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Cleavage at a V(D)J Recombination Signal Requires Only RAG1 and RAG2 Proteins and Occurs in Two Steps

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Summary

Formation of double-strand breaks at recombination signal sequences is an early step in V(D)J recombination. Here we show that purified RAG1 and RAG2 proteins are sufficient to carry out this reaction. The cleavage reaction can be divided into two distinct steps. First, a nick is introduced at the 5′ end of the signal sequence. The other strand is then broken, resulting in a hairpin structure at the coding end and a blunt, 5′-phosphorylated signal end. The hairpin is made as a direct consequence of the cleavage mechanism. Nicking and hairpin formation each require the presence of a signal sequence and both RAG proteins.

Introduction

V(D)J recombination is the process that assembles immunoglobulin and T cell receptor (TCR) genes from separate gene segments (for recent reviews see Gellert, 1992; Lewis, 1994). These gene segments are flanked by recombination signal sequences (RSSs), consisting of conserved heptamer and nonamer motifs separated by relatively nonconserved spacer regions of 12 bp (12-signal) or 23 bp (23-signal). Efficient recombination requires one 12- and one 23-signal and results in a precise heptamer-to-heptamer ligation of the two signal sequences (signal joint) and the imprecise joining of flanking coding segments (coding joint). This imprecision in the coding joint arises from short additions of self-complementary (P) or random (N) nucleotides, or small deletions, or a combination of these, and contributes to the antigen receptor diversity generated by V(D)J joining.

The occurrence of frequent deletions and additions at coding joints suggests that the V(D)J recombination reaction proceeds through a stage in which double-strand breaks (DSBs) are present. Such DSBs have been found at the TCRδ (Roth et al., 1992a), TCRβ (J. F. M. and M. G., unpublished data), and immunoglobulin loci (Schlissel et al., 1993; Ramsden and Gellert, 1995). Broken signal ends observed at these loci are blunt and contain intact signal sequences that are phosphorylated at their 5′ ends (Roth et al., 1993; Schlissel et al., 1993). A convenient system for studying these DSBs is the pre-B cell line 103/BCL-2 (Chen et al., 1994), which has been transformed by a temperature-sensitive Abelson virus. When these cells are cultured at high temperature, formation of a large number of signal ends is induced. After return to the permissive (low) temperature, signal joints are formed, indicating that broken signal ends are indeed the precursors to signal joints (Ramsden and Gellert, 1995).

Broken coding ends were first detected in mice carrying the severe combined immunodeficiency (*scid*) mutation (Roth et al., 1992b). These ends were covalently sealed in a hairpin structure. Coding ends have been more difficult to detect in the DNA of wild-type mice, presumably because coding joint formation is much faster than signal joint formation, so that coding ends are quickly consumed (Zhu and Roth, 1995; Ramsden and Gellert, 1995). However, a very low level of hairpin coding ends is detected in the 103/BCL-2 cell line (Ramsden and Gellert, 1995). The presence of a hairpinned intermediate provides a mechanistic explanation for the frequent occurrence of P nucleotides. The evidence, taken together, suggests that hairpins are normal, although short-lived, intermediates in coding joint formation. A similar reaction pathway, involving hairpin DNA intermediates, has been proposed for some plant transposons (Coen et al., 1986).

V(D)J recombination activity requires the expression of the recombination-activating genes *RAG1* and *RAG2* (Schatz et al., 1989; Oettinger et al., 1990). Together, expression of these genes is sufficient to support V(D)J recombination of reporter substrates in nonlymphoid cells. Lymphoid cells of mice lacking either of these two genes do not recombine their immunoglobulin or TCR loci (Mombaerts et al., 1992; Shinkai et al., 1992) and are devoid of V(D)J recombination activity. However, only a core portion of each RAG protein is required to induce recombination of reporter substrates in fibroblasts. Approximately one third of the RAG1 N-terminus, and one quarter of the RAG2 C-terminus, can be deleted without major loss of activity (Silver et al., 1993; Sadofsky et al., 1993, 1994; Cuomo and Oettinger, 1994).

The earliest steps of V(D)J recombination have recently been reproduced in a cell-free system (van Gent et al., 1995). Nuclear extracts of 103/BCL-2 pre-B cells were shown to cleave DNA at signal sequences. The products of this cleavage reaction were the same as those detected in cells: full-length, blunt, 5′-phosphorylated signal ends, and coding ends with a hairpin structure. The efficiency of cutting in the nuclear extract was greatly enhanced by addition of recombinant RAG1 protein, and cutting activity in an inactive extract of *RAG1* (−/−) cells was restored by this protein. These results strongly indicated that RAG1

†The first two authors contributed equally to this work.

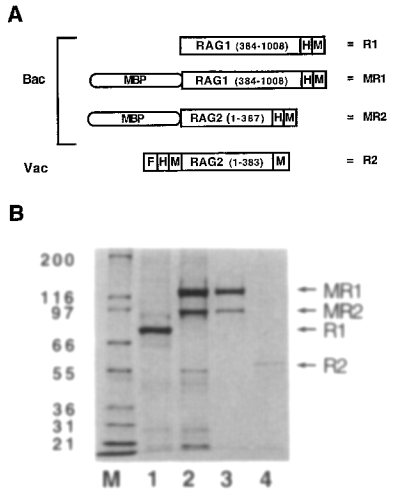

Figure 1. RAG Proteins

(A) Schematic representation of the recombinant RAG proteins described in this article. The RAG open reading frames are boxes numbered with the amino acids retained. From top to bottom, the constructs are named R1, MR1, MR2, and R2, as shown on the right. Other symbols are as follows: H, histidine tag; M, Myc epitope tag; F, FLAG epitope tag; MBP, maltose-binding protein fusion. Proteins were expressed in a baculovirus (Bac) or a vaccinia virus (Vac) expression system.

(B) Coomassie-stained 4%–12% SDS–PAGE gel of RAG1 (384–1008) purified on Ni^{2+}–Sepharose (lane 1; arrow R1), coexpressed MBP–RAG1 (384–1008) (arrow MR1), and MBP–RAG2 (1–387) (arrow MR2) purified on Ni^{2+}–Sepharose (lane 2) or Ni^{2+}–Sepharose and amylose resin (lane 3), and RAG2 (1–383) purified on a FLAG-antibody column and Ni^{2+}–Sepharose (lane 4; arrow R2). M, markers; their molecular masses are given in kilodaltons on the left.

protein directly participates in the cleavage reaction. The nuclear extract could be replaced by a fraction enriched in RAG2 protein, suggesting a direct role for this protein as well. However, it was not possible to determine the precise activities of the RAG proteins or their possible dependence on other factors in the extract.

We now report that this same cleavage reaction can be carried out by purified RAG1 and RAG2 proteins. Thus, RAG1 and RAG2 are the only proteins required for both recognition and cutting at V(D)J recombination signal sequences. Furthermore, we demonstrate that cleavage occurs in two successive steps.

Results

Expression and Purification of RAG Proteins

Formation of DSBs at signal sequences appears to be an early step in the V(D)J recombination pathway. As shown previously, reconstitution of this cleavage reaction in a cell-free system requires RAG1 protein and a pre-B cell nuclear extract (van Gent et al., 1995). This extract probably provided at least RAG2 protein, but whether additional factors were involved remained unclear.

To study the protein requirements of the cell-free system further, we overexpressed and purified derivatives of mouse RAG1 and RAG2. The solubility of the overexpressed proteins was improved by using forms of each RAG protein that were truncated. The deletion mutants used have been previously described and were shown to retain the ability to mediate recombination of substrates in tissue culture cells. The R1 and MR1 proteins (Figure 1A) contain residues 384–1008 of the 1040 amino acids in full-length RAG1 (Sadofsky et al., 1993). The R2 and MR2 proteins (Figure 1A) contain residues 1–383 and 1–387, respectively, of the 527 amino acids in full-length RAG2 (Cuomo and Oettinger, 1994; Sadofsky et al., 1994). Proteins were expressed in insect cells with a baculovirus expression system (R1, MR1, MR2) or in HeLa cells with a vaccinia virus expression system (R2). All expression constructs include a polyhistidine sequence to permit purification on a Ni^{2+}–Sepharose column, and a Myc epitope to facilitate detection. MR1 and MR2 were made as fusions with maltose-binding protein (MBP) to enhance solubility further and to allow a second purification step on amylose columns. A second affinity purification of R2 was made possible with the addition of a FLAG epitope (Hopp et al., 1988). Inclusion of the epitopes and fusion moieties did not affect the ability of these proteins to support recombination of plasmid substrates in fibroblasts (data not shown).

Proteins resulting from our purification procedures are shown in Figure 1B. After purification on Ni^{2+}–Sepharose and amylose resin, the mixture of MR1 and MR2 is approximately 95% pure (lane 3). As this is the most pure preparation available, cleavage experiments were done with this pair of proteins, unless stated otherwise. Although the preparations of R1 and R2 are slightly less pure, RAG1 and RAG2 are the major protein species present (lanes 1 and 4).

RAG1 and RAG2 Proteins Are Sufficient to Cleave DNA at Recombination Signals

We asked whether the nuclear extract used in our original cell-free system could be replaced just by RAG2 protein. A convenient method for the detection of DSBs is the ligation-mediated polymerase chain reaction (LMPCR; Figure 2A). The substrate used here is the plasmid pMS319 (Sadofsky et al., 1995), which contains both a 12- and a 23-signal. Ligation of a linker to a blunt 12-signal end followed by PCR generates a 167 bp product, with a novel ApaLI restriction site formed at the ligation border (van Gent et al., 1995). As shown in Figure 2B, the purified RAG1 and RAG2 proteins are able to cleave at the signal border in the absence of any additional proteins (lane 3). All of the LMPCR product is shortened by 25 bp following ApaLI digestion (lane 4), confirming that cleavage occurred precisely at the border between the coding sequence and the RSS heptamer and resulted in a blunt, 5′-phosphorylated signal end. DSBs were also detected at the 23-signal (data not shown). Thus, the purified RAG proteins carry out the V(D)J-mediated DSB formation seen previously with pre-B cell nuclear extracts.

It was apparent that purified RAG1 and RAG2 proteins cleaved a much larger fraction of the DNA than nuclear extracts supplemented only with RAG1. Upon Southern blotting of the cleavage products from a plasmid substrate,

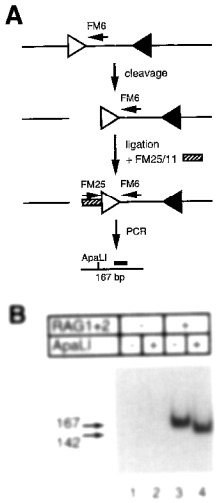

Figure 2. LMPCR Analysis of Cleavage

(A) Schematic representation of the LMPCR assay. The substrate was the plasmid pMS319. After the initial cleavage reaction (by RAG1 and RAG2 proteins MR1 and MR2), oligonucleotide linker FM25/11 is ligated onto the DSB, followed by PCR amplification. Amplification products are detected on Southern blots by using an oligonucleotide probe (depicted by a closed bar).

(B) Southern blot analysis of LMPCR products from a cleavage reaction without (lanes 1 and 2) or with (lanes 3 and 4) RAG1 and RAG2 proteins. LMPCR products were digested with ApaLI in lanes 2 and 4. The positions of the 167 bp LMPCR product and the 142 bp product resulting from ApaLI digestion are shown on the left.

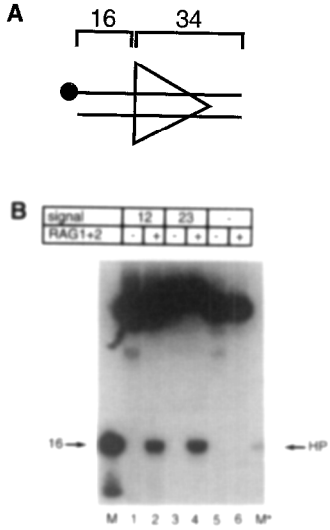

Figure 3. Oligonucleotide Cleavage Assay

(A) Schematic representation of the 12-signal substrate. The 12-signal is depicted by an open triangle, and the position of the ^{32}P label is marked by a black dot.

(B) Analysis of oligonucleotide cleavage products by gel electrophoresis under native conditions. The 12-signal substrate (lanes 1 and 2), 23-signal substrate (lanes 3 and 4), or the nonspecific 50-mer FM117/FM116 (lanes 5 and 6) was incubated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) RAG1 and RAG2 proteins MR1 and MR2. The double-stranded 16-mer (lane M) and 32-mer hairpin (lane M asterisk) oligonucleotides were included as markers. HP, 32-mer hairpin; 16, double-stranded 16-mer.

5%–10% of the DNA was found to be broken (data not shown). (For comparison, cleavage in cell extracts supplemented with RAG1 protein [van Gent et al., 1995] did not exceed 0.1%.) Thus, it seemed possible that cleavage of radiolabeled oligonucleotide substrates could be detected directly, thereby simplifying analysis of the cleavage reaction. Because cleavage took place on a plasmid containing only a single signal sequence (van Gent et al., 1995), it was likely that only one signal would be required in an oligonucleotide. We therefore made double-stranded DNA oligomers with a 12- or a 23-signal, flanked by 16 bp of coding sequence next to the heptamer and 6 bp of flanking sequence next to the nonamer (Figure 3A).

When the 12-signal substrate, radiolabeled at the 5′ end of the coding flank, was incubated with RAG1 and RAG2 proteins and the products analyzed by gel electrophoresis under native conditions, a 16 bp species was seen (Figure 3B, lane 2). This cleavage product corresponds to a DSB at the 5′ end of the heptamer sequence. Approximately 5%–10% of the oligonucleotide was cleaved. When the oligonucleotide was labeled at the 5′ end of the opposite strand, the corresponding 34 bp cleavage product was detected (data not shown); this molecule terminates precisely at the signal end. As expected, similar cleavage products were generated with a 23-signal substrate (lane 4), but not when the RSS was replaced by a scrambled sequence (lane 6).

As had been found in extracts, cleavage by the purified proteins did not require the addition of ATP or any other energy-generating cofactor. Although not required, glutamate in place of chloride stimulated the reaction and was thus routinely used as the major anion. The use of Mg^{2+} instead of Mn^{2+} as divalent cation decreased the activity, but some cleavage, including formation of hairpins, could still be observed.

Detection of Nicks and Hairpins

Coding ends produced in a nuclear extract from a pre-B cell line were previously shown to terminate in a hairpin structure. Because cleaved fragments with open ends and those with a hairpin structure are indistinguishable on a native gel, the cleavage products from a 12-signal oligonucleotide were analyzed by denaturing gel electrophoresis. Under these conditions, not one but two products were now observed (Figure 4A, lane 2). The upper product comigrates with a 32 nt hairpin molecule (marker lane, M) and the lower product with the 16 nt marker for the top strand of coding end DNA. (Throughout this paper, the top strand is defined as the strand that reads 5′ to 3′ from the coding flank into the heptamer of the RSS, e.g., the upper strand in Figure 3A.) Again as expected, both cleavage products were seen with a 23-signal substrate (lane 4), while specific cleavage was not evident in the absence of an RSS (lane 6).

The appearance of both the 32 nt and 16 nt fragments could be explained if the single cleavage product observed under native gel conditions were actually a mixture of hairpins and open coding ends. Alternatively, the cleavage product seen on the native gel could be entirely composed of hairpins, while the 16 nt molecule revealed under dena-

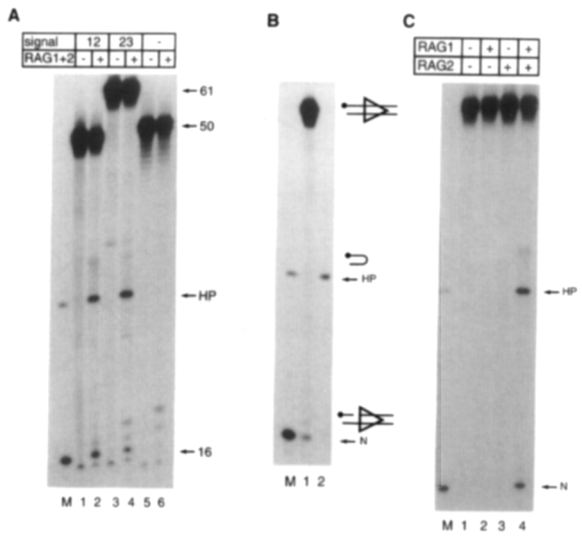

Figure 4. Characterization of Cleaved DNA

(A) Denaturing gel analysis of oligonucleotide cleavage products. The 12-signal substrate (lanes 1 and 2), 23-signal substrate (lanes 3 and 4), or the nonspecific 50-mer FM117/FM116 (lanes 5 and 6) was incubated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) RAG1 and RAG2 proteins MR1 and MR2.

(B) Analysis of cleavage products isolated from a native gel (see Figure 3B, lane 3). Products isolated from the positions of full-length oligonucleotide substrate (lane 1) and the cleavage product (lane 2) were analyzed on a 12.5% polyacrylamide gel in TBE–urea.

(C) Analysis of cleavage products in reactions without RAG proteins (lane 1), or with RAG1 only (lane 2), or RAG2 only (lane 3), or both RAG1 and RAG2 proteins (lane 4). For the assays in (C), RAG1 protein R1 and RAG2 protein R2 were used. M, marker lane containing a mixture of 16-mer coding flank fragment and 32-mer hairpin. The positions of unreacted substrates (61-mer with a 23-signal, 50-mer with a 12-signal, and nonspecific 50-mer) and the hairpin (HP) and 16-mer cleavage products are indicated on the right. In (B) and (C), the position of the nicked species (N) is also indicated.

turing conditions could represent a nick introduced at the 5′ end of the RSS. Under native gel conditions, such a nicked molecule would run at the same position as the uncut substrate, while on a denaturing gel, it would yield a 16 nt radiolabeled product. To distinguish between these possibilities, DNA migrating at the positions of full-length substrate and cleavage product was recovered from a native gel, and these two isolates were analyzed on a denaturing gel. The DNA isolated from the position of the full-length substrate (50 bp) in the native gel contained uncut molecules (50 nt), and also the 16 nt cleavage product (Figure 4B, lane 1), which must therefore result from a nick immediately 5′ of the heptamer sequence. The isolated cleavage product consisted entirely of hairpin molecules (lane 2). No open coding ends (which should be visible as a 16 nt species on the denaturing gel) were detected. DNA sequencing of the hairpin product (by the Maxam–Gilbert method) showed that the last nucleotide of the coding end in the top strand was covalently attached to the nucleotide immediately opposite in the lower strand (data not shown).

The experiments described above were all carried out with RAG1 and RAG2 fusion proteins coexpressed in insect cells (MR1 and MR2). To determine whether either protein had an activity on its own, we used RAG1 protein produced in a baculovirus expression system (R1) and RAG2 protein produced by recombinant vaccinia virus in HeLa cells (R2). The vaccinia-produced RAG2 protein was at least as active as the RAG2 coexpressed with RAG1 from baculovirus vectors. RAG2 protein expressed singly from baculovirus had a 10- to 100-fold lower activity (data not shown) and thus was not suitable for these experiments. The reasons for these differences in activity are not yet clear. When mixed together, the R1 and R2 protein preparations efficiently mediated cleavage of RSS-containing oligonucleotides (Figure 4C, lane 4). However, neither protein by itself generated either nicks or hairpins (lanes 2 and 3).

In the cleavage reactions on the 23-signal and the DNA without an RSS, a few bands could be detected above the position of the 16 nt specific cleavage product. We do not know whether this nicking activity is mediated by RAG1, RAG2, or both, or whether there is still some contaminating nuclease in these protein preparations.

Progression of Nicked DNA to Hairpins

Identification of the nicked species suggested that a nicked molecule might be a precursor of the hairpin and that nicking of DNA is the earliest event in the V(D)J recombination pathway. However, nicked products might instead result from an abortive side reaction. To distinguish these possibilities, we first investigated whether nicks appear before hairpins. The 12-signal substrate was incubated with RAG1 and RAG2 proteins, and aliquots of the reaction mixture taken at time points up to 6 hr. As shown in Figure 5A, the 16 nt product accumulated from the earliest time point tested (2 min), increasing in amount until approximately 20 min and then remaining constant for several hours. The hairpin product was not detectable until 8 min and accumulated up to 6 hr. These results support the model that a single-strand nick precedes hairpin formation.

To confirm the identity of these species, samples taken at 0, 10, and 180 min were analyzed by 2-dimensional polyacrylamide gel electrophoresis (PAGE). Samples were first electrophoresed under native conditions. The gel lane containing the cleavage products was then removed and equilibrated in buffer containing 7 M urea to denature the DNA and placed on a denaturing gel. Electrophoresis in the second dimension separates uncut oligonucleotides, nicks, hairpins, and potential open coding ends, as diagrammed in Figure 5B. Analysis of the cleavage products confirmed that the early-appearing 16 nt product arises from a nick, and the late-appearing 32 nt product is a DNA hairpin. Furthermore, no open coding ends are detected at any time. Figure 5C gives a graphic representation of the results shown in Figure 5A and demonstrates the rapid appearance of nicks followed by the slower accumulation of hairpins.

To study the conversion of nicks to hairpins further, we asked whether a preexisting nick at the border of the RSS could be converted into a hairpin. A prenicked 12-signal substrate was made by annealing three oligonucleotides:

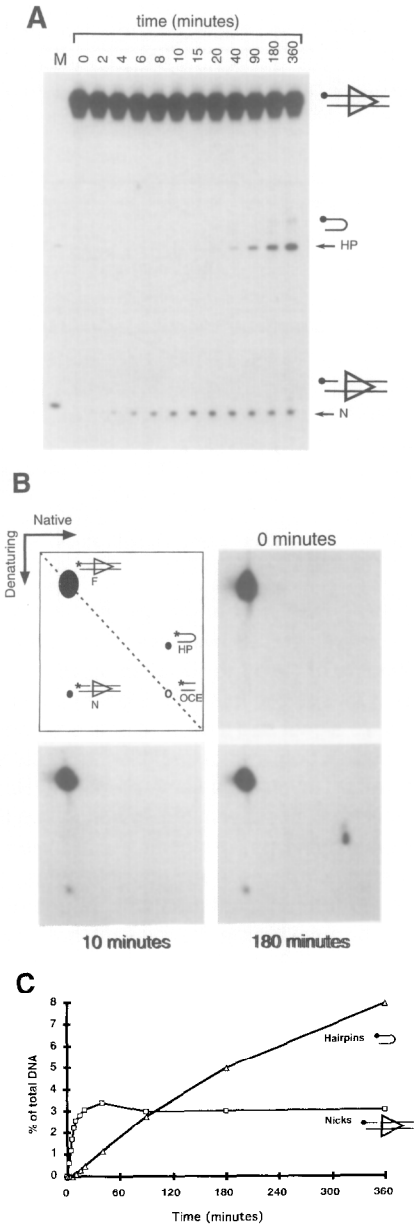

Figure 5. Nicks Precede Hairpins

(A) Time course of RAG-mediated cleavage. Cleavage products at time points between 0 and 360 min, at 30°C, were analyzed on a denaturing gel. The incubation time is shown at the top; the positions of full-length substrate, 32-mer hairpin, and 16-mer nicked product are depicted on the right. M, mixture of 32-mer hairpin and 16-mer markers.

(B) Analysis of time course by 2-dimensional gel electrophoresis. The top left corner is a graphic description of the expected relative positions of the possible DNA species. The directions of native and denaturing electrophoresis are also indicated. F, full-length substrate; N, nicked species; HP, hairpin species; OCE, open coding end species. Observed species are pictured with closed ellipses; the predicted position of the potential open coding end species is indicated by an open ellipse. Aliquots of the reaction described in (A) from 0 (top right), 10 (bottom left), and 180 min (bottom right) are shown. Only the relevant portion of each gel is presented.

(C) Graphic representation of the amounts of cleavage products in (A).

a radiolabeled coding flank 16-mer, the remaining 34 nt (including the RSS sequence) of the same strand (the top strand), and a continuous 50 nt bottom strand. This substrate was incubated with RAG1 and RAG2, and the products were analyzed on a denaturing gel. As shown in Figure 6A, the labeled nicked strand was successfully converted to a hairpin (lane 2). This activity is not due to ligation of the top strand 16-mer to the 34-mer followed by a subsequent cleavage reaction; ligation would require a phosphoryl group on the 5′ end of the 34-mer, and this is not present. The rate of hairpin formation was very similar to that of a full-length substrate, when corrected for the initial lag due to the prior nicking of the full-length DNA (data not shown). Hairpins were also formed from a prenicked substrate containing a 23-signal (lane 4), but RAG1 and RAG2 did not convert a prenicked substrate lacking an RSS into a hairpin (lane 6).

Formation of hairpins requires a phosphodiester bond in the bottom strand, opposite the site of the initial nick. When this phosphodiester bond was replaced by a nick (in a substrate with a continuous top strand), no hairpins were formed (data not shown); incubation with RAG proteins only generated the 16 nt product formed by cutting of the top strand at the RSS boundary.

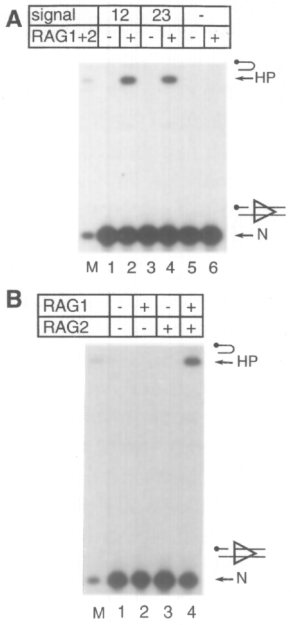

Figure 6. Nicks Are Precursors of Hairpins

(A) Analysis of cleavage products from reactions on substrates with a nick in the top strand: 12-signal (lanes 1 and 2), 23-signal (lanes 3 and 4), or the nonspecific sequence used in Figure 3 (lanes 5 and 6). DNA was incubated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) RAG1 and RAG2 proteins MR1 and MR2.

(B) Cleavage of a 12-signal substrate with a nick in the top strand, without RAG proteins (lane 1), with RAG1 only (lane 2), RAG2 only (lane 3), or both RAG1 and RAG2 (lane 4). For the assays in (B), RAG1 protein R1 and RAG2 protein R2 were used. M, marker lane with 32-mer hairpin and 16-mer markers; the positions of nicked substrate (N), hairpin (HP), and 16-mer product (16) are shown on the right.

It was shown in Figure 4C that both RAG1 and RAG2 are required for the formation of nicks and DSBs. From these results, it was not clear whether only formation of the initial nick requires both RAG1 and RAG2, or whether this is also the case for hairpin formation. We therefore incubated the prenicked 12-signal substrate with RAG1 alone (Figure 6B, lane 2), or RAG2 alone (lane 3), or a mixture of both (lane 4). Hairpins were only observed when both proteins were present, indicating that this second step in the cleavage reaction also requires both RAG proteins.

As shown above, RAG proteins purified from insect cells are active, yet insect cells do not normally carry out V(D)J recombination and would therefore be unlikely to contain factors required for this site-specific cleavage. Thus, although the protein preparations contain some minor contaminants, it is highly likely that the only relevant proteins are RAG1 and RAG2. Furthermore, the results show that no modification specific to mammalian cells is required to activate the RAG proteins.

Discussion

RAG1 and RAG2 Are Sufficient for Cleavage at Signal Sequences

V(D)J recombination initiates with the generation of DSBs at RSSs. Here, we show that only purified RAG1 and RAG2 proteins are required to carry out this site-specific cleavage reaction. The products of RAG-mediated signal cleavage are a hairpin structure on the coding side and a blunt, 5′-phosphorylated signal end. Thus, RAG1 and RAG2 proteins are sufficient to reproduce the cleavage products observed previously at TCR and immunoglobulin loci in lymphoid cells (Roth et al., 1992a, 1992b; Schlissel et al., 1993; Ramsden and Gellert, 1995), and in pre-B cell nuclear extracts at recombination signals on plasmid substrates (van Gent et al., 1995). Using purified RAG proteins and oligonucleotide substrates, we have found that the reaction occurs in two steps. In the first step, a single-strand nick is introduced at the 5′ end of the signal heptamer. In a second step, this nick is converted into a hairpin structure on the coding side and a blunt end on the signal sequence (Figure 7). Both nick and hairpin formation require an RSS, and the RAG1 and RAG2 proteins are together necessary and sufficient to carry out each step.

No specific cleavage is detected with either protein alone, even by LMPCR (unpublished data), and signal-specific binding has not yet been demonstrated by standard methods. However, the ability of purified RAG1 and RAG2 to carry out the cleavage reaction, and the requirement for a signal sequence in the substrate, implies a direct interaction between one or both RAG proteins and the signal sequence. Recent experiments show that, in a fibroblast transfection assay, recombination activity of a mutant form of the RAG1 protein is strongly influenced by mutations in the heptamer and flanking coding sequence of the substrate (Sadofsky et al., 1995), consistent with the binding of RAG1 to that region. RAG2 could also be involved in site recognition. Although both proteins are

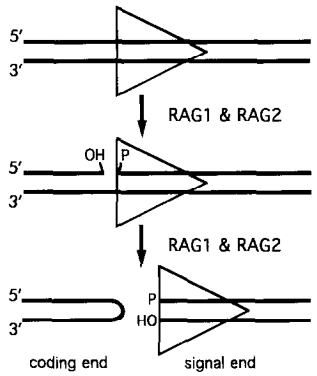

Figure 7. Model for the V(D)J Cleavage Reaction

In the first step, RAG1 and RAG2 proteins introduce a nick at the 5′ end of the signal sequence. The 3′-OH of this nicked signal is then coupled to the phosphate in the opposite strand, creating a coding end with a hairpin structure and a blunt, 5′-phosphorylated signal end. Both RAG1 and RAG2 are required for both steps.

needed for formation of an active complex with the full ability to cleave an RSS, we have not found evidence of stable binding of RAG1 to RAG2 in solution.

Earlier work showed that equivalent mutations in either the 12- or 23-signal had similar effects on recombination activity, suggesting that recognition of both signal sequences would involve the same protein(s) (Hesse et al., 1989). This is in accord with our evidence that RAG1 and RAG2 cleave both types of signals. Recognition of the two different signals may be made possible by using different numbers, or different conformations, of one or both proteins. In this context, it may be relevant that the difference in spacer lengths is approximately one turn of DNA double helix, so that the same faces of the heptamer and nonamer would be lined up in both signals.

The Cleavage Reaction

Cleavage by purified RAG1 and RAG2 proteins in the absence of extracts allows a closer look at reaction steps and energy requirements. The formation of a new phosphodiester bond at the end of the hairpin is an energy-requiring process. Because the cleavage reactions were done without addition of ATP or any other high energy cofactor, and with highly purified proteins, the source of energy would have to be either a high energy cofactor tightly bound to RAG1 or RAG2, or the conserved energy of one of the broken phosphodiester bonds. The energy of the phosphodiester bond that is broken in the initial nicking reaction is not required, because we find a prenicked substrate to be an efficient precursor for hairpin formation. However, the phosphodiester bond in the opposite strand is essential, suggesting that it is used for the formation of the new phosphodiester bond in the hairpin.

Two different methods are commonly used by recombination enzymes to conserve phosphodiester bond energy: first, a covalent intermediate can be formed between the DNA and the enzyme, which is then used to make the new phosphodiester bond, or second, the formation of the new phosphodiester bond is coupled to the breakage of the

old one in one concerted transesterification reaction. Reactions carried out by bacteriophage lambda integrase and by resolvases and invertases are examples of the first process (reviewed by Craig, 1988). In these cases, the recombination enzyme is coupled to the DNA backbone through a phosphoprotein linkage with a tyrosine or serine. Bacteriophage Mu transposition (Mizuuchi and Adzuma, 1991) and retroviral integration (Engelman et al., 1991) are examples of the second approach.

RAG-mediated hairpin formation appears to share several characteristics with this second family of transposition reactions. For instance, in the first step of Mu DNA transposition, the MuA protein introduces a pair of nicks in the transposon DNA. The exposed 3′-OH group of the transposon end is then used as the attacking nucleophile in the subsequent strand transfer step. A one-step transesterification reaction simultaneously breaks the phosphodiester bond at the target site and joins the 3′-OH of the transposon to the 5′ end of the target DNA (Mizuuchi and Adzuma, 1991). RAG1 and RAG2 could similarly use the 3′-OH of the nicked strand as the nucleophile to attack the phosphodiester bond in the other strand. With the purified system described here, it should be possible to determine whether the V(D)J cleavage pathway also involves a direct transesterification step.

V(D)J cleavage differs in one significant way from the transposition reactions, which involve a strand transfer event between different DNA molecules or different locations in the same molecule. In the V(D)J cleavage reaction, an intramolecular event must occur, with the 3′-OH of one strand attacking a phosphodiester bond in the opposite strand, resulting in hairpin formation.

The results presented here support a model in which hairpin coding ends are a compulsory intermediate on the pathway to coding joints. However, we cannot exclude that there may be an alternative pathway, involving a direct attack by the 3′-OH of the nick on the phosphodiester bond at the border of the other signal sequence (instead of the other strand of the same signal sequence), forming a coding joint without a DSB intermediate (Kallenbach and Rougeon, 1992).

Regulation of Cleavage In Vivo

V(D)J recombination is a tightly regulated event, occurring only in lymphocytes at an early stage of differentiation, at specific loci, and between pairs of gene segments flanked by suitable signal sequences (Lewis, 1994). It is likely that the specificities of cell type and differentiation stage are principally regulated at the level of RAG1 and RAG2 protein expression. The requirement of both proteins for cleavage at signal sequences may ensure that expression of either protein alone (such as the RAG1 expression observed in the brain [Chun et al., 1991]) is insufficient to cause potentially harmful DNA breaks in inappropriate cell types. The fact that both proteins are required for cleavage and are linked in the chromosome (Oettinger et al., 1990) further supports the view that they may be derived from a mobile genetic element.

Control of target site selection is probably more complicated. To assemble immunoglobulin and TCR genes, the V(D)J recombination reaction must specifically recognize individual gene segments and then cleave, process, and join them, while avoiding inappropriate joining events. By having signal sequences with different spacer lengths flanking groups of segments to be joined, and requiring that one sequence of each type be present for efficient recombination, a degree of specificity is imposed. Most evidence from in vivo experiments suggests that cleavage is a coupled reaction requiring two signals. In the TCRδ locus of mouse thymocytes, no evidence for a DSB at one signal end was found in the absence of a break at the other (Roth et al., 1992a), and in fibroblasts transfected with RAG1 and RAG2, the formation of DSBs in an integrated recombination substrate was greatly reduced when the substrate contained only a single signal sequence (D. B. Roth, personal communication). Furthermore, as noted previously (van Gent et al., 1995), cutting at one signal should leave a coding end that would persist in the absence of a partner, but these ends are rare in recombinationally active cells.

The coupled cleavage observed in vivo is not reflected in our present experiments. The RAG1 and RAG2 proteins can recognize and cleave at either a 12-signal or a 23-signal, and only a single signal is required for cleavage. Furthermore, no stimulation of cleavage is observed in the presence of a second signal. These observations suggest that cells may contain a factor that exerts negative control on the intrinsic cleavage ability of RAG1 and RAG2. Inhibition of RAG cleavage activity would then only be relieved following recognition of both a 12- and a 23-signal. If there is such a RAG inhibitor, it is unlikely to be specific to lymphoid cells or particular loci, because recombination of substrates in fibroblasts appears to follow the same coupling rule. Such an inhibitor would also prevent cleavage at randomly occurring RSS-like sequences within the genome that might otherwise be accessible to the recombination machinery. An alternative scenario is that coupling of cleavage at two signals greatly stimulates the cutting activity of the RAG proteins, allowing coupled cleavage to predominate in cells. By either model, greater control and enhanced reaction specificity would result from the requirement for two successive recognition and cleavage steps for formation of each DSB.

A further, but independent, level of control would still be required in lymphoid cells to ensure that recombination only takes place at the appropriate loci and in the appropriate order. It has been proposed that a condensed chromatin environment is itself a general inhibitor of V(D)J recombination activity, and lymphocyte-specific transcription or other processes may be required to render immunoglobulin and TCR genes open and accessible for recombination (Alt et al., 1992). In addition, a number of other proteins have been identified that possess some RSS-binding activity (reviewed by Lewis, 1994). While it is now clear that these proteins cannot themselves be the site-specific component of the recombinase, they could play some regulatory role in the reaction.

Possible Role of the RAG Proteins in Later Steps

Although RAG1 and RAG2 proteins alone can carry out

cleavage at recombination signals, their function may extend to later stages of V(D)J recombination as well. In vivo, signal ends and coding ends are joined at very different rates (Roth et al., 1992b; Zhu and Roth, 1995; Ramsden and Gellert, 1995). Hairpin coding ends are rapidly opened, processed, and joined, while signal ends are typically preserved in their initial form and are joined much more slowly. It is possible that RAG1 and RAG2, having performed the cleavage reaction, immediately dissociate from the cleaved intermediates and do not participate further, but such unprotected ends could be targets for nonstandard rearrangements such as hybrid or open-and-shut junctions, or possibly interchromosomal recombination. Thus, it seems plausible that RAG1 and RAG2 proteins may remain bound after cleavage, to exclude signal ends from coding end–specific events, and additionally to help recruit factors involved in general DSB repair. In addition, we cannot exclude the possibility that RAG1 and RAG2 may play some direct role in steps such as hairpin opening.

Experimental Procedures

DNA Techniques

Standard DNA techniques were used as described (Ausubel et al., 1989; Sambrook et al., 1989). The baculovirus transfer vector pDVG25 was constructed by ligating a PCR fragment containing the MBP–RAG2 (1–387) open reading frame from pDVG11 (van Gent et al., 1995) into the XbaI–NotI sites of pVL1393 (Pharmingen). The baculovirus expression vector AcD25 was made by using pDVG 25 and the BaculoGold Transfection Kit (Pharmingen) as described by the manufacturer. The MBP–RAG1 (384–1008) fusion construct pDVG5 was made by ligating the NcoI–XbaI fragment of pMSD27B (Sadofsky et al., 1993) containing the RAG1 open reading frame into the XmnI–XbaI sites of pMal-c2 (New England Biolabs), together with an adapter oligonucleotide. Subsequently the BsiWI–NotI fragment of pDVG5, containing part of the *malE* gene fused to the RAG1 (384–1008) open reading frame, was transferred into the BsiWI–NotI sites of pDVG25, replacing the RAG2 open reading frame. This baculovirus transfer vector pDVG26 was used to generate the baculovirus expression vector AcD26.

Construction of Vaccinia Vector and Recombinant Vaccinia

An oligonucleotide adaptor containing sequences encoding a FLAG epitope (Hopp et al., 1988) and a His_9 tag was introduced at the NcoI–EcoRI sites of the vaccinia expression vector pTM1 (Moss et al., 1990), such that the original NcoI site was destroyed, and a novel NcoI site was inserted in-frame downstream of the His_9 sequence to create pTM1FH. R2CC14M, a derivative of RAG2 containing amino acids 1–383 (Cuomo and Oettinger, 1994) and one N-terminal and three C-terminal Myc epitopes, was inserted into the pTM1FH expression vector as an NcoI–XhoI fragment such that RAG2 expression was under the control of a bacteriophage T7 promoter. Recombinant vaccinia virus was generated by using standard conditions as described (Ausubel et al., 1989). The desired recombinant RAG2 virus CRVR2.3 was identified by a two-step procedure: first, selecting for thymidine kinase–negative (TK^-) virus infecting the TK^- cell line Hu TK^- 143B (ATCC number CRL8303) by bromodeoxyuridine (BrdU) selection, and then, screening for RAG2 expression by infecting OST7–1 cells (a gift of B. Moss, National Institutes of Health), which stably express the T7 RNA polymerase in their cytoplasm, followed by immunohistochemical staining of the fixed and permeabilized cells. High titer stocks of plaque-purified virus were prepared in HeLa S3 cells.

Protein Expression and Purification

Standard baculovirus techniques were used as described (O'Reilly et al., 1994). The MBP–RAG1 and MBP–RAG2 fusion proteins (MR1 and MR2) were coexpressed by infecting Sf9 cells with the baculovirus expression vectors AcD25 and AcD26 at a 3:1 ratio. After 48 hr, cells were harvested and proteins purified on a Ni^{2+}–Sepharose column as described (van Gent et al., 1995). Fractions containing the fusion proteins were pooled, diluted with 2 vol of buffer A (10 mM Na–phosphate [pH 7.2], 500 mM NaCl, 1 mM DTT) containing 0.25% Tween 20, and loaded onto amylose resin (New England Biolabs). The column was washed with 5 column volumes of buffer A containing 0.25% Tween 20, and then 3 column volumes of buffer A. Proteins were eluted in buffer A plus 10 mM maltose. Protein-containing fractions were pooled and dialyzed against buffer R (25 mM Tris–HCl [pH 8.0], 150 mM KCl, 2 mM DTT, 10% [v/v] glycerol) for 3 hr. Aliquots were frozen in liquid nitrogen and stored at −80°C.

Truncated RAG1 protein (384–1008) (R1) was expressed and purified as described (van Gent et al., 1995).

Preparation of Vaccinia RAG2 Protein

RAG2 protein was expressed by coinfecting HeLa S3 cells with the RAG2–vaccinia virus CRVR2.3 and a T7 RNA polymerase–producing vaccinia virus, vTF7–3 (ATCC number VR-2153) at a ratio of 1:1 and a combined multiplicity of infection of 20–40. After 24 hr, the infected cells were harvested and washed twice with PBS, and the cell pellets were frozen in liquid nitrogen and stored at −80°C. Cell pellets were lysed by homogenization in 10 vol of buffer C0 (500 mM NaCl, 20 mM Tris–HCl [pH 7.5]) followed by the addition of glycerol and Triton X-100 to final concentrations of 10% and 0.1%, respectively (buffer CT). Cellular debris were removed by centrifugation at 30,000 rpm for 20 min at 4°C in a Beckman 70.1Ti rotor. The supernatant was loaded onto an anti-FLAG column (M2–agarose, IBI/Kodak) and washed with 5 column volumes of buffer CT. The RAG2 protein was eluted in buffer CT containing 100 μg/ml FLAG peptide (IBI/Kodak). The RAG2-containing fractions were pooled and brought to 10 mM imidazole and 2 mM β-mercaptoethanol (buffer I). This sample was then purified on a Ni^{2+}–Sepharose column as described (van Gent et al., 1995).

Cleavage Assays

The following oligonucleotides were used in the cleavage assays: the 12-signal substrate DAR39 (5′-GATCTGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAA CCCTGCAG-3′), annealed to its complementary strand DAR40, the 23-signal substrate DG61 (5′-GATCTGGCCTGTCTTACACAGTGGTAGTACTCCACTGTCTGGCTGTACAAAAACCCTGCAG-3′), annealed to its complementary strand DG62, and the nonspecific oligonucleotide FM117 (5′-GATCTGGCCTGTCTTAGGTCAATGCTGTAGAACTCGTCCTGTACCTGCAG-3′), annealed to its complementary strand FM116. The oligonucleotides DAR39, DG61, and FM117 were radiolabeled. Prenicked substrates were made by annealing the radiolabeled oligonucleotide DAR42 (5′-GATCTGGCCTGTCTTA-3′) to DAR40 and DG10 (5′-CACAGTGCTACAGACTGGAACAAAAACCCTGCAG-3′), DG62 and DG4 (5′-CACAGTGGTAGTACTCCACTGTCTGGCTGTACAAAAACCCTG CAG-3′), or FM116 and FM115 (5′-GGTCAATGCTGTAGAACTCGTCCTGTACCTGCAG-3′). The 12-signal substrate containing a nick in the bottom strand was made by annealing the radiolabeled oligonucleotide DAR39 to DG9 (5′-CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTG-3′) and DAR49 (5′-TAAGACAGGCCAGATC-3′); DAR49 was 5′ phosphorylated using T4 polynucleotide kinase (New England Biolabs) and nonradioactive ATP.

The standard oligonucleotide cleavage assay contained 25 mM MOPS–KOH (pH 7.0), 5 mM Tris–HCl, 30 mM KCl, 60 mM K-glutamate, 1 mM $MnCl_2$, 2.2 mM DTT, 2% (v/v) glycerol (including components contributed by the protein preparations), 0.2 pmol of double-stranded oligonucleotide substrate, and approximately 100 ng of MBP–RAG1 fusion protein and 50 ng of MBP–RAG2 fusion protein expressed in the baculovirus expression system, in 10 μl total volume. When indicated, MBP–RAG1 was replaced by 100 ng of RAG1 (384–1008), and MBP–RAG2 by 5 ng of RAG2 expressed in the vaccinia virus expression system. Reaction mixtures were incubated for 45 min at 37°C (unless stated otherwise). Negative controls in the experiments of Figures 2, 3, 4A, and 6A contained the same constituents but no RAG1 or RAG2 protein. After addition of 0.1% SDS, 4 μl of the reaction mixture was used for native gel electrophoresis. Samples were separated on a 15% polyacrylamide gel in TBE, and products were visualized by autoradiography. For markers, we used the 16-mer DAR42 annealed to its complementary strand DAR49, and the 32-mer hairpin DAR41 (5′-GATCTGGCCTGTCTTATAAGACAGGCCAGATC-3′). For analysis by denaturing gel electrophoresis, 2 μl of formamide loading dye (96% formamide, 20 mM EDTA, 0.1 mg/ml xylene cyanol XFF, 0.1 mg/ml bromophenol blue) was added to 2 μl of the reaction mixture, and

samples were heated to 95°C for 2 min and separated on 12.5% polyacrylamide gels in TBE–urea. Products were visualized by autoradiography and quantified with a phosphorimager (Molecular Dynamics).

For the 2-dimensional gel electrophoresis experiment of Figure 5B, aliquots taken at selected times from the time course experiment of Figure 5A were first electrophoresed in a 0.8 mm thick 12% polyacrylamide gel under native conditions. Each lane was cut out, equilibrated briefly (5–10 min) in 1 × TBE, 7 M urea, and laid perpendicular to the direction of electrophoresis on a 1.5 mm thick 12.5% polyacrylamide denaturing gel. To confirm the relative positions of each species, a 16 bp single-stranded DNA marker (DAR42) and a 32 bp hairpin marker (DAR41) were loaded in parallel lanes during electrophoresis of the denaturing dimension. Products were visualized by autoradiography.

Cleavage experiments on plasmid substrates were carried out in the same way as the oligonucleotide cleavage assay, but 20 ng of plasmid pMS319 (Sadofsky et al., 1995) was used instead of the oligonucleotide substrate. DSBs were detected by LMPCR as described (van Gent et al., 1995). We performed 25 cycles of PCR, resulting in a detection limit of approximately 0.001% of the input DNA.

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