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Unraveling V(D)J Recombination: Insights into Gene Regulation

Review

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V(D)J recombination assembles antigen receptor genes from component gene segments. We review findings that have shaped our current understanding of this remarkable mechanism, with a focus on two major reports—the first detailed comparison of germline and rearranged antigen receptor loci and the discovery of the recombination activating gene-1.

Overview

"The [clonal selection] theory requires at some stage in early embryonic development a genetic process for which there is no available precedent (Burnet 1957)."

B and T lymphocytes, which together comprise the adaptive arm of the vertebrate immune system, can generate specific responses to a tremendous number of antigens. At the heart of this capability are the B cell receptor (BCR) and T cell receptor (TCR) proteins, which physically bind their cognate targets and direct cellular responses to these diverse stimuli. The prescient statement above, taken from a theoretical paper by Burnet in 1957, foreshadowed by 20 years the discovery of DNA rearrangements in lymphoid cells. Burnet, modifying a theory originally put forward by Jerne, proposed that a process of "randomization," followed by antigenic selection of clones producing complementary antibodies, might account for the rapid kinetics and immense diversity of immune responses observed in vivo. Although such a genetic process was then unknown, the subsequent discoveries of V(D)J recombination and the RAG proteins, followed by intensive investigation into their mechanism and regulation, have provided an elegant molecular foundation for the clonal selection hypothesis. Elucidating V(D)J recombination has, in turn, produced novel insights into gene regulation, DNA repair, and the maintenance of genomic stability.

Historical Perspectives

The Discovery of V(D)J Recombination

In the years following Burnet's conjecture, an abundance of Ig protein sequence information made it clear that these proteins, comprised of two heavy and two light chains each, also contain subdivisions within each chain. Relatively well-conserved domains, separated by short regions of highly divergent sequence, make up the variable portion of both heavy and light chains. The constant region, in contrast, comes in only a few forms that individually do not vary in sequence; indeed, allelic constant region variants (allotypes) are inherited in a Mendelian fashion. Based on these and other observations, Dreyer and Bennett described a theoretical basis for the generation of antigen receptors through an unprecedented process involving DNA rearrangement (Dreyer and Bennett, 1965). Moreover, it had also been shown that V and C regions coexisted in a contiguous stretch of mRNA (Milstein et al., 1974), but the question remained: how were variable and constant regions incorporated into a single gene?

Answering this question was a nontrivial endeavor before the advent of molecular cloning. To approach the problem, Hozumi and Tonegawa took advantage of the newly developed techniques of restriction enzyme analysis and agarose gel electrophoresis to separate DNA fragments derived from both mouse embryos and a mouse plasmacytoma cell line. The resulting fractions were then hybridized to (largely pure) labeled κ light chain RNA derived from the cell line. While the RNA hybridized to two specific fragments from embryo DNA, it hybridized to a novel fragment from the plasmacytoma DNA (Hozumi and Tonegawa, 1976). The simplest interpretation of these results was that some distance separated variable and constant region DNA in the embryo, and that these regions had somehow been joined in the mature lymphocyte.

Direct proof that the variable and constant portions of the light chain gene had in fact been rearranged at the DNA level, however, came in conjunction with the development of molecular cloning. Working independently, Leder's group reported a comparison of germline and rearranged Igκ light chain V gene segments (Seidman et al., 1978), while Tonegawa's group, in a *Cell* paper (Brack et al., 1978), described a similar series of experiments performed on the λ light chain locus. Brack et al. generated purified phage containing germline Vλ or Cλ gene segments, as well as a recombined Vλ-Jλ-Cλ gene from a λ light chain-producing tumor line. Using restriction enzyme digestion, Southern blotting, and hybridization with known Vλ and Cλ probes, the authors confirmed that the Vλ and Cλ probes detected a single novel fragment in the cloned tumor light chain DNA. Furthermore, R loop and heteroduplex formation between the germline and rearranged fragments, coupled with electron microscopy, identified a small segment fusing the V and C sequences in the mRNA. This segment was not attached to either in germline DNA, but was attached to the V sequences a short distance upstream of the C sequences in rearranged tumor DNA. The authors named this target for V rearrangement "J," for joining (Figure 1). Thus, rather than simply appending the V region to the C region, V(D)J recombination assembles the variable region from component gene segments, with production of mature Ig light chain mRNA requiring further processing to remove the intervening J-C sequence. The Brack et al. study therefore represented an early example of intron excision through RNA splicing. Notably, the technical advances that facilitated these studies were also employed independently by other groups at about the same time to demonstrate

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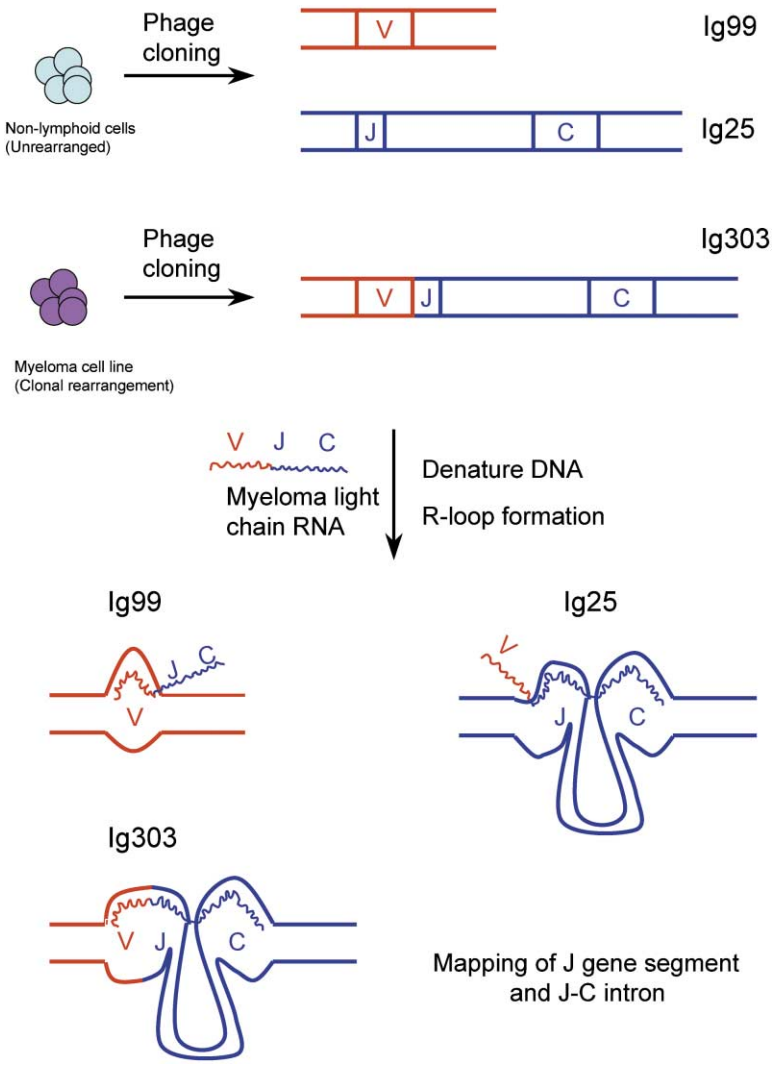

Figure 1. Mapping of Cloned Germline and Rearranged Antigen Receptor Gene Segments

See Brack et al. and text for details.

that various viral and eukaryotic genes contain intronic sequences.

From Genomic Structure to Function

With the continued development of molecular cloning and rapid methods for nucleotide sequencing, structural comparisons between cloned germline and rearranged variable region gene segments facilitated rapid progress in outlining the major features of V(D)J recombination. It was quickly appreciated that the variable region gene assembly process generates combinatorial diversity by mixing and matching V and J segments (Valbuena et al., 1978; Weigert et al., 1978). Hood's group, through protein sequence analysis, further identified a third family of gene segments (termed "D," for diversity) located between the V and J gene segments of the IgH locus (Schilling et al., 1980; Early et al., 1980). Later, the same cloning and sequencing techniques utilized to identify V(D)J recombination products of the Ig loci played a major role in identifying a similar set and organization of TCR variable region gene segments (Davis and Bjorkman, 1988). Ultimately, such work showed that the IgH, TCRβ, and TCRδ variable region exons are assembled from V, D, and J segments, while the TCRα, Igκ, Igλ, and the TCRγ variable region exons are assembled from just V and J segments (Figure 2; Lewis, 1994).

In addition to elucidating the diversity of Ig and TCR variable region gene segments, the structural studies began to shed light on the V(D)J recombination mechanism itself. Thus, recombination signal (RS) sequences, which serve as recognition sites for the recombinase machinery, were identified as short, conserved, noncoding sequences at the flanks of coding variable region gene segments. RSs consist of a relatively conserved heptamer and nonamer, with respective consensus sequences of CACAGTG and ACAAAAACC, separated by a nonconserved spacer of either 12 or 23 base pairs (Figure 2; Max et al., 1979; Sakano et al., 1979), which we will refer to as a "12RS" or a "23RS." The fact that a pair of RS sequences could generate an inverted repeat structure at the flanks of recombining coding segments raised the possibility that a RS-RS hybrid "stem-loop" might be somehow involved in synapsis (Max et al., 1979; Sakano et al., 1979), although this notion was dismissed by subsequent mechanistic studies (see below). Vκ segments are flanked with 12RSs and the Jκ segments with 23RSs, while for the Igλ locus it is the

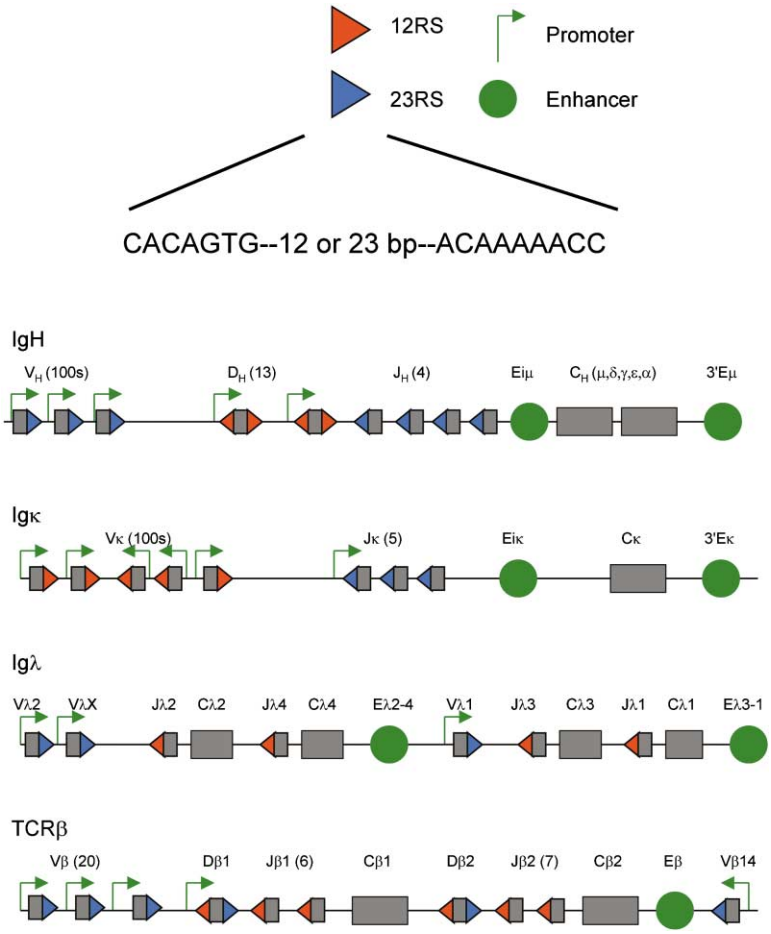

Figure 2. Genomic Structures of Selected Antigen Receptor Loci

The consensus RS sequence is listed at the top. (Adapted from Hesslein and Schatz, 2001.)

reverse. Moreover, in the IgH locus, the VH and JH segments are both flanked by 23RSs, while DH segments are flanked on either side with 12RSs. This arrangement of RSs in the various loci suggested that efficient recombination is only permitted between gene segments flanked, respectively, by a 12 and a 23RS (Figure 2; Early et al., 1980). This restriction, referred to as the "12/23 rule," directs recombination between appropriate gene segments within a given locus.

The early sequence analyses, coupled with analyses of a set of V(D)J joining events that led, by inversion, to chromosomal retention of all products of the recombination reaction, including perfectly fused RS sequences and an "imperfect" coding join, allowed derivation of a model for V(D)J recombination that incorporated several novel features (Alt and Baltimore, 1982). First, the "nonreciprocal" nature of the reaction made it apparent that V(D)J recombination involved a DNA double-stranded break (DSB) between RSs and the coding regions of a pair of recombining gene segments, followed by ligation of coding and RS ends. Second, it became possible to propose that the RS and coding ends were joined via distinct pathways with RS ends being precisely fused, while coding ends were further diversified before joining (Alt and Baltimore, 1982). In this regard, earlier sequence studies had shown that V(D)J recombination generates "junctional" diversity via loss of nucleotides at imprecisely joined borders between recombined gene segments (Max et al., 1979; Sakano et al., 1979; Seidman et al., 1979). Analyses of products of inversional recombination events led to the further proposal that junctional diversification also occurs through a novel mechanism involving addition of nontemplated "N" nucleotides via terminal deoxynucleotide transferase (Alt and Baltimore, 1982), which was subsequently proven by gene-targeted mutation (Gilfillan et al., 1993; Komori et al., 1993). Of note, the V(D)J or VJ junctional sequences encode the complementarity determining region 3 (CDR3) of Ig chains, which is a major antigen contact region (Tonegawa, 1983), and also encode an analogous antigen contact region of TCR chains (Davis and Bjorkman, 1988). Junctional diversification via the V(D)J assembly process therefore provides a major source of antibody and TCR diversity in the immune system (Davis and Bjorkman, 1988).

Beyond Sequence: Ordered Rearrangement, Allelic Exclusion, and Accessibility

Rearrangement at each antigen receptor locus is strictly regulated with respect to developmental timing (e.g., IgH before IgL), lineage specificity (e.g., VH to DJH rearrangement in B but not T cells), and allelic exclusion (see below). As predicted by the clonal selection theory, allelic exclusion ensures that a B or T cell expresses a single "specificity" of antigen receptor (Pernis et al., 1965). In this regard, a study of myeloma lines suggested that feedback regulation by a functional Ig light chain

protein, following productive assembly of a productive IgL gene on one allele, blocks further IgL gene rearrangements in the cell, thereby preventing assembly of multiple different productive IgL rearrangements (Alt et al., 1980). Relevant to the general notion of feedback regulation of allelic exclusion, subsequent analyses showed that assembly at the IgH locus is ordered, with DJH rearrangements occurring on both alleles before the initiation of VH to DJH joining. The high proportion of IgH alleles remaining in the DJH configuration after productive IgH rearrangement also provided evidence for a similar feedback regulation at the IgH locus, in which a μ heavy chain generated from a productive VH to DJH rearrangement on one allele stops further VH to DJH rearrangements (Alt et al., 1984). Proof that expression of functionally rearranged IgH and IgL genes can somehow feedback to block endogenous rearrangements came from early transgenic mouse studies (Ritchie et al., 1984; Weaver et al., 1985; Rusconi and Köhler, 1985). Moreover, gene-targeted mutation studies proved the idea, derived from earlier transgenic studies (Nussenzweig et al., 1987), that for IgH allelic exclusion, feedback is dependent on production of the membrane bound form of the IgH protein (Kitamura and Rajewsky, 1992). Many more details of how this IgH feedback process works, including a fundamental role for surrogate light chains, have been characterized, and a similar pathway of regulation has been found for TCRβ feedback regulation (Khor and Sleckman, 2002; Mårtensson et al., 2002).

The development of transfectable V(D)J recombination substrates (Blackwell and Alt, 1984; Lewis et al., 1984) greatly facilitated further investigation into the regulation of V(D)J recombination. Based on the tight correlation between transcription and V(D)J recombination, accessibility of particular V, D, and J gene segments to the V(D)J recombination machinery was proposed as the basis for developmental stage-, lineage-, and allele-specific regulation of V(D)J recombination (Yancopoulos and Alt, 1985). Further studies tested the "accessibility hypothesis" via V(D)J recombination substrates and found that introduced TCR Dβ and Jβ gene segments, but not endogenous Dβ and Jβ gene segments, rearranged in pro-B cells (Yancopoulos et al., 1986). This finding provided direct evidence that developing B and T cells utilize a common recombinase, and that, therefore, the rearrangement process must be controlled by differential accessibility. Another significant technical advance involved the development of transient V(D)J recombination substrates (Hesse et al., 1987), which could be readily assayed in many different cell lines and which were used to provide the best evidence that V(D)J recombination was a reaction that occurred specifically in developing lymphocytes. Subsequently, as discussed below, the various types of V(D)J recombination substrates were, in fact, exploited to make a truly major advance—the identification of the key tissue-specific V(D)J recombinase components (see below).

Identification of the RAG V(D)J Recombinase

To fully understand V(D)J recombination, it was clearly necessary to isolate the gene(s) encoding the V(D)J recombinase activity. Various approaches were attempted based on the known lymphoid specificity of the reaction, but Schatz and Baltimore successfully developed an elegant approach that involved activation of a chromosomally integrated V(D)J recombination substrate in a nonlymphoid cell line via transfection of sheared genomic DNA from lymphoid cells (Figure 3; Schatz and Baltimore, 1988). Inversional V(D)J recombination within the retrovirally introduced substrate conferred a drug resistance (Lewis et al., 1984), allowing specific selection of transfectants in which the V(D)J recombination process had occurred. While these studies did not prove that the transferred DNA encoded the V(D)J recombinase, the findings clearly demonstrated that the process resulted in the expression of a gene or genes sufficient to activate V(D)J recombination in a nonlymphoid cell and provided the critical finding for subsequent studies that identified the V(D)J recombinase.

Oligonucleotide tagging then was used to isolate the genes that conferred V(D)J recombination activity to the transfected fibroblast lines. Following three rounds of genomic transfections into fibroblasts, Schatz et al. (1989) identified a unique restriction fragment that cosegregated with the activity. Genomic walks within this fragment led to the coding region of the gene, named recombination activating gene-1 or RAG-1 (Figure 3; Schatz et al., 1989). The gene was given this relatively vague name because it had not been proven whether it encoded an actual V(D)J recombinase or, alternatively, acted as a transcriptional activator for V(D)J recombinase genes. Surprisingly, at the time, transfection of the RAG-1 cDNA alone failed to transfer V(D)J recombination activity. This apparent paradox was resolved by additional work demonstrating that the 18 kB phage clone containing RAG-1 also contained a second, very tightly linked gene, RAG-2, and that expression of both were necessary for the synergistic initiation of V(D)J recombination (Oettinger et al., 1990). That the two genes required for V(D)J recombinase activity should be linked within 18 kB of each other was quite fortuitous for the genomic transfection approach; indeed, had RAG-1 and RAG-2 been located further than 150 kB apart, it is highly unlikely that transfection of genomic DNA would have resulted in their cosegregation. The unique genomic structure of the RAG-1/2 locus, along with the transposase activity observed for truncated versions of the RAG proteins (see below), has been cited as potential evidence for the origin of the RAG genes from an ancestral transposon introduced into the vertebrate genome at the jawed fish (Agrawal et al., 1998; Hiom et al., 1998), a general notion first suggested based on RS structure (Sakano et al., 1979). Overall, these findings support the notion that introduction of the RAG genes may have been one of the crucial events underlying the evolution of the vertebrate adaptive immune system (Thompson, 1995).

The discovery of RAG-1 and RAG-2 has been the most important advance in the study of V(D)J recombination since the original discovery of gene rearrangement by Tonegawa and colleagues and made possible a detailed analysis of the V(D)J cleavage reaction. The discovery of the RAG genes also was of major importance in opening an entire field of study linking the repair of RAG-induced DSBs to the repair of other, environmentally induced DSBs. We now examine the post-RAG progress with respect to our understanding of V(D)J recombination and its regulation.

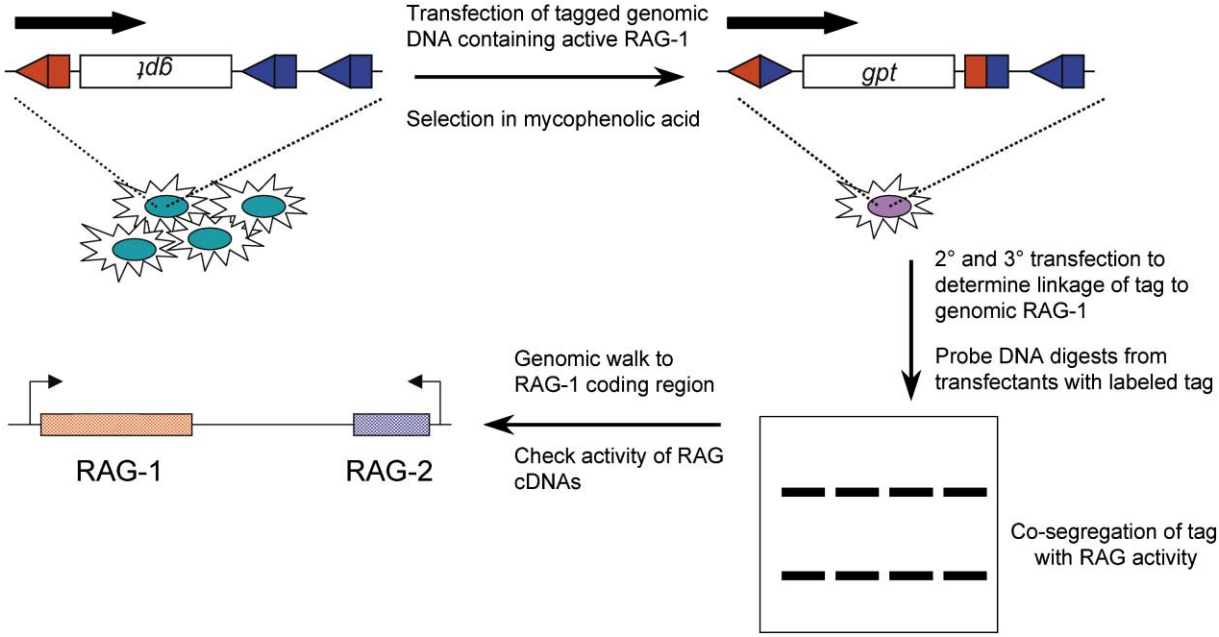

Figure 3. Cloning of the V(D)J Recombinase Genes
See Schatz et al. and text for details.

Post-RAG: Advances and Outstanding Questions Regarding V(D)J Recombination

Making the Cut—RAG-Mediated DNA Double-Strand Breaks

Following the isolation of RAG-1 and RAG-2, targeted deletion of RAG-1 (Mombaerts et al., 1992) and RAG-2 (Shinkai et al., 1992) confirmed that these genes are essential for the initiation of V(D)J recombination; RAG-1 or RAG-2-deficient animals have a severe combined immunodeficiency due to inability to assemble antigen receptor gene segments. However, the animals have no other obvious phenotypes, confounding early speculations, based on RAG-1 expression in the brain (Chun et al., 1991), that the RAG proteins may be required for some aspect of neuronal development. Thus, it appears that RAGs may have evolved to function very specifically in developing lymphocytes. Direct evidence that RAG-1 and RAG-2 form a complex (referred to as RAG) that is indeed the endonuclease that generates DSBs between V(D)J coding and RS sequences was ultimately provided by in vitro recapitulation of the cleavage reaction (van Gent et al., 1995; McBlane et al., 1995). In this regard, full-length RAG-1 and RAG-2 proteins have proven, until recently, largely insoluble in vitro. Therefore, in vitro mechanistic characterization of the RAG proteins have relied on soluble truncated "core" versions of RAG-1 and RAG-2 that are active in the cleavage/joining reaction (Figure 4A; Fugmann et al., 2000). While the use of these core proteins has been invaluable for working out the basic features of the RAG cleavage reaction, it should be noted that the non-core regions of both RAG proteins are highly conserved and are important for fully efficient V(D)J recombination in vivo (Figure 4A; Liang et al., 2002; Akamatsu et al., 2003; Dudley et al., 2003) and also may function to suppress certain "nonstandard" RAG activities (see below).

To initiate the cleavage reaction, RAG binds first to one (12 or 23) and then to a second (23 or 12) RS (Jones and Gellert, 2002; Mundy et al., 2002). Next, RAG introduces a nick precisely at the 5′ border of each RS. To complete the double-strand break, the resulting free 3′-OH on the coding strand generates a blunt, 5′-phosphorylated RS end and a closed hairpin coding end through direct nucleophilic attack on the opposite phosphodiester bond (Figure 4B; van Gent et al., 1995, 1996a; McBlane et al., 1995). The in vitro cleavage reaction reproduces the 12/23 restriction on V(D)J recombination (Eastman et al., 1996; van Gent et al., 1996b), indicating that this restriction is inherent to the basic reaction. The four free DNA ends remain associated with the RAGs in a post-cleavage complex, which may recruit general DNA repair factors (see below), protect the ends from inappropriate insertion elsewhere in the genome, and serve other functions (Figure 4B; Fugmann et al., 2000). Major unsolved problems in the RAG cleavage reaction include how RAG is targeted to chromosomal V(D)J segments in the context of higher order chromatin structure and how the RAG-cleavage complex may intersect with general DNA repair pathways during the joining phase of the reaction.

Various lines of evidence have indicated that rearrangements of particular combinations of V, D, and J segments may be restricted in a manner not explained by simple 12/23 compatibility (Lieber et al., 1994; Feeney et al., 2000). A telling example of this phenomenon is the greater ability of 3′DH 12RSs but not 5′DH 12RSs to mediate recombination to a JH 23RS in a recombination substrate (Gauss and Lieber, 1992). The TCRb locus also employs a striking restriction in V(D)J recombination that goes beyond the 12/23 rule (referred to as Beyond 12/23 or B12/23 restriction). Unlike the IgH locus, the arrangement of RS sequences in the TCRβ locus theoreti-

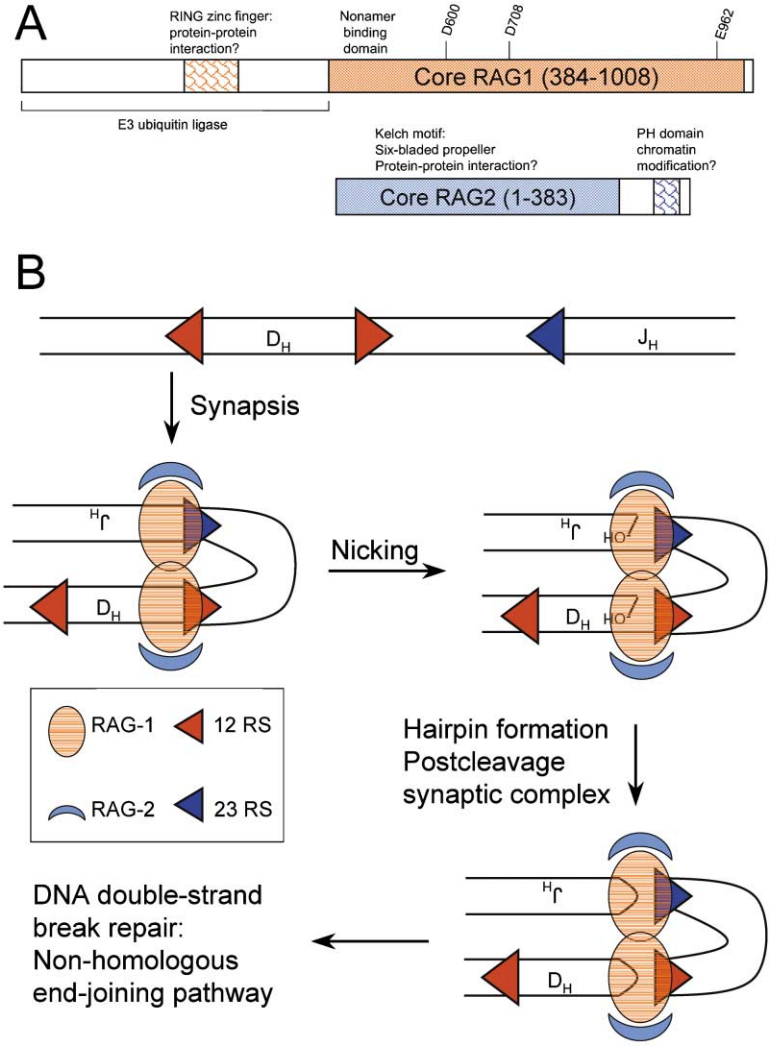

Figure 4. The RAG Proteins and the RAG Cleavage Reaction

(A) Schematic of RAG-1 and RAG-2, illustrating known motifs and the putative RAG-1 DDE active site.

(B) Summary of the RAG cleavage reaction. See text for details.

cally would permit direct Vβ to Jβ joins based simply on the 12/23 rule (Bassing et al., 2002), but such joins rarely occur. The explanation for this apparent paradox came from the finding that V(D)J recombination events mediated by Vβ 23RSs could only be targeted by the 5′Dβ 12RS and not by Jβ 12RSs, thereby ensuring that Vβ(D)Jβ joins proceed via a DJβ intermediate (Sleckman et al., 2000; Bassing et al., 2000). This beyond 12/23 restriction of V(D)J recombination is similarly maintained in nonlymphoid cells using transient recombination substrates and in vitro using purified proteins, indicating that it also is intrinsic to the RAG cleavage reaction (Jung et al., 2003; Tillman et al., 2003; Olaru et al., 2003). Further characterizing the basis for B12/23 and related phenomena may shed additional light both on the mechanisms underlying RAG-mediated cleavage and on genetic factors that effect repertoire development.

Enhancing and Promoting V(D)J Recombination

Transcriptional enhancers embedded within antigen receptor loci have been extensively documented to play a role in tissue- and stage-specific assembly of endogenous antigen receptor gene segments (Figure 2; Hesslein and Schatz, 2001; Krangel, 2003). Studies in transgenic recombination substrates first showed that an enhancer could provide V(D)J recombinational accessibility in vivo (Ferrier et al., 1990; Lauzurica and Krangel, 1994). Correspondingly, deletion of enhancer elements from endogenous mouse Ig and TCR loci blocks or significantly impairs V(D)J recombination of the corresponding loci, concomitant with impaired germline transcription (Krangel, 2003). An obvious role for enhancers in V(D)J recombination would therefore be via their role in modulating transcription. As noted above, the accessibility model was originally proposed on the basis that germline VH gene segments are transcribed only when they undergo rearrangement. Numerous studies over the years have further shown that transcription is strongly correlated with, but not necessarily sufficient for, rearrangement of adjacent antigen receptor gene segments (Krangel, 2003). In relevant recent studies, a transcriptional coactivator (OcaB; Casellas et al., 2002) and a transcription factor (Pax5; Hesslein et al., 2003) were shown to promote recombination of a subset of gene segments in the Igκ and IgH loci, respectively. However, while OcaB has been argued to exert its influence on V(D)J recombination by driving increased transcription of certain Vκ gene segments (Casellas et al., 2002), Pax5 may work differently since the subset of VH

gene segments that are not rearranged in the absence of Pax5 nevertheless undergo germline transcription (Hesslein et al., 2003). The most direct analysis to date of the role of transcription elements in V(D)J recombination has been provided by a transgenic mini-locus study that led to the conclusion that V(D)J recombinational accessibility involves promoter functions distinct from germline transcription (Sikes et al., 2002).

Taken together, the findings outlined above, along with many others (Hesslein and Schatz, 2001; Krangel, 2003), strongly imply that transcription per se through a RAG target sequence may not be directly linked to the reaction mechanism. A recent example of such a direct mechanistic linkage between recombination and transcription in the immune system comes from studies of IgH class switch recombination. In this process, transcription through IgH switch regions appears to promote class switch recombination by generating a single-strand DNA substrate for the cytidine deamination reaction catalyzed by AID (Chaudhuri et al., 2003; Ramiro et al., 2003). In contrast, the transcriptional *cis*-elements and *trans*-acting factors associated with antigen receptor variable region gene loci may play a more general role in facilitating chromatin changes that render gene segments accessible to RAG cleavage, perhaps by targeting chromatin remodeling factors (Sikes et al., 2002).

Regulation of RAG Activity

Much effort has gone into defining specific markers that relate local chromatin structure with recombinational accessibility (Hesslein and Schatz, 2001; Krangel, 2003). Over the years, various studies have associated the likelihood of endogenous and/or introduced V, D, and J segments to undergo rearrangement with markers already known to correlate with transcription at the level of chromatin structure. Such markers include DNAase sensitivity (Yancopoulos et al., 1986); methylation status of cytosine residues in DNA (Storb and Arp, 1983); and various histone modifications including hyperacetylation (McMurry and Krangel, 2000; Chowdhury and Sen, 2003). On a different scale, early replication (Mostoslavsky et al., 2001) and central subnuclear positioning (Kosak et al., 2002) are potentially important newer correlates of V(D)J recombinational accessibility. In addition, κ locus demethylation and asynchronous replication might function to provide a single allele for initial rearrangement (Mostoslavsky et al., 1998, 2001), a prerequisite for feedback regulation of allelic exclusion (Alt et al., 1980). As with transcription more generally, whether any or all of these correlates act as causes or are only effects of an open locus is an open question. In vitro studies have begun to provide additional information. For example, in vitro nucleosomal assembly on a V(D)J recombination substrate inhibits RAG cleavage in a manner reversible by histone acetylation and chromatin remodeling (Kwon et al., 2000); it further has been suggested that RAG-2 itself might have nucleosome remodeling activity and thereby potentially direct rearrangement of certain gene segments (Liang et al., 2002; Akamatsu et al., 2003). Finally, a potentially powerful assay for accessibility was developed based on the finding that isolated nuclei from cells at different developmental stages show appropriate locus sensitivity to in vitro cleavage by recombinant core RAG proteins, further solidifying the concept of differential accessibility (Stanhope-Baker et al., 1996). Ultimately, extension of such in vitro studies may offer the most direct means by which to approach these issues.

In addition to accessibility control of substrate V, D, and J segments, regulation of RAG expression provides a separate means to control V(D)J recombination. RAG-1 and RAG-2 are convergently transcribed, coordinately expressed, and accumulate to significant levels only in developing lymphocytes, providing one basis for the lymphoid-specific nature of the reaction (Nagaoka et al., 2000). A number of different elements involved in lineage- and stage-specific regulation of the RAG genes have been implicated (Yu et al., 1999b; Monroe et al., 1999b; Hsu et al., 2003). Of particular interest, both RAG-1 and RAG-2 appear to be regulated by sequences 5′ of the RAG-2 gene, an organization that has been argued to support the notion that the two genes arrived into the genome via a primordial transposon (Yu et al., 1999b). Expression of the RAG proteins in developing B and T lineage cells occurs in nonproliferating cells. For example, RAG is expressed in pro-B cells when the IgH locus is being rearranged, downregulated during the expansion stage following productive IgH rearrangement, and upregulated again in nonproliferating pre-B cells in which IgL variable region genes are assembled (Nagaoka et al., 2000). In both B and T cells, control of RAG-2 levels also is limited to G0/G1 phase of the cell cycle by RAG-2 protein degradation during transition from G1 to S phase (Desiderio and Lee, 2000). A recent report has also described an E3 ubiquitin ligase activity for RAG-1, raising the possibility that this activity may be involved in degrading proteins to regulate V(D)J recombination (Yurchenko et al., 2003). Such controls may be important to prevent the generation of RAG-induced DNA double-strand breaks (DSBs) outside of the G1 phase, for example during DNA replication, where they might induce harmful translocations and other abnormalities (Barreto et al., 2001).

RAG expression was originally thought to be permanently downregulated by expression of a complete surface IgM or TCRα/β receptor in new generated B and T cells. However, reinduction and/or continued expression of RAG in newly generated B cells was found to allow replacement rearrangements of endogenous IgL loci and, potentially, IgH loci to allow editing of self-reactive B cell receptors (Nemazee, 2000; Casellas et al., 2001). Moreover, the finding of RAG expression in splenic B lineage cells led to much excitement about the possibility that mature B cells might reinduce RAG in the context of a "receptor-revision" mechanism (Rajewsky, 1996). In this context, RAG proteins would potentially have a second role in the generation of the immune response associated with antibody affinity maturation. However, additional studies with transgenic or knockin RAG reporter constructs indicated that RAG expression cannot be reinduced in most mature B lineage cells and that the vast majority of RAG-expressing B lineage cells in the spleen represent progenitors or precursors (Yu et al., 1999a; Monroe et al., 1999a). Still, ongoing work continues to evaluate potential roles of RAG in receptor revision/editing in the periphery.

Putting the Ends Back Together Again

The cleaved coding and RS segments generated by the RAG endonuclease are joined by a ubiquitously ex-

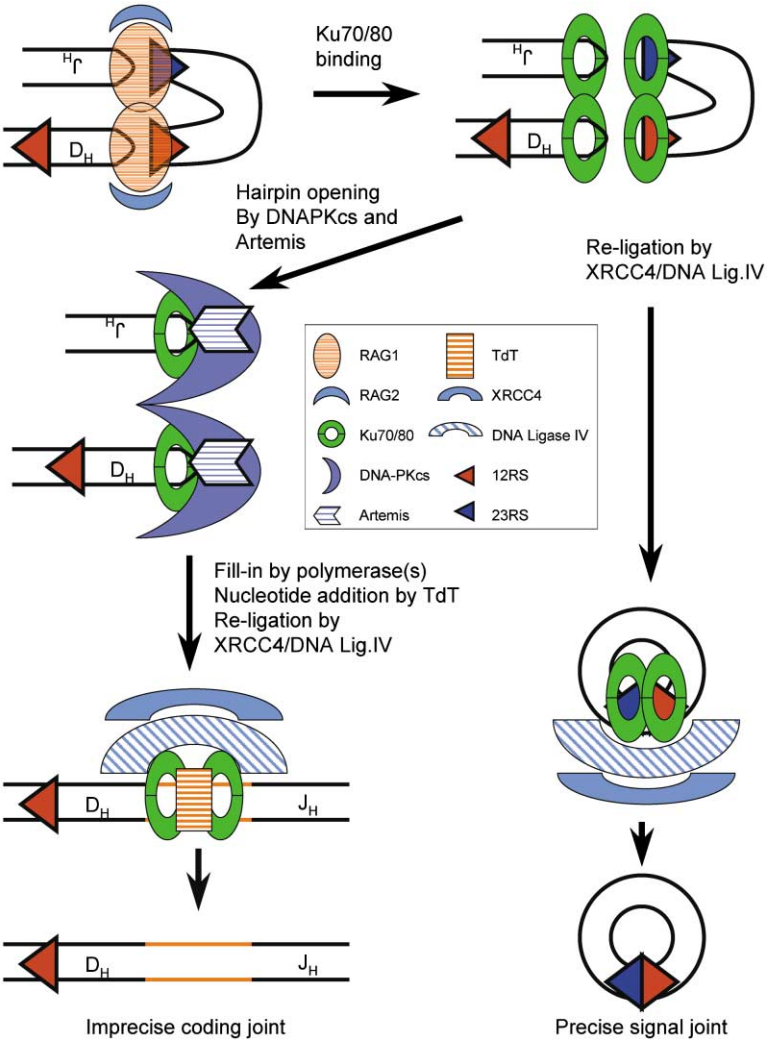

Figure 5. V(D)J Recombination and the Nonhomologous End-Joining Pathway

The known steps of the reaction are depicted, although the precise identity and/or stoichiometry of all the proteins involved at each step have not been fully characterized.

pressed set of nonhomologous end joining (NHEJ) proteins, which ligate DSBs irrespective of sequence homology (Figure 5; Bassing et al., 2002). In addition to the repair of RAG-generated DSBs in lymphocytes, NHEJ proteins also repair more generally introduced DSBs that occur in all cell types, a function necessary for the general maintenance of genomic stability (Mills et al., 2003). RAG expression vectors made possible a set of transient V(D)J recombination substrate studies of ionizing radiation-sensitive Chinese hamster ovary cell lines that firmly made the connection between V(D)J recombination and general DNA DSB repair (Taccioli et al., 1993). The six NHEJ repair proteins subsequently identified can be subdivided into two general classes. Ku70 and Ku80, which form a DNA end binding heterodimer (Ku), and XRCC4 and DNA ligase 4, which form an end-ligation complex, are evolutionarily conserved with homologs in yeast, while the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Artemis evolved more recently (Jackson, 2002). The four evolutionarily conserved NHEJ factors are required to repair all ends that are repaired by NHEJ. In this regard, Ku recognizes broken ends, where, among other things, it may recruit the XRCC4/Lig4 ligation complex (Figure 5; Jackson, 2002). On the other hand, DNA-PKcs and Artemis appear to be required predominantly for ligation of ends in need of processing before ligation.

During V(D)J recombination, the four conserved factors are needed for both coding and RS joins, while DNAPKcs and Artemis are needed for coding but, in large part, not for RS joins (Figure 5). Complex formation with and phosphorylation by DNA-PKcs activate an endonuclease activity in Artemis (Ma et al., 2002), which is necessary to cleave coding end hairpins (Rooney et al., 2002) and might also contribute to junctional deletion of nucleotides in coding joins (Ma et al., 2002; Schlissel, 2002). Of note, Ku recruits DNA-PKcs to DSBs to form the DNA-dependent protein kinase and may similarly target Artemis, although DNA-PKcs also appears to have Artemis-independent roles that might include synapsis (DeFazio et al., 2002) or activation of other factors. In mice, deficiency for any of the six known NHEJ proteins, similar to RAG deficiency, results in a SCID phenotype due to inability to join RAG-cleaved V, D, and J segments to form antigen receptor genes (Bassing et al., 2002). However, mouse mutations of these factors also lead to additional phenotypes, reflecting the role played, thus far, by NHEJ proteins in general DNA repair.

In humans, mutations in RAG and Artemis, but not the other NHEJ factors, have been found to underlie SCID (Bassing et al., 2002).

While much has been learned about the biochemistry of the V(D)J joining reaction, the apparent role of RAGs in recruiting the NHEJ machinery (Gellert, 2002) remains to be elucidated. The further development of in vitro V(D)J recombination assays could help resolve these issues. Toward this end, RS joining has been achieved in vitro (Fugmann et al., 2000), but a minimal in vitro system fully recapitulating the coding joining phase, which is more specific to V(D)J recombination, remains to be devised. Recent work also has recapitulated low efficiency RS, but not coding, joining in yeast (Clatworthy et al., 2003). Finally, it is likely that additional NHEJ and other factors associated with the V(D)J recombination reaction remain to be identified (Dai et al., 2003), and a variety of additional proteins may function more generally to recognize RAG-initiated DSBs and suppress interchromosomal V(D)J recombination and other types of genomic instability (see below).

Aberrant V(D)J Recombination May Lead to Translocations and Cancer

While impaired V(D)J recombination clearly underlies certain immunodeficiencies, this inherently dangerous recombination mechanism, which involves DSBs and a transposition-like reaction, also has the potential to generate chromosomal instability and, as a result, lymphoid malignancy (Roth, 2003). The transposition activity observed for the core RAGs in vitro led to the proposal that translocations may be generated by RAG-mediated transposition events in vivo (Hiom et al., 1998; Gellert, 2002). However, only a few potential examples of such an event (Messier et al., 2003), which would be expected to generate a telltale short target site duplication at the translocation breakpoint, have been found. With respect to the physiological relevance of RAG-mediated transposition, the non-core regions of RAG-1 and RAG-2 have been found to suppress the RAG-mediated, transposition-related hybrid joining reaction in cell lines (Sekiguchi et al., 2001). In addition, the non-core RAG regions of RAG-2 appear to suppress RAG-mediated transpositions in vitro (Tsai and Schatz, 2003; Elkin et al., 2003; Swanson et al., 2003). Thus, the full-length RAG proteins may have evolved to prevent such potentially harmful reactions, although the mechanism by which they do so remains to be elucidated.

RAGs can, however, generate oncogenic translocations via interchromosomal V(D)J recombination between an RS sequence within an antigen receptor locus and a cryptic RS elsewhere in the genome. Proof of such events came from the finding of the fused RSs on a reciprocal of the oncogenic translocation (McGuire et al., 1989; Tycko and Sklar, 1990). However, many oncogenic translocations with an antigen receptor locus as one partner do not involve sequences harboring an obvious cryptic RS on the second chromosome. Such translocations commonly appear to be derived from fusion of RAG-generated DSBs at an antigen receptor locus to DSBs generated by other processes on a second chromosome (Mills et al., 2003). In mouse models, deficiencies in NHEJ in the context of p53 deficiency inevitably lead to the occurrence of pro-B lymphomas with this type of RAG-initiated oncogenic chromosomal translocation. Defects in NHEJ and defects in p53 expression therefore cooperate to promote translocations, respectively, by leading to generation of pro-B cells with unrepaired RAG-initiated DSBs at JH loci and by failing to eliminate such cells through a normal G1 cell cycle checkpoint (Mills et al., 2003).

Histone H2AX is a histone H2A variant that is rapidly phosphorylated in the vicinity of general DSBs by ATM and other kinases and forms RAG-dependent foci at the TCRα locus (Redon et al., 2002). Notably, combined deficiency for H2AX and p53 leads to B lineage tumors with oncogenic translocations that appear to be initiated by RAG-generated DSBs in a manner analogous to those found in NHEJ/p53 pro-B lymphomas (Bassing et al., 2003; Celeste et al., 2003). While the precise role of H2AX in suppressing such DSBs is still speculative, it may well function by suppressing S phase-generated translocation targets for the RAG-generated DSBs and/or by recruiting factors that prevent DSBs from separating before their repair. In the latter context, phosphorylated H2AX has been proposed to function as an anchor for the assembly of DNA/protein complexes that prevent the dissociation and potential misrepair of the broken DNA ends at DSBs (Bassing and Alt, 2004). Likewise, defects in such processes may contribute to the high frequency of interchromosomal V(D)J recombination in human ATM-deficient cells (Taylor et al., 1996) and murine cells deficient in NBS1 (Kang et al., 2002) and with the predisposition of humans with mutations in ATM, NBS1, and MRE11 to lymphoid malignancies with translocations (Khanna and Jackson, 2001). Further studies of this intriguing potential role for checkpoint/repair proteins in suppressing RAG-initiated translocations and interchromosomal V(D)J recombination will undoubtedly reveal further insights into the overall regulation of the V(D)J recombination process.

The Future

The "two gene, one protein" hypothesis, proposed to explain the generation of antibody diversity, was experimentally proven through the discovery of V(D)J recombination. Work in V(D)J recombination, as in many other fields, has progressed hand-in-hand with the development of new technologies. Thus, molecular cloning first provided methods with which to identify the basic features of V(D)J recombination, while the application of gene transfer into cells facilitated the discovery of the RAG genes and, along with targeted mutational analysis in mice, illuminated the in vivo regulation of V(D)J recombination. In spite of the progress made in the past 25 years toward elaborating the mechanism and regulation of V(D)J recombination, one central question remains: given the complexity and size of the genome, how do the RAG proteins find their appropriate targets? The notion of accessibility control has long provided a useful framework within which to approach this question, although there remains little known about the actual physical constraints governing accessibility of individual gene segments in vivo. Advances associated with the genomics era will likely facilitate substantial progress in this area, but the development of additional technologies will also be required. For example, it will be important to further elucidate relationships between the physical localization of endogenous gene segments and recombination, as well as to perform more detailed analyses

of the kinetics of RAG localization to rearranging gene segments in vivo. In vitro experiments, perhaps at the level of whole chromosomes or chromatin-reconstituted artificial chromosomes containing modified antigen receptor loci, may help to clarify the role of promoters and enhancers in controlling V(D)J recombination within the context of higher order chromatin structure. Realizing the crystallographic structures of the various RAG-DNA complexes also remains of significant interest and should advance our understanding of RAG protein chemistry. Finally, further understanding of the interplay between V(D)J recombination, NHEJ, and other repair/checkpoint proteins should shed light on how these factors cooperate to suppress translocations and transformation. Forward progress in all of these areas will likely generate additional novel insights into control of gene expression in development and the role of chromatin dynamics in health and disease.

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