Specificity in V(D)J recombination: new lessons from biochemistry and genetics

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Recent *in vitro* work on V(D)J recombination has helped to clarify its mechanism. The first stage of the reaction, which can be reproduced with the purified RAG1 and RAG2 proteins, is a site-specific cleavage that generates the same broken DNA species found *in vivo*. The cleavage reaction is closely related to known types of transpositional recombination, such as that of HIV integrase. All the site specificity of V(D)J recombination, including the 12/23 rule, is determined by the RAG proteins. The later steps largely overlap with the repair of radiation-induced DNA double-strand breaks, as indicated by the identity of several newly characterized factors involved in repair. These developments open the way for a thorough biochemical study of V(D)J recombination.

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Abbreviations
DSB double-strand break
RAG recombination activator gene
RSS recombination signal sequence
SCID severe combined immunodeficiency

Introduction

The vertebrate immune system has evolved several strategies to diversify its repertoire of antigen receptor molecules. This review will deal with the DNA recombination events leading to the formation of mature antigen receptor genes from separate gene segments (reviewed in [1,2]). One variable (V) gene segment is coupled to a joining (J) segment (and sometimes a diversity [D] segment), forming a V(D)J exon, which encodes the variable part of the immunoglobulin or T-cell receptor protein. These gene segments are flanked by recombination signal sequences (RSSs), which consist of a conserved heptamer and a nonamer motif, separated by a 12 or 23 base pair (bp) spacer of relatively nonconserved sequence. The sites of joining are determined by the RSSs, and the well-known preference for recombination between one RSS with a 12 bp spacer and one with a 23 bp spacer (the 12/23 rule) ensures that V(D)J recombination occurs only between two coding segments of different types (eg. between V and J segments). Correct joining is of particular significance, as the consequences of a failure to accurately target V(D)J recombination include unproductive joining followed by cell death, or leukemia resulting from inappropriate linkages.

The RAG1 and RAG2 proteins (encoded by the recombination activator genes 1 and 2) are required for V(D)J recombination [3,4], and their expression pattern limits V(D)J joining to the early stages of lymphoid development. Co-expression of the RAG proteins is entirely correlated with the initiation of V(D)J recombination. In one medically significant situation that has been recently described, mutations in either human RAG1 or RAG2 have been shown to lead to severe combined immune deficiency (SCID) [5].

Another aspect of the regulation of gene rearrangement is the choice of appropriate loci in a given lineage; for example, the immunoglobulin loci rearrange in B cells and the TCR loci in T cells, but the reverse does not generally happen [2]. Newer results on the reaction mechanism, discussed below, indicate possible approaches to studying how this differential access to various sites (generally called locus accessibility) is established.

The mechanism of V(D)J cleavage

Recently, much progress has been made in understanding the molecular mechanisms underlying these rearrangements. It is now clear that the V(D)J recombination reaction can be divided into two steps: first, double-strand DNA breaks (DSBs) are made at the border between the RSS heptamer and the flanking coding segment, followed by a distinct second stage in which the signal and coding ends are processed and ultimately linked into signal and coding joints. The DSBs representing the first stage have been found in the thymus of the new born mouse [6,7], in a pre-B cell line that expresses high levels of RAG1 and RAG2, and in fibroblasts transfected with RAG1 and RAG2 expression vectors [8••,9•]. The signal ends are almost exclusively blunt, 5′-phosphorylated, and contain the complete RSS [7,10]. The coding ends, however, have a hairpin structure: the top strand is covalently coupled to the bottom strand [8••,11,12]. The observation that the signal ends can later be incorporated into signal joints provides evidence that these broken molecules are indeed intermediates in V(D)J recombination [8••]

Reconstitution of the V(D)J cleavage reaction *in vitro* has provided an even more detailed understanding of the chemical steps involved [13•,14••]. The RAG1 and RAG2 proteins first make a nick at the 5′ edge of the RSS heptamer (Fig. 1). Subsequently, the newly formed 3′-OH

on the coding DNA is coupled to the phosphate group in the opposite strand, forming a DNA hairpin on the coding end and leaving a blunt DSB on the signal end. Stereochemical analysis of the hairpin formation reaction revealed that breakage of the old phosphodiester bond and formation of the new one at the tip of the hairpin are accomplished by direct *trans*-esterification [15••]. Very similar reaction mechanisms were earlier established for two other recombination reactions, bacteriophage Mu transposition [16], and HIV integration [17]. In these reactions, the 3′-OH group at the transposon end is integrated into the target DNA via one step *trans*-esterification. Minor modifications of the DNA transposition reaction could in principle result in hairpin formation, the 3′-OH group at the border of the RSS heptamer in this instance is integrated into the other strand of the same DNA double helix instead of a different target site.

Interestingly, several other parallels exist between the RAG-mediated RSS cleavage reaction and transposition. The first step in V(D)J cleavage, nicking at the 5′ side of the RSS, shows striking similarities to the nicking step catalyzed by retroviral integrases. In both systems, this is normally a hydrolysis reaction. Under certain conditions, however, other nucleophiles (such as glycerol or ethylene glycol) can be used instead of water, resulting in a reaction product containing the alcohol covalently coupled to DNA [15••,18]. Relationships between V(D)J recombination and transposition have also been suggested in a broader immunological context as a possible explanation for the diversity of V, D, and J regions [19].

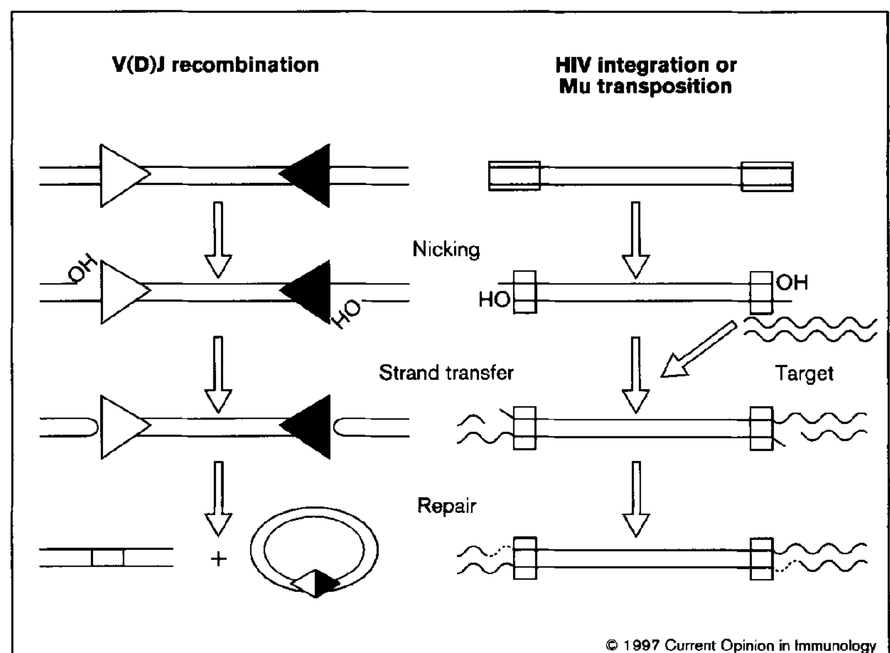
There are probably even transposons that produce hairpin intermediates: the chromosome repair following excision of some plant transposable elements is often accompanied by insertion of self-complementary nucleotide tracts very similar to the comparable tracts (P nucleotides) found in many V(D)J coding junctions [2]. This is most easily explained by the presence of hairpin intermediates.

Recognition of the recombination signal

In addition to these biochemical similarities between transposition and V(D)J cleavage, the sequence of the RSS has some resemblance to the inverted repeats found at the ends of several transposons [20]. The consensus RSS is defined by a heptamer (CACAGTG) adjacent to the coding sequence, followed by a sapcer of 12 or 23 base pairs of nonconserved sequence, followed in turn by a nonamer (ACAAAAACC). Transposons such as Tc1 and Tc3 from *Caenorhbaditis elegans*, and Uhu from *Drosophila melanogaster*, have terminal sequences with similarities to the RSS heptamer, and in some cases also have A-tracts like that in the nonamer. In addition, a CA immediately adjacent to the site of cleavage is critical for both Mu transposition and HIV integration. This sequence is present in the portion of the RSS most important for RAG protein activity (discussed in more detail below).

The conservation of RSSs was first defined by comparison of sequences at Ig and TCR loci, and later by the use of recombination substrates with defined RSS mutations [21]. It was observed that the heptamer is the most conserved sequence element of RSSs, and assays using substrates

Figure 1



Comparison of V(D)J cleavage with HIV integration (or Mu transposition).The 12-spacer signal is depicted as an open triangle and the 23-spacer signal as a closed triangle, and each end of HIV DNA as a box. Cleavage at the RSSs by the RAG proteins takes place in two steps. First a nick is made on each coding end, leaving a 3′-hydroxyl (3′-OH) group; then these hydroxyls attack the opposite strand, forming hairpins. HIV integration similarly begins with a nick that exposes a 3′-OH end on the viral DNA. In the second step, this hydroxyl then attacks the host DNA (represented by a wavy line) into which the HIV DNA integrates. The strand transfer process in Mu transposition is very similar. Note that the strand polarities are opposite in the two families, so that the 3′-OH is inside the HIV (or Mu) recognition element, but outside the V(D)J signal. The recognition element of HIV or Mu thus becomes covalently linked to the new host DNA, but the RSS cannot be similarly transferred.

mutated in either the heptamer or nonamer showed that in general mutations in the heptamer affect the level of recombination more severely than those in the nonamer. Although recombination is most efficient using substrates with both a heptamer and nonamer, even a heptamer alone is capable of mediating a low level of V(D)J recombination, and the three highly conserved residues (CAC) of the heptamer closest to the coding segment are found to be the most important ones.

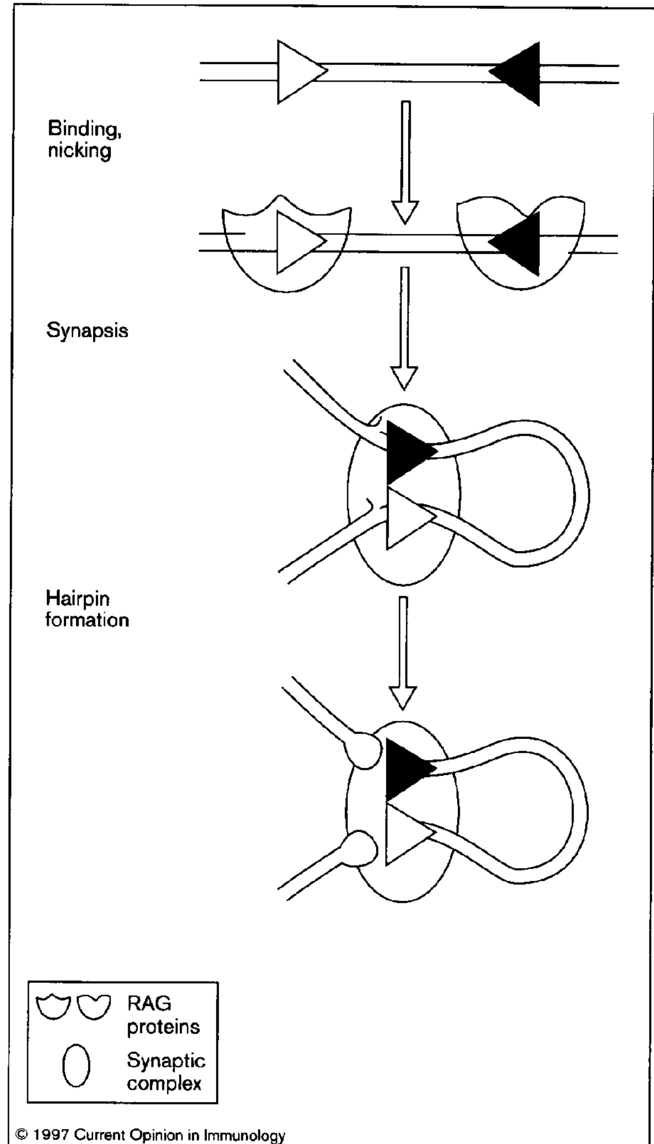
Taking into account the fact that cleavage in the cell-free reaction occurs in two steps, it has been possible to determine the DNA sequence and structure requirements of V(D)J recombination in more detail [22••,23••]. The first (nicking) step in cleavage by the RAG proteins can be mediated either by a heptamer or nonamer alone, but is done most efficiently and accurately when both elements are present. Mutations in the heptamer positions that were previously shown to be critical for V(D)J recombination in cells had little effect on nicking activity, but hairpin formation was strongly dependent on the sequence at these positions. In general the rules for *in vivo* recombination closely paralleled those for hairpin formation, rather than nicking.

Assays to study the very first steps in RSS recognition, binding of the RAG proteins, have recently been described. The first indication for binding specificity came from experiments in which various recombination substrates were used as competitors for cleavage of a wild-type substrate. Competition was observed not only when an intact RSS was used, but also with the heptamer or the nonamer alone, arguing that each element is to some extent capable of mediating binding by the RAG proteins [23••]. Substrates with only a nonamer are somewhat better competitors than those containing only a heptamer, suggesting that initial binding of the RAG proteins to the nonamer may be more effective. Recent studies suggest that the RAG1 protein by itself shows a preference for binding to the nonamer [24,25]. More recent experiments still have identified a tightly bound complex of both the RAG1 and RAG2 proteins with an RSS [26]. Efficient formation of this complex requires both the heptamer and nonamer motifs of the RSS. It can be formed in the presence of Ca^{2+}, where it does not cleave the DNA, but can then carry out the chemical steps of cleavage if 'chased' with Mg^{2+} or Mn^{2+}. Such a functional complex should be useful for distinguishing the requirements for binding from the subsequent chemical steps.

The 12/23 rule

Cleavage under cell-free conditions as described above is possible with either a 12- or a 23-spacer signal alone, whereas in cells cleavage follows the 12/23 rule previously derived from the observed recombination events [9•]. In the cell-free experiments described above Mn^{2+} was used as a divalent metal cofactor. Substitution of Mg^{2+} for Mn^{2+} alters the specificity of the RAG proteins, such that cleavage now requires both a 12- and 23-spacer signal on the same DNA molecule [27••]. Nicking in the presence of Mg^{2+} occurs on an isolated signal; it is only the final hairpin formation step that demands both partners (Fig. 2).

Figure 2



Coupled cleavage by RAG1 and RAG2. The 12-spacer signal is depicted as an open triangle and the 23-spacer signal as a closed triangle. In a first step that does not require pairing of the two RSSs (synapsis), a nick is made at the border of each RSS. After the formation of a synaptic complex, cleavage is then completed at both RSSs, with the formation of blunt-cut signal ends and hairpin coding ends.

This result implies a requirement for synapsis of 12- and 23spacer signals for cleavage to proceed. Previous experiments in cells [28] indicated that arrangement of the 12- and 23-type signals in the same orientation (resulting

in inversional recombination) required larger distances between the signals for recombination to occur than if the signals were in opposite orientation (as they are drawn in Fig. 1). Experiments in cell extracts indicated that this limitation is also observed for the cleavage reaction [29••], arguing that the signals must align (approximately) in parallel for productive synapsis to take place (as shown in Fig. 2).

Under conditions where the 12/23 rule is observed, cleavage of the two signals is simultaneous (at least within a few minutes) [27••]. Experiments with either a 12- or 23-type signal containing a mutation indicate that the mutation affects cleavage on the other signal to the same extent as it affects the mutated signal, further supporting the conclusion that the 12/23 cleavage event is a tightly coupled reaction [9•,27••,29••].

Other recombination systems (Flp, bacteriophage Mu) have been shown to require synapsis for cleavage, because the DNA is cleaved *in trans*. That is to say, proteins bound to one recombination site in a synaptic complex contribute the requisite active site for cleavage of the partner site. It will be interesting to see whether coupled hairpin formation by the RAG proteins occurs by an analogous mechanism.

Effects of sequences flanking the RSS heptamer

Cell-free hairpin formation is also dependent on the sequence of the opposite side of the cleavage site, that is the coding flank sequence. Certain coding flank sequences ('bad flanks') are found to block hairpin formation under cell-free conditions [22••,23••]. In cells, however, these sequences are recombined efficiently by wild-type RAG1. Surprisingly, a mutant version of RAG1 had previously been shown to have a very similar preference for certain coding flanks over others, in recombination of extrachromosomal substrates [30,31]. A possible explanation for these observations may be suggested by the DNA structure of RSSs. NMR and X-ray crystallography data indicate that the sequence motif CACA found in the heptamer has an unusual structure, including a disruption of normal base pairing (discussed in [22••,23••]). It was noted that the flanking sequences that permit hairpin formation (good flanks) continue this pyrimidine-purine alternation, possibly extending the unstable segment. Hairpin formation may thus be favored if the flanking sequence can contribute to base unpairing next to the cleavage site. This hypothesis is supported by the finding that the block in hairpinning can be overcome by introduction of mispaired bases in the coding flank, even if both strands have bad flank sequences. In this case the unpairing is built into the structure, and is evidently sufficient to allow hairpinning. It is also consistent with the general ability of the RAG proteins to recognize and cleave substrates with mispairs in the first two positions of the heptamer, or even substrates with an entirely single stranded signal [22••,23••].

When two RSSs are cleaved in a coupled reaction, the distinction between good and bad coding flanks is no longer apparent [27••]. It seems likely that RAG proteins bound to one RSS can loosen the DNA structure at the other sufficiently to overcome the block presented by bad flanks.

End processing and joining

In contrast to the cleavage step, the later stages of V(D)J recombination appear to be quite nonspecific. Coding ends are efficiently joined despite being unrelated in DNA sequence, and despite being further varied, before joining, by nucleotide loss and/or addition. Recent work on the pathway of joining is consistent with this view as it shows the later steps of V(D)J joining to share a number of ubiquitous factors with the repair of DNA DSBs, such as are made by X-rays (reviewed in [32]). Like the nonhomologous coding ends in V(D)J recombination, these breaks have to be repaired without the aid of extensive homology, so it is reasonable that a common pathway is used. Genetic studies have identified three factors utilized in this type of repair. One is the catalytic subunit of a DNA-dependent protein kinase (DNA-PK). This protein (DNA-PK_{CS}) has a molecular weight of 460 kDa, making it one of the largest protein chains known. Its sequence identifies it as belonging to a family of phosphatidylinositol 3 (PI3)-like kinases, a group which also includes the ATM protein implicated in ataxia telangiectasia. Some members of this family do indeed phosphorylate lipids, while others such as DNA-PK are protein kinases (reviewed in [32]).

It has now become clear that the mouse *scid* mutation, which interferes with the later stages of V(D)J recombination and thus leads to severe combined immunodeficiency, resides in the DNA-PK_{CS} gene (also known as XRCC7) [33•–35•]. The same defect renders cells from these mice defective in the repair of DNA DSBs, and hence abnormally sensitive to X-rays. When looked at in more detail, SCID cells are capable of signal joint formation in V(D)J recombination, but broken coding ends accumulate in the form of DNA hairpins and are only rarely joined. Recently, the exact site of the *scid* mutation has been mapped. It is a single base substitution that results in a stop codon and truncates the DNA-PK_{CS} protein by 83 amino acids [36,37]. The missing segment is in a region that is highly conserved among the PI3-like kinases, and may be essential for kinase activity. The CHO cell mutation V3 lies in the same gene and has very similar properties [33•], and a comparable mutation has been described in Arabian horses with the SCID phenotype [38].

Another factor utilized in DSB repair is the heterodimeric protein called Ku, a complex of 70 kDa and 86 kDa

subunits that has a strong preference for binding to DNA ends (or other structural abnormalities such as nicks or gaps). Ku is the DNA-binding component of DNA-PK, though it is not yet certain this is its only function. A DNA-end binding protein would of course be a natural participant in DSB repair. Some CHO cell derivatives defective in DSB repair have proved to have mutations in the Ku86 subunit, and these cells are also defective in V(D)J recombination; in this case, both signal and coding joints are affected (reviewed in [32]). A plausible model based on these results is that Ku protein was required to recognize and protect the broken ends resulting from the initial cleavage — both signal ends and the hairpin coding ends — and that DNA-PK was then attracted to these sites, and its action was in some way necessary to allow opening of the hairpins and later formation of coding and signal joints.

More recent work suggests that this interpretation may not be correct. In mice with a disruption of the Ku86 gene, there is a severe reduction of V(D)J recombination, as expected [39••,40••], but broken signal ends and hairpin coding ends are recovered in similar amounts to those in SCID cells [40••]. Furthermore, almost all the signal ends are intact. As it is unlikely that unprotected DNA ends would survive, protection by other factors (possibly the RAG proteins?) must now be considered. To accommodate these newer results, it has been suggested that Ku protein (and the associated DNA-PK) may act instead to disassemble the synaptic complex remaining after cleavage, and perhaps to guide correct joining [40••].

Mutations in another gene (XRCC4) have a similar defect in V(D)J recombination and DSB repair. This gene has also been cloned [41•], but the sequence is not related to any known protein, and so far no molecular function has been suggested.

Conclusions

The results described above suggest possible approaches to using the specificity of recombination for the study of developmental specificity. It appears that the specificity of V(D)J recombination is entirely determined at the initial cleavage step by the action of the RAG proteins. These proteins recognize and correctly cleave a single RSS, and RSS mutations affect this reaction very similarly to their effects on recombination. In addition, the RAG proteins are able to enforce the 12/23 rule, which is the other aspect of V(D)J specificity. The later stages of V(D)J recombination demand intricate manipulations of hairpins and broken ends, but apparently do not influence the specificity of site selection.

Experiments in cells and genetically manipulated mice have suggested that developmentally specific targeting of recombination activity occurs due to differential locus accessibility (as mentioned in the Introduction), and locus accessibility is conferred by *cis*-acting enhancer elements. Focusing on the cleavage step should now permit analysis of this accessibility in real time *in vivo*, and eventually in cell-free systems. A significant step in this direction has already been taken by the use of nuclear templates as substrates for cell-free cleavage. These templates permitted the direct demonstration that DNA in an endogenous context, that is as chromatin, can represent a barrier to cleavage by the RAG proteins [42••]. In addition to RAG1 and nuclear extract (which includes RAG2), cleavage required a permissive locus, and the pattern of permissiveness faithfully reproduced patterns of activation of V(D)J recombination observed in development.

In spite of these advances, much about the later steps of V(D)J recombination remains mysterious. For example, the hairpin opening step, which is presumably unique to V(D)J recombination, has not been characterized. It is also not clear why a protein kinase should be an essential factor in repair, and the large size of this protein suggests that additional activities or interactions are still to be discovered. Furthermore, the steps of nucleotide removal and of DNA joining do not yet have identified factors associated with them, so that much genetic and biochemical exploration is still needed.

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Together with [39••], this paper shows that the gene encoding the large subunit of the Ku heterodimer is required for efficient V(D)J recombination in mice. Surprisingly, mice lacking Ku86 retained intact signal ends, showing that these DNA termini are protected even in the absence of the DNA-end-binding Ku heterodimer.

41. • Li Z, Otevrel T, Gao Y, Cheng H-L, Seed B, Stamato TD, Taccioli GE, Alt FW: **The XRCC4 gene encodes a novel protein involved in DNA double-strand break repair and V(D)J recombination.** *Cell* 1995, **83**:1079–1089.
This paper describes the cloning of the *XRCC4* gene, whose function is required for efficient V(D)J recombination and DSB repair.

42. •• Stanhope-Baker P, Hudson KM, Shaffer AL, Constantinescu A, Schlissel MS: **Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity *in vitro*.** *Cell* 1996, **85**:887–897.
An assay to study V(D)J signal accessibility in isolated nuclei is described. In general, the pattern of cleavage (by RAG1, RAG2, and a cell extract) faithfully reproduced patterns of V(D)J recombination observed in development (e.g. certain immunoglobulin loci are cleaved only in nuclei derived from the B-cell lineage).