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RAG1/2-Mediated Resolution of Transposition Intermediates: Two Pathways and Possible Consequences

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Summary

During B and T cell development, the RAG1/RAG2 protein complex cleaves DNA at conserved recombination signal sequences (RSS) to initiate V(D)J recombination. RAG1/2 has also been shown to catalyze transpositional strand transfer of RSS-containing substrates into target DNA to form branched DNA intermediates. We show that RAG1/2 can resolve these intermediates by two pathways. RAG1/2 catalyzes hairpin formation on target DNA adjacent to transposed RSS ends in a manner consistent with a model leading to chromosome translocations. Alternatively, disintegration removes transposed donor DNA from the intermediate. At high magnesium concentrations, such as are present in mammalian cells, disintegration is the favored pathway of resolution. This may explain in part why RAG1/2-mediated transposition does not occur at high frequency in cells.

Introduction

During the development of lymphoid cells, the genes encoding immunoglobulins and T cell receptors are assembled from variable (V), diversity (D), and joining (J) gene segments by V(D)J recombination (reviewed by Lewis, 1994; Gellert, 1997). Recombination is targeted by recombination signal sequences (RSS) that flank the gene segments. Each RSS consists of conserved heptamer and nonamer motifs separated by either 12 or 23 base pairs of nonconserved sequence. Recombination only occurs between pairs of signals with dissimilar spacers, giving rise to the 12/23 rule, which ensures that only the appropriate gene segments are joined (Tonegawa, 1983). V(D)J recombination is initiated by the action of the RAG1 and RAG2 proteins, which together form a recombinase (RAG1/2) that makes double-strand DNA breaks at the borders between the RSSs and the coding DNA. The later processing and joining of signal ends and coding ends is dependent on RAG1/2 as well as other cellular factors that are involved in repair of radiation-induced double-strand DNA breaks (Bogue and Roth, 1996; Jeggo, 1998).

Several stages in the action of the RAG proteins have been defined. RAG1 and RAG2 act together to recognize an RSS, and, with the help of the nonspecific DNA binding protein, HMG1 are capable of forming a synaptic complex between RSSs with differing spacer lengths (Eastman et al., 1996; van Gent et al., 1996a, 1997). Subsequent cleavage by RAG1/2 can be divided into two distinct steps (McBlane et al., 1995). First, the RAG proteins nick the DNA at the 5′ end of a signal heptamer adjacent to the coding DNA. The nick is then converted into a hairpin in a single transesterification step, resulting in a double-strand DNA break with a blunt 5′-phosphorylated signal end and a hairpin coding end. When magnesium is used as the divalent cation, as is likely under physiological conditions, transesterification requires synaptic complex formation (van Gent et al., 1996a).

Several lines of evidence suggest that the modern immune system may have evolved from an ancient transposon. The organization of the RSSs at the immunoglobulin and T cell receptor loci is reminiscent of the sequences at the ends of transposable elements (Sakano et al., 1979; Thompson, 1995). The close linkage of the *RAG1* and *RAG2* genes, together with the sudden appearance of recombinatorial immunity in evolution also argues for derivation from a mobile DNA species (Oettinger et al., 1990). Recently, this connection has been supported by studies from our laboratory and others, showing similarities between reactions of the RAG proteins and the strand transfer reactions of retroviruses and transposons. Hairpin formation by the RAG proteins occurs in a single transesterification step (van Gent et al., 1996b), similar to the strand transfer reactions of bacteriophage Mu transposase and HIV integrase (Engelman et al., 1991; Mizuuchi and Adzuma, 1991). DNA cleavage by the bacterial transposons Tn10 and Tn5 also uses a hairpin intermediate (Kennedy et al., 1998; Bhasin et al., 1999). Furthermore, it has recently become apparent that the RAG1/2 complex has other activities in addition to cleavage. The RAG proteins can cause a signal end to attack a hairpin coding end, generating so-called hybrid joints or open-and-shut joints (Melek et al., 1998). This rejoining reaction appears to be the reversal of the initial cleavage. A related reversal of strand transfer has been observed with HIV integrase (Chow et al., 1992). The RAG proteins have also been reported to catalyze hairpin opening and carry a 3′ flap endonuclease activity (Besmer et al., 1998; Santagata et al., 1999; Shockett and Schatz, 1999). Finally, a catalytic DDE motif, similar to those found in several transposases, has been identified in RAG1 (Kim et al., 1999; Landree et al., 1999; Fugmann et al., 2000).

Most strikingly, the RAG proteins have been shown to carry out a transpositional strand transfer that is very similar to the reactions of known transposases (Agrawal et al., 1998; Hiom et al., 1998). RSS ends are inserted into target DNA either singly or pairwise; in the latter case, they insert into opposite strands with a well-defined 5 bp stagger (Hiom et al., 1998). This reaction could compete with the formation of normal signal joints and, more seriously, potentially have negative consequences for chromosomal integrity. Furthermore, RAG-mediated transpositional attack of one chromosome on another might lead to chromosomal translocations and thus be involved in the development of lymphoid tumors.

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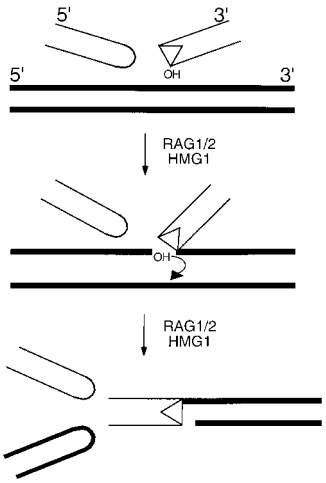

Figure 1. A Possible Mechanism for a Chromosome Translocation Catalyzed by the V(D)J Recombinase

The lymphoid-specific proteins RAG1/2 cleave an antigen receptor locus (thin lines) at an RSS to yield a blunt phosphorylated signal end and a hairpin coding end. The 3′-OH of the signal end is able to attack another DNA molecule (thick lines), resulting in a branched transposition intermediate as depicted in the middle panel. If the 3′-OH moiety, with the assistance of the RAG proteins, is able to attack the phosphodiester bond on the opposite strand, a translocated DNA species consisting of signal-bearing donor DNA and one end of target DNA is formed (after nick repair). The complementary translocation can be produced by joining of the two hairpin ends, as in normal V(D)J recombination.

If this attack is followed by a second transesterification reaction to form a hairpin in the target DNA (Figure 1), repair factors associated with coding joint formation could join the immunoglobulin and TCR promoters/enhancers to other genes leading to oncogenic transformation, as previously suggested (Hiom et al., 1998).

Here we investigate the further processing of strand transfer intermediates by the RAG proteins themselves. We find that the RAG proteins, in the absence of other cellular factors, are able to generate DNA products compatible with a chromosome translocation pathway, because a hairpin end is formed on the target DNA (Figure 1). Joining of two coding ends (one from the donor coding DNA and the other from the acceptor DNA) would complete the translocation. We also demonstrate that the RAG proteins have a competing activity that can reverse a transpositional strand transfer, removing the transposing RSS end and resealing the target DNA. The disintegration reaction, which is very similar to that described previously for HIV integrase, is extremely efficient and may partly protect cells from RAG-mediated transposition.

Results

Resolution of Strand Transfer Intermediates by Hairpin Formation on the Transpositional Target

We previously suggested that some chromosomal translocations, of the type found associated with lymphoid tumors, could result from RAG-mediated transpositional attack of an RSS onto another chromosome. This mechanism would require the branched transposition intermediate diagrammed in Figure 1 to be further processed to yield a broken target chromosome with a hairpin end, as shown. We devised a test in which radiolabeled RSS-containing DNA was first cleaved and then allowed to attack a plasmid DNA so that formation of a hairpin end in the target would generate a species of unique size. Oligonucleotides containing 12- and 23-RSSs (one of them radiolabeled) were incubated with supercoiled pBR322 DNA, RAG proteins, and HMG1 protein (Hiom et al., 1998). Deproteinized DNA was then analyzed in two-dimensional gel electrophoresis, first on a native agarose gel and then at right angles under alkaline denaturing conditions.

Two-dimensional gel analysis revealed that RAG1/2 catalyze transesterification to form a hairpin on the transpositional target DNA. After resolution of strand transfer products in two dimensions, three predominant species were observed (Figure 2). Species X, resulting from a one-ended insertion, migrates as a nicked circle in the first dimension and as a plasmid sized linear DNA in the second dimension, because the nicked strand carries the label. At the position of linear plasmid in the native gel, two species (Y and Z) are superimposed, which are then resolved in alkaline electrophoresis. Species Y corresponds to a two-ended insertion, resulting in the mobility of a linear plasmid-length molecule in both dimensions. Species Z migrates in alkali as a molecule twice the molecular weight of the linear plasmid, based on comparison to molecular weight markers. The mobility of species Z as a plasmid-length linear DNA in a native gel and as a double-length linear in alkali is expected for a linear plasmid DNA with a hairpin at one end. This product can be generated from either a double- or single-ended insertion, followed by a single hairpin formation event. As another test of this structure, the DNA was exposed to mung bean nuclease (MBN) before electrophoresis. MBN is known to digest single-stranded DNA, including the unpaired nucleotides at the tip of a hairpin (Kabotyanski et al., 1995), while leaving double-stranded DNA undigested. It would thus convert the hairpin DNA to plasmid-length linear strands. After MBN digestion, species Z was reduced by 75%, but the remainder of the electrophoresis pattern was essentially unaltered (less than 4% decrease in species X or Y). Species Z thus represents the expected product of hairpin formation from a transposition intermediate. This conversion of the branched structure requires only RAG1/2, HMG1, and divalent metal ion. It was most efficient in reactions containing 0.5 mM Mn^{2+} only or 5 mM Mg^{2+} and 0.5 mM Mn^{2+} (the condition shown in Figure 2). Although transposition proceeded efficiently in Mg^{2+}, resolution via hairpin formation did not (data not shown). As with RAG-mediated cleavage and transposition, no external energy source such as ATP was needed.

Hairpin formation by RAG1/2 was also monitored by use of a preassembled branched substrate, resembling a transposition intermediate. The substrate (Figure 3) was radiolabeled at the 5′ end of the 30 nt fragment such that hairpin formation would yield a DNA chain 60 nt long. After reaction with RAG1/2, HMG1, and various metal ions, the products were resolved by denaturing electrophoresis (Figure 3). In the presence of Mn^{2+} (but not Mg^{2+} or Ca^{2+}), we observed a DNA species that

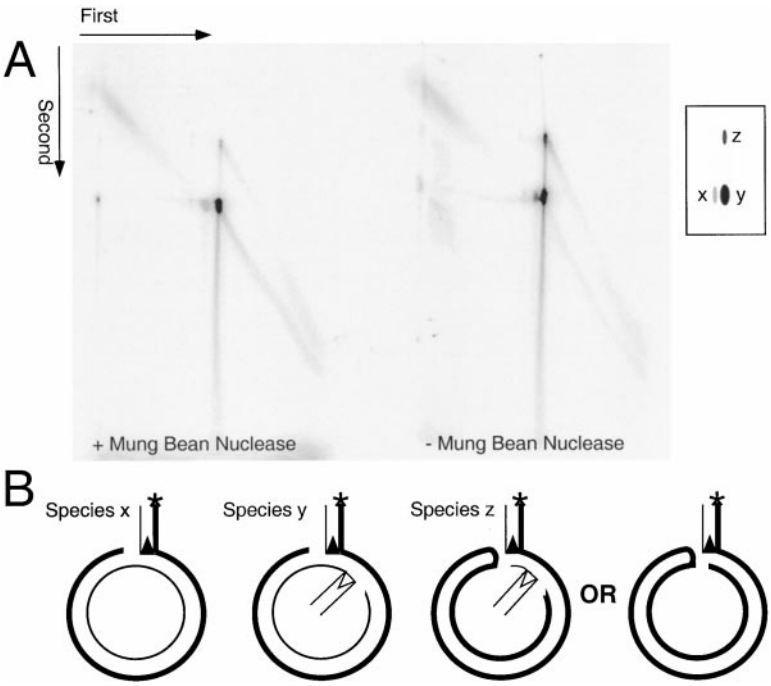

Figure 2. RAG-Mediated Hairpin Formation from a Transposition Intermediate

(A) Transposition reactions containing 0.5 mM Mn^{2+}, 5 mM Mg^{2+}, RSS-containing oligonucleotides, and plasmid target DNA were assembled and incubated at 37°C for 1 hr. Reactions were digested with mung bean nuclease (left side) or mock treated (right side). Two-dimensional agarose gels were run to identify large hairpin-containing DNA species. The first dimension separation was carried out under native conditions on a 1% agarose gel. The second dimension was run under alkaline denaturing conditions. Three predominant species observed are schematized to the right of the gel.

(B) Predicted DNA species formed via RAG-mediated transposition. Species X results from one-ended transposition, while species Y results from double-ended transposition. A hairpin-ended DNA molecule has the mobility of species Z, running as a linear plasmid monomer in a native gel and a plasmid dimer in alkali. Radiolabel is indicated by an asterisk.

migrated as a 60 nt hairpin molecule (compared to a labeled MW marker). Although the efficiency of hairpin formation on this preformed transposition intermediate was significantly lower than observed with the plasmid assay described above, this experiment confirmed that a hairpin could be made directly from the transposition intermediate.

Interestingly, the predominant product in this experiment was a very abundant species 80 nt long (Figure 3, lanes 2–4) that appeared to result from the removal of signal DNA and the simultaneous rejoining of the top strand of the remaining DNA. This reaction appears to be analogous to the disintegration process observed with HIV integrase (Chow et al., 1992), as is described more conclusively below. Unlike the conversion to hairpin, this product was made equivalently in Mn^{2+} or Mg^{2+}. Although the disintegration product was also observed in Ca^{2+}, it was only at 4% of the levels seen in Mn^{2+} or Mg^{2+}.

Disintegration of Transposition Intermediates by RAG1/2

Experiments with the preformed strand transfer intermediate revealed a novel activity for RAG1/2. To confirm that the 80 base species observed in Figure 3 resulted from disintegration of the RSS from target, we incubated RAG1/2 with substrates labeled in various positions. As above, incubation of RAG1/2 with the 5′ labeled 30 base DNA substrate, in the presence of Mg^{2+}, resulted in the formation of the 80 nt species (Figure 4A, lane 1). When the 5′ terminus of the 95 base DNA strand carrying the signal was labeled, a 45 nt band was observed, at about the same intensity (lane 4). This species corresponded to cleavage of the RSS at the branch point. Thus, both expected products of disintegration, the released RSS-bearing DNA and the rejoined target strand, were formed. This reaction is consistent with attack by the 3′-OH at the branch point on the phosphodiester bond connecting the RSS to the DNA.

The transposed RSS end can be detached from the strand transfer intermediate by either transesterification or hydrolysis. The 3′-OH group at the branch is required for transesterification. A substrate assembled with a 3′-deoxy end at the branch point cannot rejoin the target DNA to yield the 80 nt species (compare lanes 1 and 2 of Figure 4A). However, when the 95 nt strand is labeled, release of a small amount of the 45 nt RSS fragment is seen even in the absence of a terminal 3′-OH (lane 5), suggesting that the product could be generated by hydrolysis of the phosphodiester bond adjacent to the RSS. Labeling the substrate at the 3′ end of the 95 nt strand allowed us to monitor both transesterification and hydrolysis (Figure 4B). Incubation with RAG1/2 yielded two species. Appearance of the 80 nt species was dependent on the 3′-OH at the branch, while the 50 base species was not (compare lanes 7 and 8). Furthermore, the absence of the attacking 3′-OH enhanced the amount of the species generated by hydrolysis. Similarly, resection of the attacking 3′ end by two or three nucleotides of terminal end decreased rejoining via transesterification and increased the amount of hydrolysis product (lanes 11 and 12). Removing these 3′ nucleotides, and thereby altering the location of the 3′-OH within the active site, evidently renders the 3′-OH less available as a nucleophile for transesterification.

Relaxed Requirements for Disintegration

The coupled cleavage of RSS DNA that initiates V(D)J recombination by releasing coding ends is a highly regulated process that relies, under biologically relevant conditions, on the presence of both a 12- and 23-RSS, the integrity of the signal sequences, HMG1, and RAG1/2 (Eastman et al., 1996; Ramsden et al., 1996; van Gent et al., 1996a, 1997). The requirements for disintegration

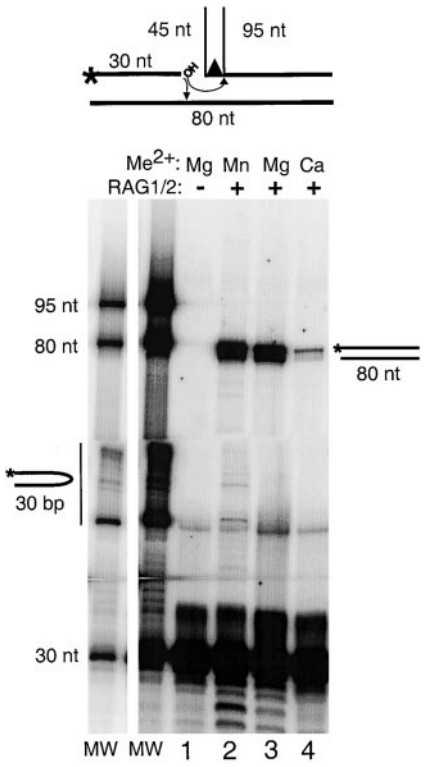

Figure 3. Disintegration of Transposition Intermediates by the RAG Proteins

The branched substrate depicted at top was labeled at the 5′ end of the 30 base oligonucleotide. Reactions were carried out with varying metal ion concentrations and with or without RAG1/2, as indicated. Reaction products were resolved on a 15% urea-acrylamide gel. Lane 1, 5 mM $MgCl_2$; lane 2, 0.5 mM $MnCl_2$; lane 3, 5 mM $MgCl_2$; lane 4, 5 mM $CaCl_2$. Molecular markers (MW) to the left of the figure were overloaded, so a lighter exposure is shown adjacent to this lane. Migration of a 5′-labeled hairpin marker is indicated by a bar at the left side of the figure.

were less strict. We incubated a branched intermediate containing a 12-RSS with RAG1/2 in the presence of Mg^{2+}, with or without a DNA fragment carrying a 23-RSS. As before, disintegration resulted in the efficient generation of an 80 nt species, and the presence of the 23-RSS had very little effect on this process (Figure 5A). A 23-RSS with no coding flank had equally little effect (data not shown). In contrast, an oligonucleotide cleavage assay confirmed that conversion of a nicked 12-RSS to a hairpin was greatly stimulated by the presence of the partner RSS (data not shown; van Gent et al., 1996a). Both 12 and 23 signals are also required for efficient transposition (Hiom et al., 1998). In addition, HMG1 did not stimulate disintegration (Figure 5A), while it does stimulate cleavage (van Gent et al., 1997). By these criteria, disintegration is less demanding than either cleavage or transposition.

Disintegration is sensitive to the sequence of RSS within the transposition intermediate. Disintegration is greatly reduced when the sequence of either the heptamer or nonamer is completely altered, though less extensive mutations are tolerated quite well (Figure 5B). The effects of these changes on disintegration parallel those reported for RSS cleavage (Ramsden et al., 1996). For example, scrambling of the 7-mer consensus from CACAGTG to ACTGCGT abolishes both nicking and hairpin formation at RSSs, as well as disintegration. Changing the final residues of the 7-mer has moderate effects on hairpin formation and disintegration. Hairpin formation occurs at about 75% of consensus levels (Ramsden et al., 1996), while disintegration proceeds at about 60%.

Competition between Transposition and Disintegration of the Strand Transfer Intermediate

A further distinction between RAG-mediated cleavage, transposition, and disintegration was found in their response to metal ion concentration. Although cleavage efficiency was relatively constant over the range of 0.5–25 mM Mg^{2+}, transposition was most efficient at low Mg^{2+} and declined sharply at higher metal ion concentrations, while disintegration continued to increase (Figure 6A). The difference between disintegration and transposition could occur at several possible steps: RSS binding, removal of coding DNA by cleavage, target DNA binding, strand transfer chemistry, or disintegration chemistry. The data in Figure 6A indicate that RSS binding and cleavage are not inhibited at the high Mg^{2+} concentrations and that disintegration proceeds efficiently. Gel shift analysis, in which nonspecific target DNA is labeled while the RSS DNA is not, demonstrates that binding is only moderately reduced at 25 mM Mg^{2+} compared to 5 mM Mg^{2+}. In 25 mM Mg^{2+}, binding occurred at 67% of the level observed in 5 mM Mg^{2+} (data not shown). Therefore, reduced binding does not account for the significant difference between transposition and disintegration.

Time course experiments, under conditions that favor disintegration (25 mM Mg^{2+}), show that the transposition products peak at an early time and then diminish (Figure 6B). The amount of transposition product is greatest at 30 min and then decreases. By 240 min, transposed DNA is barely detectable, while the cleaved product remains unchanged (data not shown). In contrast, the time course in 5 mM Mg^{2+} is very different. Transposition peaks at about 60 min, but it does not diminish over time. These reactions were performed with a 12-RSS labeled at the 5′ terminus of the bottom strand, such that cleavage and transposition could be monitored simultaneously. The significant decrease in transposition is not the result of a nuclease in the RAG1/2 preparation, as branched structures that do not contain an RSS are quite stable when incubated with RAG1/2 (Figure 6A; data not shown). The data also suggests that the lower transposition observed in 25 mM Mg^{2+} is not due to poor target binding. We speculate that under some conditions, disintegration and the completion of transposition may compete to resolve transposition intermediates.

Discussion

It was recently shown that the RAG proteins are capable of performing transpositional strand transfer and covalently inserting RSS ends into various positions in a target DNA (Hiom et al., 1998; Agrawal et al., 1998). The resulting branched molecules are analogous to those made by well-known “cut-and-paste” transposons such

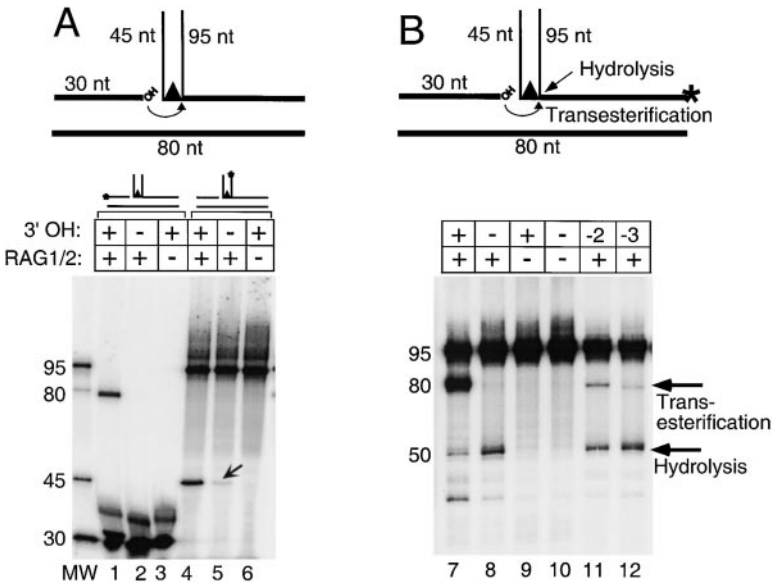

Figure 4. Two Mechanisms of Disintegration by RAG1/2

(A) Disintegration can proceed by transesterification. Oligonucleotides were labeled at the positions indicated and annealed to form the species depicted. Transposition reactions were carried out in 5 mM $MgCl_2$ with and without RAG1/2. Substrates lacking a 3′-OH on the 30 base oligonucleotide were also tested. A putative hydrolysis-mediated disintegration product is indicated by an arrow. Molecular weight markers (MW) are as indicated.

(B) Disintegration proceeds either by hydrolysis or transesterification. Disintegration reactions were performed as in (A) except the substrate was radiolabeled at the 3′ terminus of the 95 nt DNA molecule. Where indicated, the 3′-terminal two or three bases of the 30 nt species were removed. Products of transesterification and hydrolysis are indicated.

as *Tn7* and *Tn10* (Mizuuchi, 1992). Transpositional attack by the purified RAG1/2 proteins can be either one ended or two ended, leading to the single or pairwise insertion of RSSs. Similarities to other transposons include the observation that pairwise attack of two RSS ends takes place on opposite strands with a defined stagger of 5 bp. However, transposition is evidently not the usual outcome of RAG activity. Normally, cleavage leads to the formation of coding joints and signal joints. Thus, the observation of transpositional strand transfer by the RAG proteins led us to ask how transposition intermediates are resolved if they are formed. This work describes two modes of resolution that are distinct from the completion of transposition.

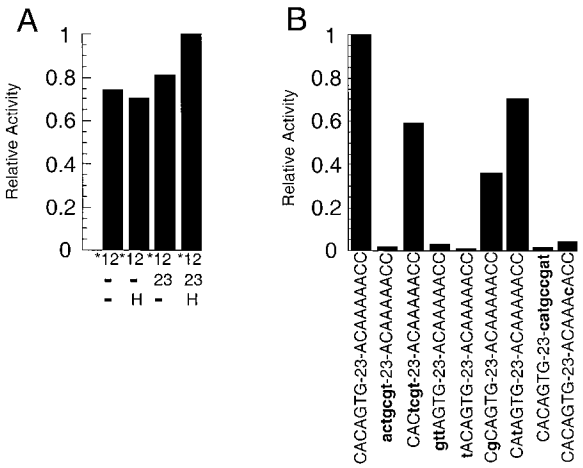

Figure 5. Requirements for Disintegration

(A) Disintegration is independent of two signals and HMG1. Reactions were assembled with or without unlabeled 23-RSS and with or without HMG1 (as indicated). The 12-RSS disintegration substrate was radiolabeled at the 5′ terminus of the 30 base species so that disintegration resulted in an 80 base species (as in Figure 4A, lane 1). Products were resolved on a 10% denaturing acrylamide gel, and the amount of 80 nt DNA was quantitated. Activities are reported relative to the reaction containing both HMG1 and unlabeled 23-RSS. Cleavage reactions were carried out in parallel in which the top strand was 5′ labeled.

(B) Disintegration is sensitive to signal sequence. Disintegration reactions were assembled with the 23-RSS varied from consensus (first bar) as indicated by lowercase letters. The substrates were labeled at the 5′ terminus of the 30 base species. Reactions were resolved on a 10% denaturing gel, and the 80 base disintegration product was quantitated. Activity is reported relative to disintegration with consensus signal sequence. The data shown is the average of two experiments.

The first reaction described provides a possible molecular model for a chromosomal translocation event (Figure 7). We previously suggested that translocations could result from an unusual mode of resolution of a transpositional intermediate. If the exposed 3′-OH in the target DNA at the branch point can attack the opposite strand, it will convert it to a hairpin end that is similar to the hairpins made at coding ends in normal RAG cleavage. This could then be followed by the joining of the newly created end to the hairpin end from the original cleavage at the antigen receptor locus. The process would be similar to normal coding joint formation but would result in the joining of ends from two different chromosomes. The reciprocal translocation would have the attacking RSS joined to the remaining part of the target chromosome. We show here by use of a plasmid-based transposition assay that the RAG proteins can indeed act on a strand transfer intermediate to produce the hairpin required in this model. Once these two species are formed by the RAG proteins, the usual DNA double-strand break repair pathway could complete the joining of both products (Bogue and Roth, 1996; Jeggo, 1998). Although the existence of such translocations is still uncertain, these experiments show that it is biochemically plausible.

Some translocations are at least consistent with this mechanism. One predicted product of this process would join the RSS from an Ig or TCR locus to non-RSS DNA on the partner chromosome. Such junctions have been identified (Hollis et al., 1984; Bernard et al., 1988). In a larger number of translocations, there was no RSS in the target chromosome sequence at or near the break point (see, for example, a collection of translocation

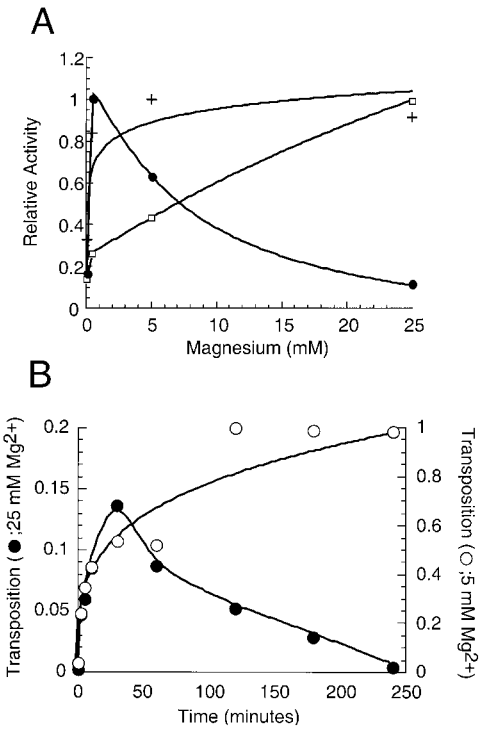

Figure 6. Examination of Disintegration and Transposition under Varying Reaction Conditions

(A) Effect of Mg^{2+} concentration on signal cleavage, transposition, and disintegration. Disintegration or cleavage reactions were assembled in the presence of Ca^{2+} and incubated for 10 min at 37°C. Acceptor plasmid and the indicated amounts of Mg^{2+} were then added, and the incubation continued for 60 min further. Reactions were extracted, precipitated and resuspended in TE. Half the reaction was resolved on a 6% urea-polyacrylamide gel, and the remainder on a 1% agarose gel. The 12-RSS oligonucleotide was labeled at the 5′ terminus of the bottom strand so that cleavage and transposition could be monitored simultaneously. For disintegration, the 5′ end of the 30 base oligonucleotide was labeled, as described above. Activities are reported relative to the maximum observed for that reaction. Closed circles, transposition; open squares, disintegration; plus signs, RSS double-strand cleavage.

(B) Removal of RSS DNA from strand transfer intermediates. Transposition reactions were assembled as described and initiated in 25 mM or 5 mM $MgCl_2$. Reactions contained 5′ bottom strand–labeled 12-RSS, unlabeled 23-RSS, pBR322, RAG1/2, and HMG1. Aliquots were removed at 0, 2, 5, 10, 30, 60, 120, and 180 min. Reactions were phenol-chloroform extracted. Products were resolved on a 6% urea-polyacrylamide gel, and cleavage and transposition were quantitated. Activity is reported relative to the maximum observed for transposition in 5 mM $MgCl_2$. The scale to the left of the graph is for transposition in 25 mM $MgCl_2$ (closed circles), and the scale to the right is for transposition in 5 mM $MgCl_2$ (open circles).

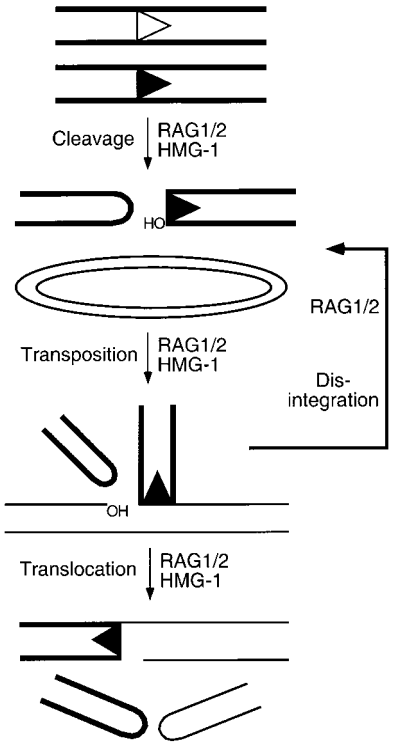

Figure 7. RAG1/2 Can Resolve Transposition Intermediates by Two Competing Pathways

As demonstrated previously, the RAG1/2 complex binds signal bearing DNA in a coupled manner and cleaves both signals. Only one signal will be followed for clarity. After cleavage, the signal DNA can attack a new molecule (a supercoiled plasmid in this case) resulting in a transposition intermediate. In Mn^{2+}, this intermediate can be resolved through hairpin formation on the target DNA. The result is a pair of hairpin-ended molecules that are substrates for resolution by the standard V(D)J joining machinery, and a fusion of an RSS-bearing DNA to target DNA. Alternatively, the transposition intermediate can be resolved by elimination of RSS donor DNA via disintegration. Under some conditions, the disintegration reaction is favored.

sequences [Limpens et al., 1995] and our earlier discussion [Hiom et al., 1998]). Such translocations could well have been produced by transpositional events, but definite evidence will require development of a system for in vivo RAG-mediated transposition.

RAG-Mediated Disintegration

Although neither RAG1 nor RAG2 has significant sequence homology to bacterial or eukaryotic transposases, connections at the level of reaction mechanism are becoming more obvious. The previously described similarities include a direct transesterification mechanism for strand transfer (van Gent et al., 1996b), as described for bacteriophage Mu transposase and HIV integrase, the formation of hairpin intermediates during cleavage (McBlane et al., 1995), and, of course, the RAG-mediated cut-and-paste transposition itself; (Agrawal et al., 1998; Hiom et al., 1998). Another resemblance was pointed out in the analogy between the disintegration reaction of HIV integrase (Chow et al., 1992) and RAG-mediated formation of hybrid and open-and-shut joints (Melek et al., 1998). In the present work, the direct observation of disintegration sharpens this analogy. In fact, the RAG proteins disintegrate a branched intermediate much more efficiently than they make hybrid or open-and-shut joints. Essentially all of a branched substrate can be disintegrated, whereas less than 1% of DNA was converted to hybrid or open-and-shut joints, and their detection required a PCR assay. Hybrid joint formation and disintegration also differ in that HMG1 and a pair of RSSs are required for the former but not the latter.

We find that disintegrative excision of the RSS-ended fragment is usually accompanied by resealing of the

target DNA, but not always. Sometimes, the target DNA remains nicked, implying that hydrolytic cleavage of the branchpoint competes with transesterification. Hydrolysis can be greatly enhanced by recessing the 3′-OH several nucleotides from the branch, or by substituting a 3′-deoxy group (Figure 4B). For disintegration to occur, the sequence of the branch has to closely match an RSS. This sequence specificity (and the coupled rejoining of the target DNA) distinguish this reaction from the nonspecific RAG flap endonuclease recently described (Santagata et al., 1999). The other requirements for disintegration are quite different from those for transposition. Transposition is only efficient when a 12/23-RSS pair is present, but for disintegration a second RSS is irrelevant (Figure 5). Metal ion requirements are also not as strict for disintegration. Transposition is most efficient in low Mg^{2+}, whereas disintegration is still efficient at high Mg^{2+} concentrations and also proceeds in Mn^{2+}. Unlike cleavage and transposition, disintegration is also not stimulated by the presence of HMG1 protein. It is interesting to note that the average total Mg^{2+} concentration in a mammalian cell is 20–25 mM (Alberts et al., 1989). Our data suggest that high Mg^{2+} concentrations favor both signal cleavage and disintegration but disfavor transposition. The intracellular environment may thus contribute to minimizing transposition and maximizing disintegration.

Our results suggest that disintegration by RAG1/2 is not simply the chemical reversal of transpositional strand transfer. First, disintegration and strand transfer differ in their ionic requirements. Strand transfer of a precleaved substrate proceeds as efficiently in Ca^{2+} as in Mg^{2+}, but disintegration is extremely inefficient when carried out in Ca^{2+} (Figure 2). Second, Mg^{2+} titration reveals that transposition activity is optimal at relatively low concentrations, while disintegration is most efficient at much higher metal concentrations. Finally, the time course of transposition at the higher Mg^{2+} conditions is not consistent with a simple equilibrium between the forward strand transfer and the reverse disintegration reaction. If the two reactions were direct reversals, one would expect to see transposition increasing over time and then reaching a steady state where the rates of disintegration and strand transfer are equal. Instead, we observed the amount of transposed product peaking at an early time and then decreasing. It is possible that the active site of RAG1/2 has different conformations for the two reactions and, therefore, requires different reaction conditions. Determination of the stereochemistry of strand transfer and disintegration should be informative regarding the mechanisms of both reactions. This type of analysis has proven valuable for understanding the mechanisms of action of HIV integrase, Mu, and Tn10 transposase (Mizuuchi and Adzuma, 1991; Kennedy et al., 1998; Gerton et al., 1999).

Another factor that may play a role in preventing a disintegrated transposition intermediate from retransposing is the fact that a pair of cleaved signal ends is required for effective binding by the RAG proteins. It was shown that a single cleaved signal end is not bound by RAG1/2 (Hiom and Gellert, 1998). However, a single-ended transposition intermediate is efficiently bound and disintegrated by these same proteins. Therefore, after a single-ended strand transfer, the RAG proteins are able to remove the signal DNA from target DNA very efficiently, but the removed signal cannot support further signal binding nor strand transfer (in the absence of the second signal).

Competing Pathways for Resolving Transposition Intermediates

There are many functional similarities between the RAG proteins and other transposases (including the presence of a DDE motif of active site residues) (Kim et al., 1999; Landree et al., 1999; Fugmann et al., 2000). However, the ability to reverse the strand transfer reaction differs greatly in this family. While RAG1/2 carries out disintegration very efficiently, Tn10 has not been shown to perform this reaction (A. Kennedy, K. Mizuuchi, and D. Haniford, personal communication). If one considers the function of this transposase, the ability to disintegrate or not may correspond to the biological role. For example, Tn10 requires transposition for its spread, so a bias in favor of strand transfer as against disintegration is efficient. In contrast, the principal biological action of RAG1/2 is RSS cleavage to initiate V(D)J recombination, and transpositional strand transfer can have potentially deleterious effects. It is thus advantageous to efficiently eliminate any transposition intermediates that may be formed.

With the two novel activities of the RAG proteins described in this paper, there are now three possible ways of resolving a RAG-produced transposition intermediate. The DNA insertion could be completed in the fashion typical for transposable elements, using the DNA repair machinery to fill in and seal the gaps at the ends of the mobile segment. Alternatively, a branched intermediate can be resolved by hairpin formation to generate a translocation, as we previously suggested. However, before either of these possibly deleterious DNA species can be completed, it is possible for the transposition intermediate to be reversed via disintegration, restoring the intact target chromosome, or, in the case of disintegration via hydrolysis, leaving it nicked. Disintegration would be expected to oppose potential RAG-mediated DNA mobility. In a sense, the high level of disintegration activity may serve as a protective mechanism, safeguarding the genome from the deleterious effects of transposition or translocation.

Experimental Procedures

Proteins

RAG1 and RAG2 fusion proteins (MR1 and MR2) were prepared as previously described (McBlane et al., 1995). HMG1 protein was a gift from R. Johnson (UCLA).

DNA Donors, Disintegration Substrates, and Targets

All oligonucleotides were purchased from Oligos Etc. and purified by polyacrylamide gel electrophoresis under denaturing conditions. Oligonucleotide substrates were 3′ end labeled with ^{32}P-cordycepin (NEN) using recombinant terminal deoxynucleotidyl transferase (GIBCO Life Technologies) or 5′ end labeled with γ-^{32}P-ATP (NEN) using T4 polynucleotide kinase (New England Biolabs). Substrates with a 16 bp coding flank and a 12-spacer RSS or 23-spacer RSS were previously described (McBlane et al., 1995). Cesium chloride purified pBR322 DNA was a gift from Mary O'Dea. Disintegration substrates were formed by annealing the indicated oligonucleotides in 100 mM potassium glutamate. Disintegration oligonucleotides used were: 30 nt top strand, ccggctcgagccatgatcgaagaaggtaac (if

indicated the terminal 3′ nucleotide was 2′-OH, 3′-deoxy); 80 nt bottom strand, ggcggccgcattggcctgtcgacagtactgcctatggattctcgag tagggttaccttcttcgatcatggctcgagccgg; 95 nt 23-RSS oligo, cccgagg gtttttgtacagccagacagtggagtactaccactgtgcctactcgagaatccataggc agtactgtcgacaggccaatgcggccgcc; 45 nt 23-RSS, DG4 (Hiom and Gellert, 1998); 84 nt 12-RSS oligo, ctgcagggtttttgttccagtctgtagcact gtgcctactcgagaatccataggcagtactgtcgacaggccaatgcggccgcc; 34 nt 12-RSS, DG10 (Ramsden et al., 1996); MM571, gatctccgggaacg taactgcgtcctcgat.

Transposition Reactions

Transposition was carried out in two stages. First, 0.02 μM each of 12-RSS and 23-RSS substrates was incubated with 100 ng coexpressed RAG1/2 in binding buffer (25 mM MOPS [pH 7.0], 5 mm $CaCl_2$, 75 mM potassium glutamate, 4 mM dithiothreitol, 100 mg/ml bovine serum albumin [New England Biolabs], 3 μg/ml HMG1 protein) for 10 min at 37°C to form paired complexes. Next, DMSO was added to a final concentration of 10%, and 100 ng pBR322 and metal ion were added as indicated. Incubation was continued for 30–60 min as indicated. Reactions were phenol-chloroform extracted, ethanol precipitated, and resuspended in either glycerol or formamide dye, as required for native or denaturing electrophoresis. For samples requiring quantitation of transposition products as well as signal cleavage and disintegration, reactions were divided into two separate tubes after extraction. They were precipitated and resuspended in the appropriate loading buffer prior to resolution on denaturing polyacrylamide gels for disintegration and cleavage or native Tris-acetate-EDTA (TAE) agarose gels for transposition.

Two-Dimensional Gel Analysis of Transposition

Transposition reactions (20 μl) were carried out at as described above. Reactions were boiled for 2 min, and products were then incubated with 1 μl of a 1/50 dilution of a 50 U/μl stock of mung bean nuclease (GIBCO-BRL) or mock treated for 10 min at 37°C (in a 50 μl reaction volume). After phenol-chloroform extraction and ethanol precipitation, the samples were resolved on a 1% TAE agarose gel. Products were further resolved in the second dimension on a 1.2% alkaline agarose gel. The gel was neutralized, dried down, and exposed to phosphorimaging plates.

Disintegration Reactions

Disintegration was carried out as described for transposition, except that a labeled branched substrate was used (see Figure 3). Samples were phenol-chloroform extracted and resolved on urea-polyacrylamide gels as indicated. To maintain uniformity between reaction conditions for transposition and disintegration, 100 ng pBR322 was included in the reactions.

Gel Shift Assays

Gel shift assays were performed as previously described (Hiom and Gellert, 1998) except for the DNA substrate, which was 5′ top strand radiolabeled, non-RSS oligonucleotide MM571, and its complement. Unlabeled DAR39/DAR40 (Hiom and Gellert, 1998) was present as the RSS donor. The DNA mixture was preincubated with RAG1/2 in binding buffer containing 5 mM $CaCl_2$ for 10 min at 37°C. $MgCl_2$ was then added to the indicated concentration, and the incubation was continued for 15 min.

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