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Promoters, enhancers, and transcription target RAG1 binding during V(D)J recombination

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V(D)J recombination assembles antigen receptor genes in a well-defined order during lymphocyte development. This sequential process has long been understood in the context of the accessibility model, which states that V(D)J recombination is regulated by controlling the ability of the recombination machinery to gain access to its chromosomal substrates. Indeed, many features of "open" chromatin correlate with V(D)J recombination, and promoters and enhancers have been strongly implicated in creating a recombinase-accessible configuration in neighboring chromatin. An important prediction of the accessibility model is that cis-elements and transcription control binding of the recombination-activating gene 1 (RAG1) and RAG2 proteins to their DNA targets. However, this prediction has not been tested directly. In this study, we use mutant Tcra and Tcrb alleles to demonstrate that enhancers control RAG1 binding globally at Jα or Dβ/Jβ gene segments, that promoters and transcription direct RAG1 binding locally, and that RAG1 binding can be targeted in the absence of RAG2. These findings reveal important features of the genetic mechanisms that regulate RAG binding and provide a direct confirmation of the accessibility model.
process, the immunoglobulin loci undergo little or no recombination. Tcra locus assembly is itself a strictly ordered process, with D-to-J joining occurring before V-to-DJ joining. This precise regulation is achieved despite the use of the same enzymatic machinery for all recombination events and the conserved sequence features shared by all RSSs.

Our understanding of the mechanisms that dictate ordered V(D)J recombination has for many years been guided by the accessibility model (Yancopoulos and Alt, 1985), which proposes that the access of chromatinized RSSs to the V(D)J recombinase is modulated by developmental and stage-specific mechanisms. The model has received support from a wide range of experiments. V(D)J recombination of specific gene segments strongly correlates with features reflecting an open configuration at associated chromatin, including nuclease sensitivity, germline transcription, activating histone modifications, and DNA hypomethylation (Cobb et al., 2006; Jung et al., 2006; Krangel, 2007). Both in vivo (Stanhope-Baker et al., 1996) and biochemical studies (Kwon et al., 1998; Golding et al., 1999) have demonstrated that chromatin represents a significant barrier to the initiation of V(D)J recombination, and numerous findings indicate that promoters, enhancers, transcription factors, and transcription itself play key roles in overcoming this barrier. A central prediction of the accessibility model is therefore that transcriptional control elements and transcription are critical for allowing the recombination machinery to gain access to RSSs. However, this prediction has not been tested directly because methods for measuring RAG binding to DNA in vivo were unavailable.

We recently demonstrated, using chromatin immunoprecipitation (ChIP), that RAG1 and RAG2 bind to a focal region (termed the “recombination center”) containing some or all of the J gene segments within the Ig heavy chain (Igh), Igk, Tcrb, and Tcra loci (Ji et al., 2010). Importantly, RAG1 and RAG2 were found to be recruited independently of one another into Igk, Tcrb, and Tcra recombination centers. Although RAG2 binding closely mirrored the distribution of H3K4me3 throughout the entire genome, RAG1 binding was suggested to be strongly dependent on direct recognition of the RSS (Ji et al., 2010). How RAG1 binding is targeted and how this relates to the mechanisms that control accessibility is not known.

Here, we demonstrate that promoters, enhancers, and transcription are critical regulators of RAG1 binding to the Tcrb and Tcra loci, thereby validating a central tenet of the accessibility model.

RESULTS AND DISCUSSION

Control of RAG1 binding in the Tcra locus

The 1.6-Mb Tcra locus contains 61 J gene segments distributed throughout a 65-kb region near its 3' end and ~100 V gene segments scattered over a large 5' region of the locus (Fig. 1 A). We recently found that RAG binding to Tcra chromatin occurs in DP but not DN thymocytes and focuses on the most 5' Jx gene segments (Ji et al., 2010), which are strongly preferred in initial Tcra gene rearrangements (Krangel, 2007). Little or no binding was detected to Vx gene segments, leading us to propose that RAG proteins bind first to Jx segments, forming a “recombination center,” within...
which the RAG proteins capture a V\(\alpha\) segment for recombination (Ji et al., 2010).

To investigate how T\(\alpha\)n locus assembly is controlled, we determined the pattern of RAG1 binding to six mutant T\(\alpha\)n alleles in which transcriptional control elements were deleted or repositioned, or in which transcriptional elongation was blocked (Fig. 1 A; shading indicates regions in which recombination is inhibited as a result of the mutation). WT and mutant alleles were analyzed in thymocytes from mice that were deficient in RAG2 and that expressed a rearranged T\(\alpha\)b transgene. The absence of RAG2 ensured that all T\(\alpha\)n alleles remained in their unarranged configuration while the T\(\alpha\)b transgene allowed for the development of DP thymocytes, the cellular subset in which T\(\alpha\)n recombination takes place. The T\(\alpha\)n enhancer (E\(\alpha\)), which lies 3’ of the C\(\alpha\) constant region, is critical for T\(\alpha\)n locus recombination, germline transcription from the TEA promoter (Sleckman et al., 1997), and histone acetylation across a 500-kb region that spans all of the J\(\alpha\) gene segments and the 3’ portion of the V\(\alpha\) cluster (Hawwari and Krangel, 2005; McMurry and Krangel, 2000). As expected (Ji et al., 2010), the WT T\(\alpha\)n allele showed strong binding of RAG1 at the most 5’ J\(\alpha\) gene segments analyzed (TRAJ61 and TRAJ58) and substantial acetylation of histone H3 across the majority of V\(\alpha\) (TRAJ61 and TRAJ58) and substantial acetylation of histone H3 across the majority of V\(\alpha\) gene segments analyzed (TRAJ61–TRAJ52; Villey et al., 1996; Hawwari et al., 2005), and the J\(\alpha\)52 promoter, which is located within the 56R allele displays robust binding upstream of the termina
tor (TRAJ61, TRAJ58, and TRAJ56), and weak binding at TRAJ61–TRAJ52 interval (Fig. 3 A and B), whereas the 56R allele displayed robust binding upstream of the terminator (TRAJ61, TRAJ58, and TRAJ56), and weak binding at TRAJ53 and TRAJ52 (Fig. 3 C). These findings strongly argue that transcripts initiating at the TEA promoter facilitate V(D)J recombination by virtue of their elongation through the TRAJ61–TRAJ52 region, thereby rendering RSSs in the transcribed region accessible to RAG1 binding.

T\(\alpha\)n alleles typically undergo multiple V(D)J recombination events that use progressively more 3’ J\(\alpha\) gene segments, with each secondary event deleting the previously formed V\(\alpha\)\(\alpha\) segment. The current model to explain the targeting of secondary T\(\alpha\)n recombination events proposes that the promoter of the V\(\alpha\)\(\alpha\) segment renders proximal downstream J\(\alpha\) segments accessible for recombination (Hawwari and Krangel, 2007). Evidence for this model derives from a T\(\alpha\)n allele engineered to contain a TRAV17–TRAJ57 junction (HY\(\alpha\) allele) in which the earliest subsequent recombination events are focused strongly on the region immediately downstream of the HY\(\alpha\) segment, from TRAJ56 to TRAJ52 (Fig. 3 D), as previously reported (Hawwari and Krangel, 2007). When we examined the HY\(\alpha\) allele, we found that RAG1 binding was highest in this interval (Fig. 3 D), as previously reported (Hawwari and Krangel, 2007). We conclude that the presence of a V\(\alpha\)\(\alpha\) segment promotes secondary recombination by enhancing the accessibility of immediately downstream RSSs for binding by RAG1.

Control of RAG1 binding in the T\(\alpha\)b locus

The T\(\alpha\)b locus contains two D\(\beta\)-J\(\beta\) clusters in a 10-kb stretch and 31 V\(\beta\) gene segments, 30 of which lie in the 380-kb region at the 5’ end of the locus, as well as a single V\(\beta\)3 (TRBV31) that resides at the 3’ end of the locus, downstream of the T\(\alpha\)b enhancer (E\(\beta\); Fig. 1 B). We previously showed that RAG protein binding focuses on the two D\(\beta\)-J\(\beta\) clusters and that binding of RAG1 occurs in the presence or absence of RAG2 (Ji et al., 2010). Transcriptional control elements play a critical role in controlling T\(\alpha\)b assembly. Deletion of E\(\beta\) dramatically inhibits recombination of the entire T\(\alpha\)b locus (Bories et al., 1996; Bouvier et al., 1996) and strongly reduces measures of T\(\alpha\)n alleles in which a transcriptional terminator was inserted immediately downstream of TEA (TEA-T allele) or immediately downstream of TRAJ56 (56R allele; Abarrategui and Krangel, 2006; Abarrategui and Krangel, 2007; Fig. 1 A). The TEA-T allele displays a strong reduction in activating histone marks and recombination in the region spanning TRAJ61–TRAJ52, which is very similar to that caused by complete deletion of TEA (Abarrategui and Krangel, 2007). In contrast, the 56R allele displays defective recombination only in a small region downstream of TRAJ56, including TRAJ53 and TRAJ52 (Abarrategui and Krangel, 2006). When we assessed RAG1 binding to these two alleles, defects closely paralleled those observed for recombination: the TEA-T allele showed greatly diminished RAG1 binding throughout the TRAJ61–TRAJ52 region, thereby rendering RSSs in the transcribed region accessible to RAG1 binding.
Figure 2. The effect of enhancer or promoter deletion on RAG1 binding to Tcra. (A–D) Binding of RAG1 (left) or levels of H3 acetylation (H3-Ac, right) at the indicated gene segments or regions were assessed by ChIP in primary thymocytes (almost entirely DP cells) from Rag2<sup>−/−</sup> Tcrb transgenic mice homozygous for a WT Tcra allele (A), the ΔEx allele (B), the ΔTEA allele (C), or the ΔTEA ΔJ49 allele (D). DNA recovery in immunoprecipitates and in input DNA samples was assessed by qPCR and relative immunoprecipitation/input<sub>corr</sub> values were calculated as described in Materials and methods. These values have been corrected for background and are expressed relative to the signal obtained at the TRBD1 (Dβ1) gene segment, which was set arbitrarily to a value of 100. TRBD1 binds RAG1 robustly and exhibits substantial H3 acetylation in Rag2<sup>−/−</sup> x Tcrb-transgenic thymocytes (Ji et al., 2010 and not depicted). Data are the mean of four (A, RAG1), five (A, H3-Ac), three (C, RAG1), or two (all others) independent experiments involving individual mice, with bars indicating the mean and error bars representing the SEM. ND, not done.
chromatin accessibility across both $\text{D}\beta\text{-J}\beta$ clusters (Mathieu et al., 2000). In contrast, deletion of $\text{PD}\beta 1$, the germline promoter associated with the TRBD1 gene segment, strongly reduces recombination and measures of accessibility at the first $\text{D}\beta\text{-J}\beta$ cluster, but not the second (Whitehurst et al., 1999, 2000). To determine whether $\text{E}\beta$ and $\text{PD}\beta 1$ regulate V(D)J recombination by controlling RAG protein binding, we performed RAG1 ChIP on WT, $\Delta\text{E}\beta$, and $\Delta\text{PD}\beta 1$ alleles in DN thymocytes from Rag2$^{-/-}$ mice (WT and $\Delta\text{PD}\beta 1$ alleles) or Rag2$^{+/-}$ mice ($\Delta\text{E}\beta$ allele). RAG2 deficiency was used to maintain the WT and $\Delta\text{PD}\beta 1$ alleles in germline configuration and arrest development at the DN stage, but was not

Figure 3. The effect of transcription termination or a rearranged V,D,J segment on RAG1 binding to Tcra. (A–D) Binding of RAG1 (left) or levels of H3 acetylation (H3-Ac, right) at the indicated gene segments or regions were assessed by ChIP in primary thymocytes from Rag2$^{-/-}$ Tcrb transgenic mice homozygous for a WT Tcra allele (A), the TEA-T allele (B), the 56R allele (C), or the HY$\alpha$ allele (D). Data in A for the WT allele are reproduced from Fig. 2 A to facilitate comparisons. Data in B–D are the mean of two independent experiments involving individual mice and are presented as in Fig. 2. Asterisk: two copies of TRAV17 are present in the HY$\alpha$ allele (its germline location and the V,D,J segment) and both copies are detected by the qPCR assay, which amplifies sequences upstream of the TRAV17 RSS. ND, not done.
required for ΔEβ homozygous mice, which have developmental and recombination defects similar to those of Rag2−/− mice (Bories et al., 1996; Bouvier et al., 1996).

As expected (Ji et al., 2010), the WT Tcα allele exhibited RAG1 binding at both the first and second Dβ-Jβ clusters, but not at the three Vβ gene segments assayed (Fig. 4 A). Deletion of Eβ eliminated RAG1 binding and reduced H3 acetylation across both Dβ-Jβ clusters (Fig. 4 B), whereas deletion of PDb1 only affected RAG1 binding and H3 acetylation at the first Dβ-Jβ cluster (Fig. 4 C). Hence, in both the Tcα and Tcβ loci, enhancers exert global control of V(D)J recombination, whereas promoters operate in a local manner, and they do so by enabling the recombination machinery access to RSSs. A previous study found that TRBJ1.6 retains substantial nuclease sensitivity on a ΔPDb1 allele (Oestreich et al., 2006). Our data indicate that this is not sufficient to allow detectable RAG1 binding (Fig. 4 C), and hence that Eβ is not sufficient in the absence of PDb1 to support RAG1 binding to TRBJ1 gene segments.

In the mutant Tcα or Tcβ alleles analyzed, we observed a striking spatial correspondence between the region of the locus that suffers a recombination defect, the region in which RAG1 binding is defective, and the region in which H3 acetylation is reduced. Given the numerous important functions of RAG2, it is remarkable that RAG1 binding in the absence of RAG2 reflects so accurately the recombination defects of the mutant alleles. We infer that transcriptional control elements and transcription elongation directly facilitate RAG–DNA binding, perhaps by disrupting RSS–nucleosome contacts (Du et al., 2008; Kondilis-Mangum et al., 2010) in a manner that is not dependent on RAG2. There are, however, two examples where the correlations are imperfect. First, in the HYα allele, early recombination events are higher at TRAJ49 and TRAJ48 than at TRAJ56–TRAJ50 (Hawwari and Krangel, 2007). In contrast, RAG1 binding (Fig. 3 D) and H3 acetylation (Fig. 3 D; Hawwari and Krangel, 2007) were strongest at TRAJ56, TRAJ53, and TRAJ52. The basis of this discrepancy, which is particularly marked at TRAJ56, is unclear (Hawwari and Krangel, 2007). Second, for all Tcα alleles analyzed, except ΔEα (most notably ΔTEA), RAG1 binding and H3 acetylation were not correlated at TRAJ48 and TRAJ37, with TRAJ48 exhibiting higher H3 acetylation but lower RAG1 binding than TRAJ37 (Fig. 2 and Fig. 3). We hypothesized that this discrepancy might be explained by better binding of RAG1 to the TRAJ37 RSS than to the TRAJ48 RSSs. However, competition gel shift experiments demonstrated that these two RSSs bind equally well to RAG1 in the presence of HMGB1 (which was included to more closely mimic the conditions found in RAG2-deficient cells; Fig. S1). We do not currently have an explanation for the discrepancy between histone acetylation and RAG1 binding at TRAJ48 and TRAJ37.

Figure 4. The effect of enhancer or promoter deletion on RAG1 binding to Tcβ.

(A–C) Binding of RAG1 (left) or levels of H3 acetylation (H3-Ac, right) at the indicated gene segments or regions were assessed by ChIP in primary thymocytes (almost entirely DN cells) from Rag2−/− mice homozygous for a WT Tcβ allele (A) or the ΔPDb1 allele (C), or Rag2−/− mice homozygous for a ΔEβ allele (B). Relative immunoprecipitation/input_corr values have been normalized to the signal obtained at the TRDD2 gene segment (arbitrarily set to a value of 100), which we have found binds RAG1 and RAG2 strongly in thymocytes (not depicted). Data are the mean of 3 (A) or 2 (B, C) independent experiments involving thymocytes pooled from 5–10 mice and are presented as in Fig. 2.
Although it was not possible to assess RAG2 binding in our experiments, we expect that the pattern of RAG2 binding would closely resemble that of RAG1 in these mutant Tna and Terb alleles, for two reasons. First, we have not previously observed a substantial difference between RAG1 and RAG2 binding patterns in antigen receptor loci (Ji et al., 2010). And second, for the mutant Tcra alleles for which it has been determined (ΔTEA, TEA-T, and 56R), H3K4me3 patterns (which should accurately predict RAG2 binding) are similar to those we observe for RAG1 (Abarrategui and Krangel, 2006, 2007), and clearly depend on transcription. Because the ΔEα and ΔEβ alleles are transcriptionally silent (Bories et al., 1996; Bouvier et al., 1996; Sleckman et al., 1997), they almost certainly lack substantial levels of both H3K4me3 and RAG2 binding, as we have shown is the case for RAG1 binding (Fig. 2 B and Fig. 4 B). The absence of RAG2 was unlikely to compromise RAG1 analysis because RAG1 binding to Tna and Terb was very similar in the presence or absence of RAG2 (Ji et al., 2010).

The accessibility model grew out of observations that transcription of germline gene segments correlated developmentally with their recombination (Yancopoulos and Alt, 1985). Subsequently, the model has been strengthened by numerous findings that link V(D)J recombination to transcriptional control elements, transcription factors, transcription elongation, activating histone modifications, nucleosome hypersensitivity, DNA hypomethylation, chromatin structure and chromatin remodeling enzymes (Cobb et al., 2006; Jung et al., 2006; Krangel, 2007). At the core of the model is the idea that all of these processes operate together to achieve a single goal: to allow a common recombination machinery (RAG1/RAG2) access to the appropriate DNA substrates (RSSs) so that binding can take place. Our experiments provide the first direct test of this idea and demonstrate that enhancers, promoters, and transcription elongation indeed control the binding of RAG1 to RSSs—and hence are critical for the formation of recombination centers, within which V(D)J recombination has been proposed to take place (Ji et al., 2010). Although regulated accessibility of RSS substrates is not the only means by which V(D)J recombination is controlled (e.g., higher order chromatin architecture plays a significant role; Jhunjhunwala et al., 2009), our findings emphasize the fundamental importance of the accessibility model in understanding the biology of V(D)J recombination.

ChIP. The antibodies and procedures used for the ChIP assay have been described in detail previously (Ji et al., 2010). In brief, total thymocytes were harvested, cross-linked with 1% HCHO, and after quenching with 0.125 M glycine, cells were washed and frozen as cell pellets. Cell pellets were resuspended in RIPA buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1% Triton X-100, and 0.1% sodium deoxycholate, 0.1% SDS) containing 0.8 M NaCl and sonicated to achieve a DNA length of approximately 300–500 bp. The resulting chromatin was incubated with anti-RAG1 polyclonal antibody (Ji et al., 2010), anti-acetylated H3 antibody (recognizing H3 acetylated on K9 or K14; Millipore), or normal rabbit IgG (Millipore), and immune complexes were isolated with Protein A agarose beads (Millipore). Input and immunoprecipitated DNAs were quantitated by duplicate qPCR, and after correction for the background signal obtained with normal rabbit IgG, the immunoprecipitation/input values were calculated as described previously (Ji et al., 2010). These values were then divided by those obtained for the TRBJ1 gene segment (Fig. 2 and Fig. 3) or the TRD2 gene segment (Fig. 4), and multiplied by 100 to yield the plotted values. Most PCR primer and Taqman probe sequences have been described previously (Ji et al., 2010). For TRD2, the following oligonucleotides were used: forward primer, 5′-GGGATAC- GACACAGTGTGG-3′; reverse primer, 5′-GGGCTGTGTTTACCTTCCAT-3′; and probe, 5′-TCTCCCCAGGCTCTTGCCCTG-3′.

MATERIALS AND METHODS

Mice and alleles. The ΔEα allele (Sleckman et al., 1997), ΔTEA allele, ΔTEAΔ49 allele (Hawwari et al., 2005), TEA-T allele (Abarrategui and Krangel, 2007), 56R allele (Abarrategui and Krangel, 2006), and HYe allele (Buch et al., 2002) were bred to homozygosity on the Rag2−/− Tcrb−/− background as described previously (Hawwari et al., 2005). The ΔPDβ1 allele (Whitehurst et al., 1999) was bred to homozygosity on the Rag2−/− background and the ΔEβ allele (Bouvier et al., 1996) was bred to homozygosity on the C57BL/6 background (Oestreich et al., 2006). All animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University Medical Center and Washington University School of Medicine.

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