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Characterization of broken DNA molecules associated with V(D)J recombination

(double-strand breaks/coding ends/signal ends/recombination signal sequences/DNA hairpins)

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ABSTRACT **We previously demonstrated that DNA molecules with double-strand breaks at variable-(diversity)-joining [V(D)J] recombination signal sequences are relatively abundant in mouse thymocytes. This abundance strongly suggests that the mechanism of V(D)J recombination involves double-strand cleavage at recombination signals. As a first step toward understanding the mechanism of cleavage, we used a sensitive PCR assay to characterize the structure of one class of cleavage products, the signal ends, in detail. Here we demonstrate that most of these ends are blunt and terminate in 5′ phosphoryl groups. Virtually all of the flush signal ends are full length. A minor subpopulation of broken ends terminates in short single-strand extensions. We have found no evidence for covalent DNA–protein linkages involving the signal ends. These data allow further refinement of the double-strand cleavage model for V(D)J recombination.**

Immunoglobulin and T-cell receptor (TCR) variable-region genes are encoded by multiple DNA segments that are assembled in developing lymphocyte precursors by a process known as variable-(diversity)-joining [V(D)J] recombination (1). The recombination machinery recognizes short DNA sequences, termed signal sequences, that consist of conserved heptamer and nonamer elements separated by non-conserved spacer regions of 12 or 23 nt. Recombination occurs at the borders between signals and their neighboring coding regions and generates two types of junctions—a rather imprecise fusion of the two coding sequences, termed a coding joint, and a heptamer-to-heptamer fusion of the signals, called a signal joint.

The molecular details of V(D)J recombination are unknown, largely due to the lack of a cell-free system capable of carrying out the reaction. The recent discovery of DNA molecules with double-strand breaks at TCRδ recombination signal sequences in mouse thymocytes (2, 3) has allowed us to study the mechanism by characterizing potential reaction intermediates. The identification of molecules with signal ends (2) and molecules with coding ends (3) strongly supports a double-strand cleavage model for recombination.

If molecules with signal ends are intermediates in the formation of signal junctions, most of these ends should retain all of the sequence of the recombination signal on one or both strands, because the junctions almost always contain all of the nucleotides from both signals. The simplest expectation is that broken signal ends would be blunt, although any configuration allowing conservation of all sequence information in both recombination signals would be compatible with formation of perfect signal junctions.

In this study we have used ligation of double-stranded oligonucleotides, both with and without subsequent PCR amplification, to probe the structure of signal ends in detail. Our results address the following issues: (*i*) the configuration of the cleaved termini (blunt, 5′-extension or 3′-extension), (*ii*) the chemical nature of the termini, and (*iii*) the extent of nucleotide loss.

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MATERIALS AND METHODS

Genomic DNA Preparation. DNA from 2- to 3-day-old BALB/c mouse thymocytes was prepared as described (2). All DNA samples were treated with proteinase K.

Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer and were purified by PAGE. Double-stranded ligation primers were annealed as described (4). Blunt ligation primers for ligation-mediated PCR (LMPCR) were prepared by annealing the oligonucleotides DR19 and DR20. Ligation primers containing single-strand extensions were prepared from the following pairs of oligonucleotides: 5′ extensions: 1 nt, DR36/DR20; 2 nt, DR37/DR20; 3 nt, DR38/DR20; 4 nt, DR41/DR20; and 3′ extensions: 1 nt, DR19/DR42; 2 nt, DR19/DR43; 3 nt, DR19/DR44; 4 nt, DR19/DR45. The oligonucleotides used as probes in the bulk ligation assay were as follows: blunt end, DR39 + DR40; 2-nt 5′ extension, DR47 + DR40. DR20 and DR6 were used as PCR primers. The 5′ end of DR6 anneals 93 nt upstream of the first nucleotide of the Dδ2 element. The 5′ end of DR2, which was used as a hybridization probe, anneals 53 nt upstream of the first nucleotide of the Dδ2 element.

Sequences of oligonucleotides are as follows: DR2, 5′-GACACGTGATACAAAGCCCAGGGAA; DR6, 5′-TGGCTTGACATGCAGAAAACACCTG; DR19, 5′-CACGAATTCCC; DR20, 5′-GCTATGTACTACCCGGGAATTCGTG; DR39, 5′-CACGAATTCCCGGGTAGTACATAGCGTACTTAGCGGAGAGTGACCGGTTG; and DR40, 5′-CTCTCCGCTAAGTACGCTATGTACTACCCGGGAATTCGTG. DR36, -37, -38, and -41 have the same sequence as DR19 with 5′-terminal extensions of G, TG, GTG, and CGTG, respectively. DR42, -43, -44, and -45 have the same sequence as DR20 with 3′-terminal extensions of C, CA, CAC, and CACG, respectively. DR47 has the same sequence as DR39, with a 5′-terminal extension of TG.

Ligation-Mediated PCR Assay. Undigested thymus DNA was mixed on ice with 60 pmol of annealed ligation primers in a solution containing 50 mM Tris·HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, and 3 units of T4 DNA ligase (GIBCO/BRL). Ligations were performed for 12–16 hr at 15°C. Reactions were terminated by phenol extraction. After ligation, 50–100% of each sample was amplified in a 50-μl reaction containing 25 pmol of each primer (DR6 and DR20), 50 mM Tris·HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 0.01% gelatin, and 1.2 units of *Taq* polymerase (Amplitaq; Perkin–Elmer). Amplification was carried out in a PE 9600 thermal cycler (Perkin–Elmer).

Abbreviations: LMPCR, ligation-mediated PCR; TCR, T-cell receptor; V(D)J, variable-(diversity)-joining.

Initial denaturation was performed for 2 min at 94°C, followed by 17 cycles consisting of 15 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C. Amplified products were purified by ethanol precipitation, separated by electrophoresis through 6% polyacrylamide gels, and electrophoretically transferred to nylon membranes, which were probed by hybridization to a ^{32}P-labeled oligonucleotide (DR2). In some experiments, PCR products purified by ethanol precipitation were incubated with 30 units of *Apa*LI (New England Biolabs).

Mung Bean Nuclease Treatment. Four micrograms of thymus DNA was treated with 5–50 units of mung bean nuclease (New England Biolabs) in a buffer containing 10 mM Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 1 mM $ZnSO_4$ at 37°C for the indicated times. Reactions were terminated by phenol extraction, followed by ligation and PCR as described above. To demonstrate the activity of the mung bean nuclease, 5 μg of BALB/c testis DNA was digested with 50 units of *Pst* I (GIBCO/BRL) or *Rsa* I (New England Biolabs). Half of each sample was treated with 50 units of mung bean nuclease for 1 hr. Both treated and untreated samples were used for ligation and PCR amplification as described above.

RESULTS

To study the structure of signal ends, we used a modification of the LMPCR procedure of Mueller and Wold (4). To detect flush double-strand breaks, a ligation primer is constructed by annealing two complementary oligonucleotides, 11 and 25 nt in length, so that one end is blunt. This configuration ensures that the oligonucleotide can only ligate in one orientation. Ligation of undigested thymus DNA with such a ligation primer results in the formation of one phosphodiester bond between the 5′ phosphate of a preexisting flush double-strand break and the long strand of the ligation primer. The first round of extension produces a completely double-stranded product that can be amplified using the TCRδ primer and the long strand of the ligation primer. Because ligation precedes extension, this procedure detects only double-strand breaks. The specificity of the ligase reaction allows the strand configuration of the ends (flush, 5′ overhang, or 3′ overhang) to be probed by varying the configuration of the double-stranded ligation primer, as discussed below. The ligation step also provides information about the chemical structure of the ends.

Most Signal Ends Are Blunt and Terminate in 5′ Phosphates. We chose to study molecules with signal ends located to the left of the TCR Dδ2 element (2). About 2% of DNA in thymocytes of newborn BALB/c mice is broken at this site. We initially explored the possibility that the ends were blunt by testing their ability to ligate to blunt-ended primers. The amount of product obtained from LMPCR using such primers is roughly proportional to the amount of thymus DNA input into the reaction (Fig. 1*A*). The primers were designed so that blunt-end ligation onto a full-length signal end creates a site for the restriction endonuclease *Apa*LI. PCR products arising from blunt-ended, full-length signal ends should lose 25 nt after digestion with *Apa*LI. As shown in Fig. 1*B*, >90% of the PCR products are sensitive to *Apa*LI, demonstrating that the vast majority of the molecules capable of ligating to the blunt-ended primer (that is, molecules with flush termini) contain full-length signal ends.

The presence of a PCR product also shows that some fraction of the signal ends terminates in 5′ phosphates. Ligation requires the presence of a 5′ phosphate on the broken end, as the oligonucleotides end in 5′ hydroxyls. To determine whether molecules with 5′ phosphates compose a significant proportion of the total pool of signal ends, genomic DNA was treated with polynucleotide kinase before LMPCR. Fig. 1*C* shows that this treatment did not increase

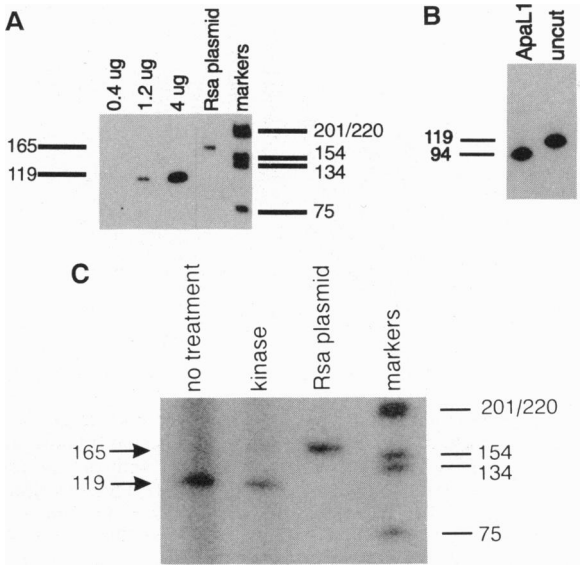

FIG. 1. The majority of signal ends are blunt and full-length and terminate in 5′ phosphoryl groups. (*A*) To test responsiveness of the PCR assay to the number of broken molecules available for ligation, the indicated amounts of undigested thymus DNA were mixed with uncut liver DNA up to a total of 4 μg and subjected to LMPCR. As a control, plasmid DNA containing TCR Dδ2 genomic sequences was digested with *Rsa* I (which leaves blunt ends) and used to program a LMPCR reaction. The expected sizes of LMPCR products resulting from the Dδ2 signal end and the *Rsa* I-digested plasmid are shown. The marker lane contained radiolabeled size standards. (*B*) LMPCR products generated from 4 μg of undigested thymus DNA were either digested with *Apa*LI (ApaL1 lane) or not treated after PCR (uncut lane). The expected sizes of full-length and *Apa*LI-digested PCR products are indicated. (*C*) Thymus DNA (4 μg) was ligated with blunt ligation primers either with or without prior T4 polynucleotide kinase treatment (20 units of enzyme for 1 hour at 37°C in a buffer containing 1 mM ATP). The blot was visualized by using a Molecular Dynamics PhosphorImager.

the yield of products, indicating that most blunt signal ends contain 5′ phosphates. The presence of a PCR product also indicates that at least some of the flush termini are not blocked by covalently linked peptides.

Because the LMPCR procedure does not provide information about the fraction of ends that contribute to product formation, we considered the possibility that only a small proportion of signal ends might be capable of ligating to the blunt primer. To address this question, we devised a bulk ligation assay capable of probing the structure of signal ends without PCR amplification. This assay measures a change in the length of the entire DNA population after ligation, allowing the fraction of ends capable of ligating to the primers to be measured directly. Fig. 2 shows that ligation of uncut thymus DNA with 1200 pmol of the blunt probe results in an appropriate shift in the mobility of ≈50% of molecules with signal ends. This result provides a minimum estimate of the fraction of ligatable termini, due to the technical difficulties inherent in achieving complete ligation in mixtures containing large amounts of uncut genomic DNA. Ligation to a control primer containing a 2-nt 5′ overhang resulted in little, if any, mobility shift, indicating that only a small fraction of signal ends has recessed 3′ termini capable of ligating to this probe (see below). These data confirm our results with LMPCR, indicating that most signal ends are blunt, unblocked, and terminate in 5′ phosphates.

A Minor Subpopulation of Signal Ends Has Lost Nucleotides from 5′ or 3′ Termini. The LMPCR assays described above detect only flush ends. We considered the possibility that 5′ or 3′ single-stranded termini might be produced, either as a

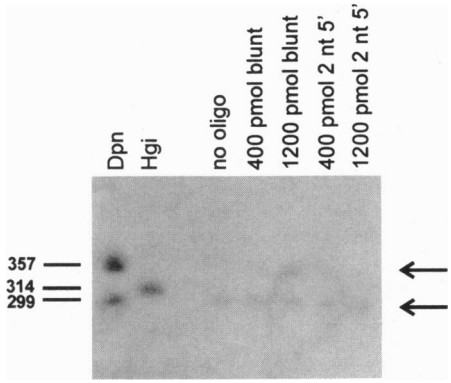

FIG. 2. A bulk ligation assay shows that a significant fraction of signal ends are blunt, with 5′ phosphates. Undigested thymus DNA (70 μg per lane) was ligated for 2 days at 14°C with the indicated amount of double-stranded oligonucleotides (see text for description) and 4000 units of T4 DNA ligase. Reactions were terminated by phenol extraction, and samples were digested with 120 units of *Xmn* I (New England Biolabs) for 30 hr at 37°C. Samples were analyzed by electrophoresis through a 20 × 25 cm 3% NuSieve agarose gel followed by transfer to a nylon membrane and hybridization to radioactively labeled 5′ Dδ2 probe, as described (2). Autoradiography was done for 8 days using an intensifying screen. Size standards (Dpn and Hgi lanes) have been described (2). Arrows mark positions of the signal end fragment (299 nt) and the expected size of the signal end fragment after ligation to the oligonucleotide (350 nt).

direct consequence of the cleavage reaction or as a result of secondary nucleolytic processing events. To test this possibility, thymus DNA preparations were treated with mung bean nuclease to convert single-strand extensions to blunt ends, which were then ligated with blunt primers and subjected to PCR. Fig. 3*A* shows the results of treatment with various levels of mung bean nuclease. Even at the higher enzyme concentrations, no shorter products are detected (compare lanes marked −Apa). Products shorter than those observed in the absence of nuclease treatment should be detected if single-stranded regions of >5 nt were present at a significant fraction of the signal ends (a difference of 8 nt is easily resolved by the electrophoresis conditions used in these experiments, as shown in Fig. 3*B*).

To detect short single-strand remainders with even greater sensitivity, we took advantage of the fact that removal of any extension, even a single nucleotide, renders the resulting blunt end incapable of generating an *Apa*LI site after ligation with the blunt primer. Only a small fraction of the PCR product becomes resistant to *Apa*LI, even at high concentrations of nuclease (Fig. 3*A*), indicating that only a minority of the signal ends contain single-strand protrusions. Similar results were obtained by using S1 nuclease (data not shown). As a control for the activity of mung bean nuclease, mouse testis DNA was digested with either *Rsa* I (blunt ends) or *Pst* I (4-nt 3′ overhang) and treated with mung bean nuclease before ligation. Fig. 3*B* shows that no PCR products were obtained from *Pst* I-digested DNA in the absence of nuclease treatment. After treatment, *Pst* I-digested DNA produced an amount of PCR product similar to *Rsa* I-digested DNA. As expected, treatment with mung bean nuclease did not affect the ability of *Rsa* I-digested DNA to ligate to the blunt primers.

Polarity and Length of Single-Strand Extensions at Signal Ends. The presence of a small amount of *Apa*LI-resistant products after digestion with mung bean nuclease suggested that a minor population of signal ends (<5%) has short single-stranded termini. To investigate these molecules in more detail, ligation primers with 1- to 4-nt 5′ extensions and 1- to 4-nt 3′ extensions were used to assess the distribution of single-strand extensions at signal ends. Each of these

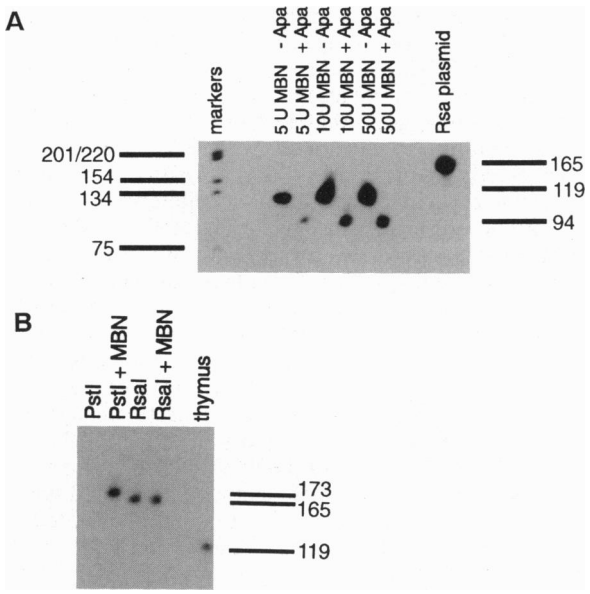

FIG. 3. Treatment with mung bean nuclease before LMPCR confirms that most signal ends are blunt. (*A*) Each lane contains the products of LMPCR using 4 μg of thymus DNA treated with mung bean nuclease as described in text. Samples were either loaded directly on the gel (−Apa) or digested with *Apa*LI before electrophoresis (+Apa). Size standards were prepared as described in the legend to Fig. 1. Expected sizes of PCR products are shown on right. (*B*) Five micrograms of testis DNA from BALB/c mice was digested with *Pst* I or *Rsa* I. +MBN lanes contain samples treated with 50 units of mung bean nuclease for 1 hr before LMPCR. The thymus lane contains LMPCR products from signal ends present in 4 μg of undigested thymus DNA, used as a size standard. Expected sizes of the PCR products are indicated. The amount of PCR product generated from restriction enzyme-digested testis DNA is not much greater than the amount of product from similar amounts of undigested thymus DNA, where only 2% of the DNA is specifically broken. We routinely observed much less efficient LMPCR amplification in restriction enzyme-digested genomic DNA than expected, presumably because the large number of broken ends titrated out the ligation primers.

primers should only generate a PCR product when ligated to signal ends with the corresponding complementary extension, as shown in Fig. 4*A*. The only 3′ extension that generated significant amounts of PCR product was the 1-nt extension (Fig. 4*B*). Small amounts of product were produced using all four 5′ extension primers. The 2-nt 5′ extension primer generated the most product, although the amount was significantly less than that observed by using the blunt primer. This assay appears to overestimate the abundance of ends with single-stranded extensions, possibly because ligation of ends with complementary extensions is more efficient than blunt-end ligation. The low abundance of signal ends missing 2 nt from the 3′ end was confirmed by using a ligation probe terminating in a 2-nt 5′ overhang in the bulk ligation assay (Fig. 2). No product was detected, indicating that the abundance of this species must be at least severalfold less than that of the flush signal end.

DISCUSSION

In this report we examine the molecular structure of the signal ends in detail. The results obtained using LMPCR, in conjunction with bulk ligation assays, show that most signal ends are flush and terminate in 5′ phosphoryl groups. There is an extremely high degree of conservation of nucleotides at these blunt ends, as >90% of the PCR products are sensitive to digestion with *Apa*LI. A very small percentage of signal

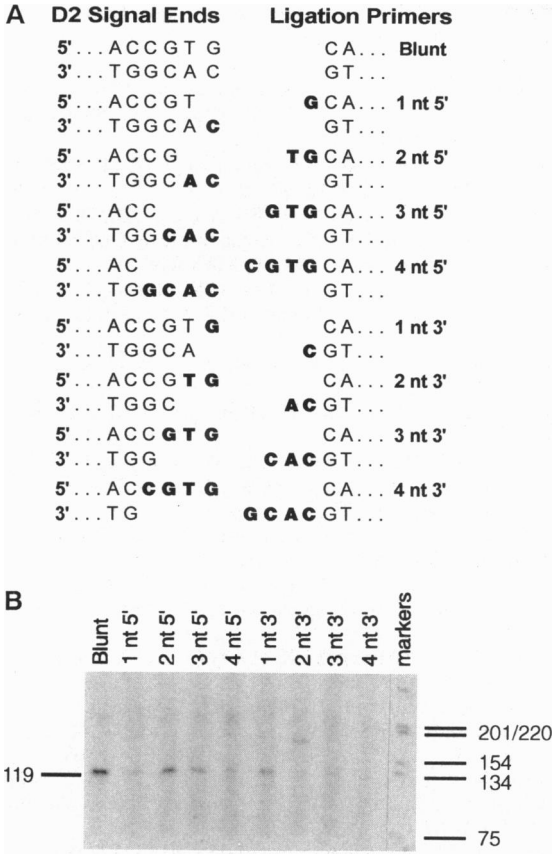

FIG. 4. Length and polarity of single-strand extensions at signal ends determined with a series of ligation primers. (*A*) Configuration of the ends of the ligation primers used, along with sequence of the signal ends they are designed to detect. (*B*) Each ligation primer shown in *A* was ligated with 2 μg of thymus DNA, followed by 17 cycles of PCR. This experiment was done on three different thymus DNA preparations, yielding similar results. All products seen by using the primers containing single-strand extensions were sensitive to digestion with *Apa*LI (data not shown), indicating that these products resulted from ligation of complementary ends, as shown in *A*.

ends contains single-strand termini resulting from loss of nucleotides from either the 5′ or 3′ end. We do not know whether these molecules are minor, perhaps aberrant, products of the original cleavage event or whether they are produced by secondary nucleolytic processing.

Our data strongly suggest that cleavage produces a flush double-strand break at the boundary between a recombination signal and the adjacent coding element, generating a flush signal end. Flush cleavage is consistent with the low frequency of nucleotide loss in signal joints (5, 6). Loss of nucleotides might be expected to occur more frequently if cleavage created single-stranded extensions requiring filling-in or removal of nucleotides before joining. Indeed, previous studies on the processing of DNA ends in fibroblasts showed that joining of mismatched ends is much more likely to involve loss than filling-in of nucleotides (7, 8). If the processing of signal ends were similar, single-stranded termini made by the primary cleavage event should often result in signal joints with deletions, but these are rare.

Although this paper shows that most signal ends are formed as blunt double-strand breaks, we have previously found that the coding ends in *scid/scid* thymocytes are covalently sealed in the form of hairpins (3). This latter observation, in conjunction with the presence of the P nucleotide sequences that form inverted repeats in coding joints (9, 10), suggests that hairpins are normal intermediates in the formation of coding joints. In Fig. 5 we present a model indicating how a flush double-strand break at the signal end, and a hairpin at the coding end, could result from a single cleavage reaction. This model is based on previously described reactions where the production of hairpin ends often involves the formation of covalent DNA–protein intermediates. For example, proteins of the λ integrase family create staggered single-strand nicks and remain covalently attached to the broken ends by 3′-phosphodiester linkages to a tyrosine residue (11–14). A 5′ hydroxyl appropriately positioned on the strand opposite the DNA–enzyme complex can act as a nucleophile attacking the DNA–protein phosphodiester linkage, releasing free enzyme and forming a covalently sealed hairpin structure (15, 16). In the initial cleavage of V(D)J recombination, protein could be linked either through a 3′-phosphodiester linkage, as with λ integrase, or through a 5′-phosphodiester linkage, as in the resolvase/invertase family (17, 18). Only 5′ linkages are shown in Fig. 5, for purposes of illustration. Although this discussion focuses on covalent DNA–protein intermediates, other alternatives are possible.

In principle, cleavage could involve covalent protein–DNA intermediates at both the coding and signal ends (symmetric model) or only at the coding ends (asymmetric model), as shown in Fig. 5. Both of the above models account for hairpins at coding ends by postulating interstrand attack of a free hydroxyl group on the DNA–protein phosphodiester bond, liberating the enzyme and producing a hairpin. The hairpins must later be resolved, presumably by nicking, to allow coding joint formation. The essential difference between the models lies in the handling of the signal ends. By the asymmetric model, covalent protein–DNA complexes are formed only at the coding ends. If the linkage is through the 5′ end, cleavage results in a signal end with a 5′ phosphate and a 3′ hydroxyl. If both nicks occur precisely at the signal-coding junction, cleavage will produce the observed flush signal end.

According to the symmetric model, the protein would be covalently attached to both coding and signal ends. Protein at signal ends is then removed by nucleophilic attack of a hydroxyl group from the other signal end, resulting in precise joining. This mode of resolution might be facilitated (and hairpin formation at signal ends disfavored) by geometric

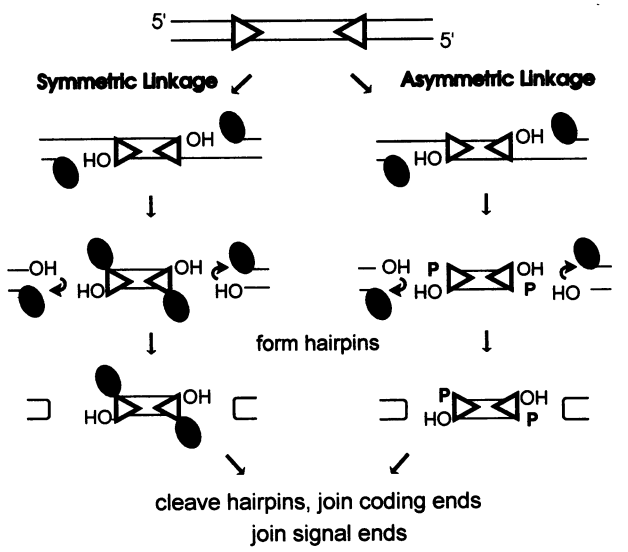

FIG. 5. Possible mechanisms for V(D)J recombination involving covalent DNA–protein intermediates. The triangles represent recombination signal sequences, with the vertical face of the triangle signifying the first nucleotide of the heptamer. The black ellipses represent protein molecules. For illustration purposes, only 5′ linkages are shown (see text for details).

constraints imposed by formation of a complex involving both signal ends. In this model, the presence of a covalently attached protein could easily explain the apparent stability of signal ends as well as their conservation of nucleotides. However, our analysis of signal ends strongly argues against the presence of covalently bound protein. If a significant fraction of signal ends were covalently associated with protein, the DNA samples, even though they are all protease treated, should contain peptides blocking either the 5′ or 3′ termini. Linkage to the 5′ end would completely block ligation to nonphosphorylated oligonucleotides, and the presence of a 3′-linked peptide would inhibit ligation through steric effects. The results of bulk ligation experiments provide strong evidence against covalent modification of signal ends, as ≈50% of the abundant, intact signal ends present in thymocytes could be ligated. Thus, the observations in this paper are most consistent with the asymmetric model. Our results would also be consistent with a process exemplified by transposition in the bacteriophage Mu, which involves a direct one-step esterification without the need for a covalent protein–DNA bond at any stage (19).

Our data provide strong evidence that the signal ends in thymocytes are not stabilized or protected by covalent modifications. The observed protection of signal ends from nucleotide loss could be conferred by noncovalently associated proteins. Perhaps the signal ends are protected by proteins that specifically recognize the recombination signals and may ultimately participate in the joining of one signal to another.

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