The Journal of Immunology

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J Immunol 2015; 195:5103-5105; ;
doi: 10.4049/jimmunol.1502150
http://www.jimmunol.org/content/195/11/5103

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Pillars of Immunology

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Beyond Hypothesis: Direct Evidence That V(D)J Recombination Is Regulated by the Accessibility of Chromatin Substrates

Michael S. Krangel

The pioneering work of Hozumi and Tonegawa (1) revealed that B lymphocytes can assemble *Ig* genes via somatic DNA recombination. This process, V(D)J recombination, is essential to create the highly variable and clonally distributed AgR repertoires of B and T cells that are central to adaptive immunity. Ig (*Igh*, *Igk*, *Igl*) and TCR (*Tcra-Tcrd*, *Tcrb*, *Tcrg*) loci contain arrays of V, D (in some cases), and J gene segments, and in individual precursor lymphocytes, one V and one J segment, or one V, one D, and one J segment, are joined to create a functional gene encoding an AgR protein. We know now that this work is performed by the lymphoid-specific recombinase proteins RAG1 and RAG2, which create DNA double-strand breaks (DSBs) at V, D, and J recombination signal sequences (RSSs), and by nonhomologous end-joining proteins, which repair those DSBs (2).

In 1985, with the molecular machinery of V(D)J recombination still 4 y from discovery (3), Yancopoulos and Alt (4) proposed a hypothesis to explain how V(D)J recombination could be developmentally regulated in vivo. What these investigators were trying to explain was why Ig genes rearranged in B cells and TCR genes in T cells; why, at the *Igh* locus, D_H to J_H joining preceded V_H to DJ_H joining; and why *Igk* rearrangement followed *Igh* gene assembly. Such developmentally regulated rearrangement was likely critical for the logic of early B and T cell development and of particular importance to the enforcement of allelic exclusion. Yancopoulos and Alt detected tissue-specific and developmentally regulated transcription of unrearranged, germline-configured V_H gene segments and noted that this transcription was associated with those V_H gene segments becoming substrates for V(D)J recombination in developing B lymphocytes. Based on this and other lines of reasoning, they proposed that lineage-specific, developmental stage–specific, and ordered recombination of Ig and TCR genes was accomplished by a single recombinase whose accessibility to Ig and TCR locus chromatin was tightly controlled during lymphocyte development (4). The transcription detected in their assays was interpreted as an indicator of accessibility, or perhaps causal in creating accessibility (5). Yancopoulos and Alt's proposal, widely known as the "accessibility hypothesis," has guided research in this area for decades. However, in 1996, despite formal proof that a single recombinase controlled V(D)J recombination in B cells and T cells (3, 6–9), and despite many studies correlating "germline transcription" with recombination and demonstrating an important role for transcriptional enhancers as regulators of V(D)J recombination (10), accessibility remained a hypothesis for which there was no formal evidence; accessibility per se had not been directly measured. This month's *Pillars of Immunology* article by Stanhope-Baker et al. (11) provided the first direct evidence that accessibility was real, and that chromatin served as the critical "gatekeeper" for V(D)J recombination in vivo.

What made this work possible? Earlier papers by Schlissel et al. (12) and Roth et al. (13) adapted the approach of ligation-mediated PCR (LM-PCR) (14) to develop a highly sensitive assay for pre-existing DSBs in purified genomic DNA (gDNA) of developing lymphocytes. V(D)J recombination–associated DSBs were first identified in thymus DNA by Roth et al. (15) who used Southern blots to detect a linear DNA fragment resulting from cleavage at the Dδ2 and Jδ1 RSSs. However, this approach required large amounts of DNA and was most effective for unusually abundant DSBs. Using LM-PCR, Schlissel et al. (12) were able to detect RAG-dependent Ig RSS signal ends as blunt, 5′-phosphorylated molecules that had been cut precisely at the RSS heptamer. These DSBs were detectable only in cell populations known to support recombination of the gene segments in vivo (D_H and J_H DSBs in bone marrow and thymus, but Jκ DSBs in bone marrow only). Thus, Schlissel could readily detect likely V(D)J recombination intermediates in gDNA and concluded that developmental regulation in vivo was enforced at or prior to the cleavage step at which they were generated.

Equally important were developments in the Gellert and Oettinger laboratories. Van Gent et al. (16) showed that nuclear extract (NE) from a recombinase-expressing cell line, supplemented with rRAG1 protein, could cleave RSS-containing plasmids in vitro. Endogenous RAG1 and RAG2 present in the NE contributed to this activity, although rRAG2 could not. Notably, under the conditions used, cleavage could occur at a single RSS, meaning it did not occur in coupled fashion as in vivo. This would be important when subsequently applied to nuclei by Stanhope-Baker et al. (11)

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Abbreviations used in this article: DSB, double-strand break; gDNA, genomic DNA; LM-PCR, ligation-mediated PCR; NE, nuclear extract; RSS, recombination signal sequence.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1502150

because it allowed the cleavage assay to serve as a measure of accessibility of a single RSS rather than a more complex measure of the status of RSS pairs. This work was followed by that of McBlane et al. (17) showing that cleavage of RSSs in naked DNA could be achieved using preparations of purified rRAG1 and rRAG2 proteins in vitro. Taken together, these studies established that RAG1 and RAG2 function as a nuclease rather than by activating recombination indirectly.

Armed with this knowledge, Stanhope-Baker et al. (11) set out to test the accessibility hypothesis. The laboratory supplemented NEs prepared from either a recombinatorially active pre–B cell line or bovine thymus with rRAG1, and then used these reagents to probe nuclei prepared from RAG1- or RAG2-deficient pro–B cell lines, RAG1-deficient thymocytes, or pre–B cells from RAG1-deficient, μ-transgenic mice. Using LM-PCR, Stanhope-Baker et al. (11) detected DSBs at J_H2 but not Dδ2 in pro–B cells, and at Dδ2 but not Jκ1 in thymocytes, indicating lineage specificity. Jκ1 DSBs occurred in LPS-stimulated pro–B cells or in pre–B cells isolated from RAG1-deficient, μ-transgenic mice, but not in RAG-deficient pro–B cells, indicating temporal or developmental stage specificity. No DSBs were detected using nuclear templates from other cell lineages, but purified gDNA could be cleaved regardless of the source. These observations led the authors (11) to conclude that lineage- and developmental stage–specific features of chromatin structure determine whether RAG can access and cleave particular RSSs.

The authors then extended their analysis to the question of *Igh* allelic exclusion. They observed DSBs at V_H RSSs and at 5′ D_H RSSs when RAG-deficient pro–B cells were treated with supplemented NE, indicating that V_H and D_H gene segments were both accessible in pro–B cells, when V_H to DJ_H rearrangement occurs (11). However, only 5′ D_H RSSs were cleaved when the same treatment was applied to mature B cells. Stanhope-Baker et al. (11) concluded that unrearranged V_H gene segments are selectively packaged into inaccessible chromatin at later stages of B cell development, providing a mechanism for feedback inhibition of V_H to DJ_H recombination. The authors' conclusions about V gene segment chromatin structure and allelic exclusion, much like their overall conclusions about chromatin structure as the developmental regulator of V(D)J recombination, have been validated in numerous subsequent studies, and they have certainly stood the test of time (18).

In light of more recent developments, it is interesting to re-examine what the authors (11) could not fully understand. Why were rRAG proteins unable to replace the NE? This was almost certainly because the endogenous RAG proteins were full-length and contained the "non-core" regions that the rRAG proteins lacked. The truncated proteins were later shown to be less effective than the full-length proteins when assayed in vivo (19, 20), and truncated RAG2 lacks the plant homeodomain that is now understood to localize RAG2 to accessible chromatin by interaction with trimethylated histone H3 lysine 4 (21–24). The authors (11) also questioned the role of transcription, because the nuclei used as templates were not transcriptionally active. They concluded that accessibility must reflect a "stable element of chromatin structure" and conjectured that this might be unrelated to transcription (11). Of course, this was just before the explosion of information about epigenetic regulation via the covalent modification of histones (25), and about the role of transcription in depositing histone modifications, including histone acetylation (26) and H3 lysine 4 methylation (27, 28). Indeed, subsequent work would document developmental regulation of AgR locus histone modifications (29) and a direct role for germline transcription in chromosomal V(D)J recombination in vivo (30, 31). However, it was Stanhope-Baker et al. (11) who gave substance to the notion of accessibility and set the stage for the major focus on AgR locus epigenetics that has continued to this day (32, 33).

Disclosures

The author has no financial conflicts of interest.

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