleavage (Golding et al. 1999), suggesting that the developmental and cell type specificity of V(D)J recombination is regulated at the level of recombinase accessibility to individual RSS substrates. Though first proposed by Alt and colleagues in 1986 (Alt et al. 1986; Golding et al. 1999), a molecular definition for the so-called recombinational accessibility hypothesis has proved elusive.

Initially viewed as static docking sites for the RAG proteins, RSS elements have received considerable attention as recombinational accessibility control elements (ACEs). All functional RSS elements share relatively conserved sequences including A/T-rich nonamer and palindromic heptamer elements, separated by a nonconserved spacer sequence of either 12 or 23 bp. In support of an accessibility model in which RAG binding requires

chromatin “opening” and histone displacement, consensus RSS nonamers have been shown to contain elements that effectively position nucleosomes across the RAG binding sites, thereby limiting recombinase access until nucleosome removal (Baumann et al. 2003). In contrast, in vivo analyses of D and J gene segments in *Tcra* and *Tcrb* found no consistent nucleosomal positioning across individual RSSs (Kondilis-Mangum et al. 2010), a distinction the authors attribute to the dominant nucleosomal positioning effect of neighboring transcription control elements (discussed below). Consequently, though RSS nonamers may possess intrinsic nucleosome positioning activity, they are unlikely play more than a minimal role in establishing gene segment chromatin organization.

Efficient recombination only occurs under physiological conditions between RSSs of differing spacer size. This 12–23 restriction ensures D segment inclusion in *IgH* rearrangements, where V and J segments are each flanked by 23-RSSs, and D is flanked 5′ and 3′ by 12-RSSs. In contrast, *Tcrb* is organized such that 12-RSSs are found 5′ of each D and J β segment, and 23-RSSs are positioned 3′ of the V and D β segments. Despite the 12–23 compatibility of V and J β RSSs, no V-to-J β rearrangements are observed on wild-type *Tcrb*, nor do V β segments rearrange with germline D β s. Studies aimed at dissecting this restricted assembly regulation, dubbed “beyond 12–23” (B12–23), suggest a critical role for the D β RSSs and their flanking sequences (Tillman et al. 2004). For example, V-to-J β assembly can be induced when either RSS is replaced in kind with a D β RSS (e.g. replacing the V β 23-RSS with the D β 1 23-RSS (Bassing et al. 2000, 2002; Hughes et al. 2003; Sleckman et al. 2000; Tillman et al. 2004)). However, studies using extrachromosomal substrates clearly demonstrate that much of the B12–23 restriction is directly encoded in the RSS components and flanking sequences themselves rather than through regulation of higher order epigenetic assemblies. Specifically, the Schatz laboratory has shown that RSS naked DNA templates recapitulate B12–23 assembly in non-lymphoid cells or in cell-free extracts with purified RAG proteins (Jung et al. 2003), and that D β 1 12-RSS is nicked much faster than J β 12-RSSs (Drejer-Teel et al. 2007).

More recent analyses of RAG-mediated RSS nicks on in vitro and in vivo substrates suggest a mechanism for the B12–23 restriction whereby D β 23-RSS and 12-RSS initiate recombination, binding RAGs and then capturing either J β or V β RSSs to form respective DJ β or V-DJ β synaptic complexes (Franchini et al. 2009). These same studies further indicated that the D β 1 23-RSS impedes RAG-mediated cleavage at the D β 1 12-RSS, thereby restricting V β assembly to DJ β joints. Finally, binding of the AP-1 transcription factor, c-Fos, to conserved sites in the 3′ RSS of both D β segments has been shown to impair

cleavage at the 5′ D β 1 12-RSS on extrachromosomal plasmids and thereby limit V β -to-D β recombination (Wang et al. 2008). Collectively then, the various studies that have assessed B12–23 would suggest a primarily genetic role for the D β RSS elements in guiding *Tcrb* recombination through preferential and sequential recruitment of RAG proteins, first the D β 23-RSS and then the D β 12-RSS, relative to the V and J β RSSs.

Because c-Fos is a transcription factor, remains unclear whether AP-1 binding at D β simply contributes to an allosteric mechanism of D β 23-RSS preference or whether it helps enforce D β usage by activating associated D β germline promoters. When chromosomal D β 1 and J β 1.1 segments were moved away from the D β 1 promoter and into the location of downstream V β 14, their assembly in mutant mice was delayed until V β segments gained accessibility (Yang-Iott et al. 2010), suggesting proximity to PD β 1 is critical. Sleckman and colleagues recently found that deletion of the J β 1.1 RSS from a highly modified *Tcrb* allele severely attenuated transcription through the DJ β 1 cassette, suggesting the presence of an unidentified transcription control element within the J β 1.1 RSS (Khor et al. 2009). Finally, we have recently found that upstream stimulatory factor binds a conserved E-box positioned within the 5′D β 2 12-RSS spacer, repressing the activity of a promoter positioned immediately upstream of D β 2 (McMillan et al. in preparation). Given the role of promoters in directing recombinational accessibility (discussed below), these separate findings suggest that beyond their genetic impact on recombinational control, the ACE activities associated with *Tcrb* RSSs may primarily derive from their contributions to germline transcription.

Transcriptional Control of Recombinational Accessibility

The onset of recombination correlates with germline transcription of D and/or J gene segments in both Ig and TCR loci (Lennon and Perry 1990; Royer et al. 1985; Van Ness et al. 1981; Yancopoulos and Alt 1985). At individual loci, where one or a small number of enhancers must regulate the activity of multiple V-, D-, or J-associated promoters, targeted enhancer deletion impairs germline transcription (Bories et al. 1996; Bouvier et al. 1996; Chen et al. 1993; Monroe et al. 1999; Serwe and Sablitzky 1993; Sleckman et al. 1997; Xiong et al. 2002; Xu et al. 1996) and leads to a reduction in the levels of histone acetylation over large portions of the targeted locus (Mathieu et al. 2000). Significantly, enhancerless loci also show marked decreases in V(D)J recombination (Bories et al. 1996; Bouvier et al. 1996; Chen et al. 1993; Monroe et al. 1999; Serwe and Sablitzky 1993; Sleckman et al. 1997; Xiong et al. 2002;

Xu et al. 1996), suggesting that enhancers help coordinate the epigenetic modifications that permit recombinase recruitment. For example, deletion of the *Tcrb* enhancer *E β* leads to a two- to threefold drop in histone acetylation levels over a 30 kb domain that contains both D-J-C β gene segment clusters (Mathieu et al. 2000) and blocks D β transcription and rearrangement (Bories et al. 1996; Bouvier et al. 1996).

Gene targeting experiments suggest a similar though more localized role for promoter activity in directing V(D)J recombination (Chowdhury and Sen 2004; Krangel et al. 2004; Oltz 2001; Schlissel 2004). Whereas *E β* deletion abolishes all *Tcrb* assembly (Bories et al. 1996; Bouvier et al. 1996), deletion of the germline promoter associated with D β 1 (Sikes et al. 1998) impairs recombination of the DJ β 1 cassette (Sikes et al. 1999; Whitehurst et al. 1999), but not recombination at DJ β 2 (Whitehurst et al. 1999), which is under transcriptional control of separate promoters that flank D β 2 (McMillan and Sikes 2008, 2009). The mechanism(s) through which promoters regulate recombinational accessibility are as yet undetermined. Individual promoters assemble distinct and complex networks of regulatory factors and stimulate very different levels of transcription in response to homeostatic, metabolic, or developmental cues. Despite such diversity, a consensus framework for promoter activation generally involves transcription factor binding, recruitment of histone modifying enzymes and chromatin remodelers, assembly of the transcriptosome and subsequent transcription. Krangel et al. showed that a blockade of transcriptional elongation in the *J α* array leads to suppression of TCR α recombination and to chromatin remodeling of *J α* segments thus establishing a role for germline transcription in assembly of the *Tcra* locus (Abarrategui and Krangel 2006). Conversely, rearrangement of a *Tcrb* minilocus transgene was unaffected by inversion of the D β 1 promoter, which dramatically attenuated transcription through the D β 1 and J β 1 segments (Sikes et al. 2002).

Apparent differences between promoter contributions to *Tcra* recombination and that of *Tcrb* are also observed at the epigenetic level. Deletion of the D β 1 germline promoter had no measurable impact on histone acetylation levels of chromosomal *Tcrb* miniloci (Sikes et al. 2002), and only modest impact on H3 acetylation in PD β 1-deficient mice despite inhibition of D β 1 recombination and transcription (Whitehurst et al. 2000). In contrast, H3 and H4 acetylation levels across a region of *Tcra* that contains the most 5' *J α* segments were dramatically impaired in DP thymocytes of mice that lack the T early alpha (TEA) germline promoter (Hawwari et al. 2005).

Do promoters have discrete routes toward augmenting recombinational accessibility, or does accessibility derive from actions central to transcriptional initiation by

combinatorially regulated promoters? By replacing the D β 1 promoter in *Tcrb* miniloci with a variety of alternative promoters, we previously showed that promoter activation rather than activity of a specific promoter is required for D-to-J β recombination (Sikes et al. 1999). Indeed, Oltz and colleagues found that recruitment of SWI/SNF to promoter-less miniloci was sufficient to restore D β 1 transcription and recombination (Osipovich et al. 2007). The need for promoter recruitment of SWI/SNF to displace histones from the D β 1 RSS could explain both the apparent transcriptional independence of PD β 1 recombinational control and the sensitivity of DJ β 1 recombination to PD β 1 repositioning away from the D. When PD β 1 was moved from its native position 5' of D β 1 to locations between D β 1 and J β 1.1 or downstream of J β 1.2 in a *Tcrb* minilocus transgene, levels of transgene recombination were progressively attenuated with distance from the D (Sikes et al. 2002). The physiological impact of this position effect on promoter-dependent recombination is illustrated in D segment usage during *Tcrb* assembly, where rearrangement of the DJ β 1 cassette outpaces that of DJ β 2 (Born et al. 1985; Haars et al. 1986; Lindsten et al. 1987; Uematsu et al. 1988). Like D β 1, D β 2 is flanked by a 5' promoter that presumably enforces DJ β accessibility during V-to-DJ recombination. However, in the germline locus, the 5'PD β 2 is held inactive and transcription of the DJ β 2 cassette is instead dependent on a second promoter positioned between D β 2 and J β 2.1 (Fig. 1) that initiates transcription several hundred base-pairs downstream of the D β 2 RSS (McMillan and Sikes 2008). Because DJ β 2 joints accumulate more slowly than do DJ β 1 joints, initial V β arrangements preferentially target the upstream DJ β , allowing DJ β 2 joints to act as a reserve substrate for secondary V β rearrangements to rescue frame shifted V(D)J joints. This maximizes both β -selection survival and *Tcrb* repertoire diversity.

The downstream *E β* enhancer loops back to make physical contact with both D β 1 and D β 2 (Oestreich et al. 2006). Even longer range looping has been shown to reversibly contract both *Ig* and *Tcr* loci during lymphocyte development (Fitzsimmons et al. 2007; Fuxa et al. 2004; Sayegh et al. 2005; Skok et al. 2007), and facilitate reorganization of distal V elements away from or toward heterochromatic perinuclear or pericentromeric regions during rearrangement or allelic exclusion (Kosak et al. 2002; Roldan et al. 2005; Schlimgen et al. 2008). Short-range looping caused by *E β* contact with D β promoters might facilitate general chromatin opening across J β , while also stimulating D β promoter activity. However, such a model raises interesting questions. How would V β chromatin be recruited to newly formed DJ β assemblies if *E β* remains tethered to DJ β ? Assuming V β promoters act like their D β counterparts to govern gene segment accessibility,

are their actions similarly dependent on E β or on undefined enhancers in the upstream portion of the locus? Additionally, whereas V elements are generally hundreds of kb removed from D or J elements, the D β s are only no more than three or four nucleosomes upstream of their most proximal J β elements. Is epigenetic regulation of such proximal gene segments distinct from the mechanism that regulates assembly of widely separated segments?

RAG2 contains a C-terminal PHD-finger domain that sensitizes RAG binding to histone octamers in which histone 3 is covalently trimethylated on lysine 4 (H3K4me3) (Matthews et al. 2007), an epigenetic mark exclusively associated with transcriptionally active genes and predominantly deposited near the transcriptional start site. Paradoxically, while RAG2 has been shown to localize at chromatin sites enriched in H3K4me3 (Ji et al. 2010), in vitro chromatinization studies have demonstrated the inhibitory nature of nucleosomal assembly on RAG binding (Golding et al. 1999). In their recent survey of each antigen receptor locus, Schatz and colleagues definitively showed that RAG2 binding directly correlates with H3K4me3 enrichment (Ji et al. 2010). For example, binding of RAG2 at sites along the *Tcrb* locus of pro-T thymocytes from RAG1-deficient mice is essentially restricted to the D β and J β gene segments, which unlike the V β segments in these cells are strongly enriched for H3K4me3.

How then, if histone occupancy is inhibitory for RAG binding, does H3K4me3 enrichment mesh with recombinational accessibility? The answer may lie in the nucleosomal architecture assumed across the rearranging gene segments. Transcriptional activation leads to the reorganization or phasing of nucleosomes proximal to the transcription start site. Using tiled PCRs to examine mononucleosomes, Krangel and colleagues found that both D β elements and J α 61 assume an ordered nucleosomal structure in the presence of enhancer activity, leaving their RSS elements relatively nucleosome-free (Kondilis-Mangum et al. 2010). Moreover, transcription from the upstream TEA promoter induces nucleosome repositioning relative to the J α 61 RSS. By extension, as polymerase passes through an RSS, recombinational accessibility could derive from the transient displacement of histones from the RSS and the recruitment of RAG complexes by H3K4me3-marked histones in newly reconstituted or repositioned nucleosomes.

Outlook

The current epigenetic model of accessibility involves initial nucleation of histone modifying and chromatin remodeling proteins at germline promoters and enhancers, likely through recruitment by defined transcription factor

assemblies. Subsequent germline transcription would then extend histone reorganization, particularly H3K4me3 deposition downstream of the transcription start site, relieving any final epigenetic barriers to accessibility at transcribed RSS elements. Though individual RSS elements show clear and remarkable sequence-specific impact on the order of gene segment accessibility, such genetic regulation is imposed within the context of a chromatin dynamism that appears to be primarily governed by the components of germline transcription. It should be noted that even prior to germline transcription and locus assembly, separate studies have demonstrated that promoters and enhancers of *Tcra* and *Tcrb* are already marked by transcription factor binding and histone modifications (Hernandez-Munain et al. 1999; Weishaupt et al. 2010), suggesting that the pathway to recombinational accessibility starts before lineage identity is established. Such epigenetic priming is common among developmental control genes, and its persistence at the TCR genes is consistent with the regulatory role recombination plays in thymocyte development. In light of the various chromatin and transcriptional regulators currently implicated in accessibility control, a true understanding recombinational accessibility will require dissecting their mechanistic hierarchy at separate loci, and perhaps for individual rearrangements. Consequently, the rich regulatory landscape that has been defined for D β 1 and D β 2 make *Tcrb* assembly a powerful model system for unraveling the epigenetics of V(D)J recombination.

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