grinding frozen tissue with a mortar and pestle in liquid N_2 and homogenizing in a hypotonic buffer [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% Triton X-100, leupeptin (2 μg/ml), pepstatin (2 μg/ml), and 1 mM each of dithiothreitol (DTT), benzamidine, and 4-(2-aminoethyl)-benzenesulfonyl fluoride] in a 15-ml Dounce homogenizer. Extracts were centrifuged at 25,000*g* at 4°C for 30 min. Unless indicated otherwise, the cleared lysate was used in the experiments.
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13. PCR-generated fragments corresponding to the specified regions of AKAP79 were cloned into a pET-16b vector (Novagen) in frame with a histidine$_{(10)}$-Tag (His-Tag) following standard cloning protocols. The complete nucleotide sequence of each fragment was confirmed by automated sequencing. Recombinant proteins were expressed in *Escherichia coli* BL21 cells and purified from the soluble fraction under nondenaturing conditions by nickel affinity chromatography (Novagen). Protein concentrations were determined by the DC protein assay (Bio-Rad).
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16. RII overlays were carried out as described [S. M. Lohman, P. DeCamilli, I. Einig, U. Walter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6723 (1984)].
17. PKC was assayed as described [C. House, R. E. H. Wettenhall, B. E. Kemp, *J. Biol. Chem.* **262**, 772 (1987)] in a reaction containing 50 mM tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM DTT, 100 μM [γ-^{32}P]adenosine triphosphate (ATP) (200 to 500 cpm/pmol), phosphatidylserine (20 μg/ml), and either epidermal growth factor receptor peptide (VRKRTLRRL) (*29*) or myelin basic protein (residues 4 to 14) peptide as substrates at 30°C. PKC (50 nM) was diluted in 20 mM tris (pH 7.7), 1 mM DTT, and bovine serum albumin (BSA; 1 mg/ml). Inhibition constants (IC_{50}) were determined over an inhibition concentration range of 0.1 to 10 μM. K_i values were obtained from secondary plots of K_m/V_{max} versus inhibitor concentration, after determination of K_m and V_{max} for four concentrations of inhibitor from Lineweaver-Burk plots assayed over the same substrate concentration range.
18. PKA was assayed as described [J. D. Corbin and E. M. Reiman, *Methods Enzymol.* **38**, 287 (1974)].
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20. HEK 293 cells were collected in hypotonic buffer containing protease inhibitors and sonicated three times for 5 s. Total lysates (1.2 mg in 300 μl) were incubated overnight in the absence or presence of 2 μg of purified recombinant AKAP79. Samples were incubated with 4 μg of affinity-purified polyclonal antibody to AKAP79 (2503) and precipitated with 40 μl of a 50% solution of protein A–Sepharose (Sigma). Complexes were washed twice in hypotonic buffer, twice in low-salt buffer [50 mM Hepes (pH 7.4), 0.15 M NaCl, 0.1% NP-40, 1 mM EDTA, and 0.1% SDS], twice in high-salt buffer (50 mM Hepes, 0.5 M NaCl, 0.1% NP-40), and twice in low-salt buffer. The precipitated complexes were boiled for 5 min in 20 μl of SDS–sample preparation buffer and immunoblotted.
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22. For confocal microscopy, HEK 293 stable cell lines expressing pcDNA3 or AKAP79 were grown on cover slips overnight, rinsed in phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde in PBS, treated with acetone (−20°C) for 1 min, rehydrated, and preblocked with 0.1% BSA in PBS. An antibody mixture containing mouse antibody to PKC (M4; 1:200 dilution) and rabbit antibody to AKAP79 (2503; 1:200 dilution) was applied for 1 hour. The cover slips were then washed three times in 0.1% BSA in PBS. Fluoroscein isothiocyanate–conjugated donkey antibody to rabbit immunoglobulin G (IgG) (1:50) and Texas red–conjugated donkey antibody to mouse IgG (1:100) were applied for 1 hour. The cover slips were then washed three times in 0.1% BSA in PBS, mounted with Slow Fade Antifade (Molecular Probes), and analyzed with the Leitz Fluovert FU confocal photomicroscope with a 63/1.4 N.A. OEL PL APO lens. Specific staining was not detected in control experiments with secondary antibody alone.
23. HEK 293 cells were transfected with Lipofectin Reagent (Life Technologies) with either pcDNA3 or pcDNA3 containing a 1312–base pair Hind III–Not I fragment encoding AKAP79. After 24 hours, G418 (0.5 mg/ml; Sigma) was added to select for stable cells overexpressing AKAP79. Cells were maintained in media containing G418 (0.2 mg/ml). Protein immunoblot analysis demonstrated increased expression of AKAP79, whereas PKCα expression remained constant.
24. Regulatory subunits of PKA were purified with the use of cAMP-agarose as described [R. L. Potter, P. H. Stafford, S. S. Taylor, *Arch. Biochem. Biophys.* **190**, 174 (1978)] with modifications (*4*). The complex from bovine brain lysates (*8*) was eluted from cAMP-agarose with 75 mM cAMP.
25. Immunoprecipitation of CaN was achieved by incubating bovine brain lysates (1 mg protein) (*8*) with affinity-purified antibodies to the CaN A subunit (8 μg) for 4 hours at 4°C. Proteins were precipitated as described (*20*).
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29. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; F, Phe; G, Gly; K, Lys; L, Leu; M, Met; P, Pro; R, Arg; S, Ser; T, Thr; and V, Val.
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Similarities Between Initiation of V(D)J Recombination and Retroviral Integration

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In the first step of V(D)J recombination, the RAG1 and RAG2 proteins cleave DNA between a signal sequence and the adjacent coding sequence, generating a blunt signal end and a coding end with a closed hairpin structure. These hairpins are intermediates leading to the formation of assembled antigen receptor genes. It is shown here that the hairpins are formed by a chemical mechanism of direct trans-esterification, very similar to the early steps of transpositional recombination and retroviral integration. A minor variation in the reaction is sufficient to divert the process from transposition to hairpin formation.

Functional immunoglobulin and T cell receptor genes are assembled during vertebrate lymphoid development from separate gene segments. This DNA rearrangement, called V(D)J recombination, takes place at recombination signal sequences (RSSs) that specify the border of the coding segments (*1*). Double strand breaks (DSBs) at the RSS border depend on expression of the *RAG1* and *RAG2* genes (*2*) and are probably intermediates in this recombination reaction (*3*, *4*). After DSB formation, a pair of signal ends or coding ends is coupled to form a signal joint or coding joint, respectively. These joining reactions require several genes that are also involved in general DSB repair (*4*).

We recently developed a cell-free assay in which specific cleavage at RSSs requires only the RAG1 and RAG2 proteins (*5*, *6*). An oligonucleotide containing one RSS serves as a substrate and is cut efficiently. A DNA species containing a nick in the top strand (as drawn in Fig. 1) at the border of the RSS and the flanking ("coding") sequence was found to be the precursor to a DSB. The products of the cleavage reaction are a blunt, 5′-phosphorylated signal end and a coding end with a hairpin structure. These are the same species detected in recombinationally active cells.

Formation of the new phosphodiester bond at the tip of the hairpin requires energy. Because no adenosine triphosphate (ATP) or other high-energy cofactor is present in the cleavage reaction, the energy is likely to be derived from one of the broken phosphodiester bonds. The energy of the bond broken in the initial nicking reaction does not appear to be conserved: DNA substrates with a preexisting nick at this position are efficiently converted into hairpins (*6*). Thus, the energy of the phosphodiester bond in the bottom strand opposite the nick must be conserved, either through a covalent protein-DNA intermediate or by direct trans-esterification. A

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reaction of the first type would involve a nucleophilic attack by the recombinase protein on the phosphodiester bond, for example through a serine or tyrosine residue, followed by a nucleophilic attack on the phosphoprotein linkage by the 3′ hydroxyl produced in the initial nicking event. Examples of such (topoisomerase-like) reactions are bacteriophage lambda integration and reactions catalyzed by bacterial resolvase and invertase proteins (*7*). The other possibility is a direct attack on the phosphodiester bond by the 3′ hydroxyl group formed in the nicking reaction (Fig. 1B). Such direct trans-esterification reactions have been demonstrated in bacteriophage Mu transposition (*8*) and retroviral integration (*9*).

A stereochemical experiment can distinguish between these two possibilities (*10*). A nucleophilic attack on a chiral phosphate by an in-line S_N2 mechanism will invert its chirality. Direct trans-esterification involves one nucleophilic attack and would therefore result in inversion of chirality, whereas a reaction through a covalent protein-DNA intermediate would involve two nucleophilic attacks, resulting in overall retention of chirality. As a normal phosphodiester bond is not chiral, a modified linkage has to be made. A phosphorothioate group (in which one of the nonbridging oxygen atoms of the phosphodiester is substituted by a sulfur) is commonly used as a chiral linkage. Such a phosphorothioate linkage was built into an oligonucleotide substrate at the position of the bond to be attacked for hairpin formation (Fig. 1A). The hairpin product will then contain a phosphorothioate linkage between the A in the top strand and the T in the bottom strand.

Two different substrates were constructed, with the phosphorothioate linkage in either of the two stereochemical configurations, R_p or S_p (*11*). Hairpins were formed by the RAG1 and RAG2 proteins only on molecules in the S_p configuration. These hairpins were isolated from a denaturing gel, and their stereochemical configuration was analyzed. Two nucleases were used for this analysis: snake venom phosphodiesterase (PDE), which digests the R_p but not the S_p form (*12*), and nuclease P1, which has the opposite specificity (*13*). P1 will thus leave a labeled dinucleotide from the R_p isomer, whereas PDE will leave a labeled dinucleotide from the S_p isomer. As shown in Fig. 1B (lanes 1 and 2), the unreacted substrate was fully digested to mononucleotides by nuclease P1 but not by PDE, which demonstrates that it is indeed in the S_p configuration. The hairpin product, however, was fully digested by PDE, but nuclease P1 left a labeled dinucleotide (Fig. 1B, lanes 4 and 5). Thus, the hairpin formation reaction resulted in inversion of chirality, which is indicative of direct trans-esterification.

Similar trans-esterification reactions have been described previously only for bacteriophage Mu transposition and retroviral integration. In these systems, the recombinase [MuA or integrase (IN), respectively] first nicks the donor DNA, and the resulting 3′ hydroxyl is then used as the nucleophile to attack a phosphodiester bond in the target DNA (Fig. 2). The hairpin formation reaction mediated by RAG1 and RAG2 differs in that it involves strand transfer to the opposite strand of the same DNA, instead of to an external DNA. However, even this difference may only be apparent; MuA and IN have both been shown to produce DNA hairpins under certain circumstances (*14*).

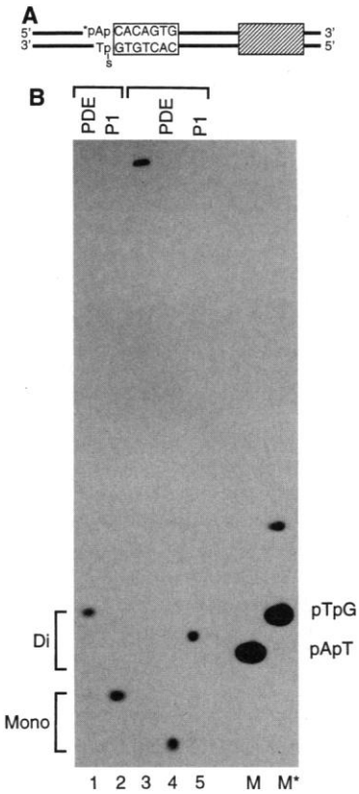

Fig. 1. (**A**) Possible mechanisms of DNA hairpin formation. The oligonucleotide substrate used for stereochemical analysis of the hairpin formation reaction is shown here, with the heptamer and nonamer represented by open and hatched boxes, respectively. The position of the ^{32}P label is depicted by an asterisk, and the phosphorothioate group by an "s." (**B**) Inversion of chirality in hairpin formation. The substrate shown was incubated with RAG1 and RAG2, and hairpin products were purified from a 12.5% TBE-urea gel. This DNA was digested with snake venom PDE (lane 4) or nuclease P1 (lane 5), and products were separated on a 22.5% TBE-urea gel. Lane 3 shows the undigested hairpin product. Lanes 1 and 2 show an unreacted substrate digested with snake venom PDE and nuclease P1, respectively. In this case, the ^{32}P label was inserted in the bottom strand, 5′ of the G at the border of the heptamer for detection of the products of nuclease digestion. Lanes M and M* contain DNA dinucleotide markers dAdT and dTdG (which migrates close to dGdT), respectively. A mononucleotide is left after digestion of the hairpin by PDE, but a dinucleotide is left after digestion by P1, whereas the reverse is true of the substrate DNA. This demonstrates the inversion of chirality at the phosphorothioate. The dinucleotide derived from the hairpin product has a slightly slower mobility than the dAdT marker, because it contains a phosphorothioate linkage instead of a phosphodiester bond.

We have also found other similarities. The initial nicking reaction carried out by IN is normally a hydrolysis that releases a dinucleotide with a 5′ phosphate and a 3′ hydroxyl from the end of the viral DNA. However, under certain reaction conditions, this nucleophilic attack can be carried out by the 3′ hydroxyl of the viral DNA molecule rather than by water, resulting in a circular dinucleotide (*9*). Alternatively, some alcohols such as glycerol or 1,2-ethanediol release a dinucleotide with a covalently attached alcohol, in a reaction termed alcoholysis (*15*).

We investigated whether the nicking reaction carried out by RAG1 and RAG2 could also use an alcohol as the nucleophile. The standard oligonucleotide substrate is not convenient for this test, because it is technically difficult to separate a 34-nucleotide product from its alcohol adduct. Thus, an oligonucleotide substrate was constructed with a nick two base pairs inside the heptamer of the RSS. A ^{32}P label was introduced at the border of the heptamer and adjacent coding sequence to allow detection of the predicted dinucleotide resulting from nicking at the signal-coding border (Fig. 3A) (*16*). Cleavage by RAG1 and RAG2 generated the expected dinucleotide (Fig. 3B, lane 2), but when 1,2-ethanediol was present in the reaction, an additional product with slower mobility was

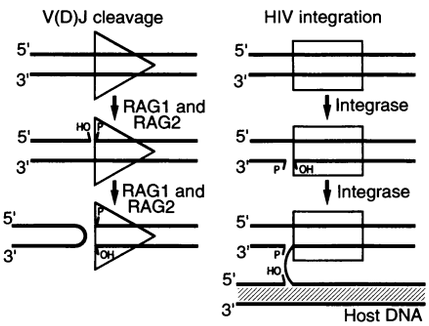

Fig. 2. Comparison of the V(D)J cleavage reaction and HIV integration. The RSS is depicted as a triangle and the HIV recognition sequence as a box. The host DNA (into which HIV DNA integrates) is represented by a hatched bar. The reactions are shown for only one recognition sequence.

also formed (Fig. 3B, lane 3). A similar product, but with a different mobility, was obtained by the addition of glycerol. The identity of this latter product as the alcohol adduct was confirmed by its resistance to calf intestine alkaline phosphatase and by the generation of a slow-moving species (presumably a glyceryl-mononucleotide) after nuclease P1 digestion. The alcohol adducts formed by HIV-IN (human immunodeficiency virus–IN) behave very similarly under these conditions (*15*). Thus, RAG1 and RAG2 can also mediate alcoholysis.

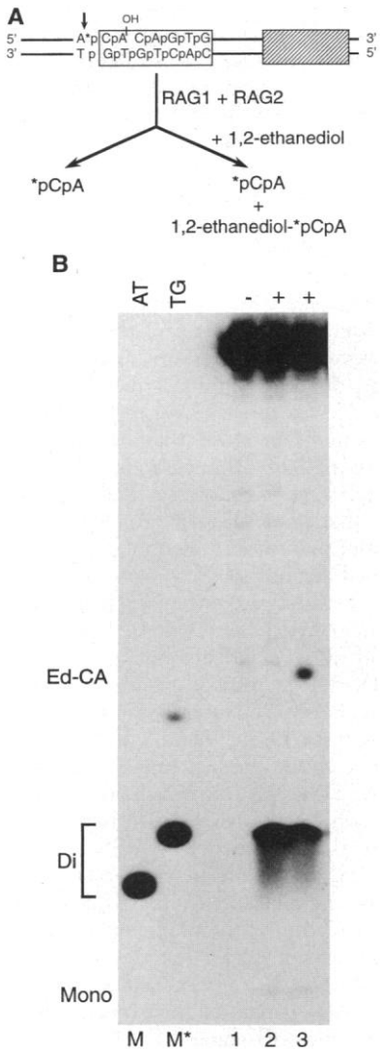

Fig. 3. RAG1 and RAG2 mediate alcoholysis. (**A**) Schematic representation of the alcoholysis substrate. The heptamer and nonamer elements are represented by an open and a closed box, respectively. Note that a nick is present in the top strand 3′ of the second nucleotide within the heptamer. Hydrolysis is expected to produce a dinucleotide with a 5′ phosphate and a 3′ hydroxyl. In the presence of 1,2-ethanediol, a dinucleotide product with an ethanediol group coupled to the 5′ phosphate may be expected. For convenient analysis of the products, a ^{32}P label (depicted by an asterisk) was incorporated between the last two nucleotides of the coding flank in the top strand (*11*) (see Fig. 1A). (**B**) Analysis of cleavage products on a 22.5% TBE-urea gel without (lane 1) or with RAG1 and RAG2 (lanes 2 and 3, respectively). Cleavage reactions were done without (lane 2) or with (lane 3) 20% (v/v) 1,2-ethanediol added. dAdT and dTdG were included as dinucleotide markers (shown on the left). Mono, mononucleotides; di, dinucleotides; Ed-CA, 1,2-ethanediol adduct. Lanes M and M* are as in Fig. 1B.

As most nucleases do not permit the substitution of alcohols for water in their reactions, these results establish a further resemblance between the catalytic properties of RAG1 and RAG2 and those of HIV-IN. There is convincing evidence that the same active site of MuA or IN mediates both nicking and strand transfer (*17*); mutational analysis of the MuA and IN proteins was unable to separate the two reactions, and the chemistry of the nicking reaction is very similar to the strand transfer reaction. As the properties of the RAG-catalyzed reactions are so similar to those of these reactions, it seems likely that there will be one active site for both nicking and hairpin formation. This putative active site could be contained within either RAG1 or RAG2, or it could be shared between both. At present, the location of the active site is unknown, as both proteins are required for all activities.

These findings provide support for the speculation that the antigen receptor genes, and the RAG1 and RAG2 proteins that mediate their rearrangement, may have evolved from an ancestral transposon (*18*). The presence of RSSs facing in opposite directions (the most usual arrangement in the antigen receptor loci) is similar to the inverted repeat architecture of many transposon ends, and the RAG proteins could have developed from genes encoded by the former transposon. After the initial invasion, such a transposon must have lost its ability to reintegrate after excision. We note a significant difference between the chemistry of the reaction mediated by RAG1 and RAG2 and that of the MuA-IN family of transposons. In the reactions mediated by MuA and IN, the initial nick leaves a 3′ hydroxyl on the transposon end, so that transfer of this group to a second DNA leads to integration of the transposon at a new site (Fig. 2). In V(D)J cleavage, the strand polarity is reversed, so that the 3′ hydroxyl at the nick is not in the recognition element (the RSS) but in the coding flank, thereby precluding transposition by a mechanism similar to Mu or retroviral integration. Nicking of this strand resembles the cutting reaction of the nontransferred strand of Tn*7* or Tn*10* (*19*). The strand transfer reaction may have been redirected to formation of hairpins at the coding ends.

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