Intermediates in V(D)J recombination: A stable RAG1/2 complex sequesters cleaved RSS ends

Jessica M. Jones and Martin Gellert*

Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 5, Room 241, Bethesda, MD 20892

Contributed by Martin Gellert, September 5, 2001

Rearrangement of gene segments to generate antigen receptor coding regions depends on the RAG1/2 recombinase, which assembles a synaptic complex between two DNA signal sequences and then cleaves the DNA directly adjacent to the paired signals. After coupled cleavage of complementary signal sequences, virtually all of the cleaved signal ends remained associated with RAG1/2 in stable complexes. These signal end complexes were distinct from various precleavage RAG1/2 signal complexes in that they were resistant to treatment with heparin. A mammalian joining apparatus consisting of purified Ku70/86, XRCC4, and DNA ligase IV proteins was sufficient to join deproteinized cleaved ends, but retention of signal sequences within the signal end complex blocked access to the DNA ends and prevented their joining by these proteins. Sequestration of cleaved ends within the signal end complex would account for the persistence of these ends in the cell after cleavage and may explain why they do not normally activate the DNA-damage-dependent cell cycle checkpoint.

Recombination of V(D)J gene segments to form intact Ig and T cell receptor coding regions is a necessary part of B and T cell development and contributes to the enormous repertoire of the vertebrate specific immune system (reviewed in ref. 1). V, D, and J gene segments are flanked by recombination signal sequences (RSS) comprising conserved heptamer and nonamer motifs separated by nonconserved spacers of 12 or 23 bp. Recombination almost invariably brings together gene segments flanked by "complementary" RSSs with spacers of different lengths, a phenomenon known as the 12/23 rule (2). At the biochemical level, V(D)J recombination can be divided into two phases: cleavage and joining. Cleavage is carried out by the lymphoid- and development-specific recombinase composed of the RAG1 and RAG2 gene products 1 and 2 (RAG1/2) (3–5). Joining requires proteins involved in the nonhomologous end joining (NHEJ) pathway of DNA repair (6, 7). The transition from cleavage to joining is not well understood, and evidence suggests that the cleaved ends are never free in the cell.

The cleavage phase of V(D)J recombination has been reconstituted with purified proteins. RAG1/2 with the assistance of the chromatin-associated proteins high-mobility group (HMG)1 or HMG2 (8) recognizes the RSS and, in the presence of divalent metal cation (Mg^{2+} or Mn^{2+}), introduces a single-stranded nick between the heptamer and coding region (3). The 3′ hydroxyl at the end of the coding DNA then attacks the phosphodiester bond on the opposite DNA strand in a direct transesterification reaction (3, 9). This generates a double-strand DNA break, with a hairpin on the end of coding DNA and a blunt-cut RSS or signal end (SE). If Mg^{2+} is the cation, nicking can take place at a single RSS, and the 12/23 rule is implemented at the second step, with transesterification requiring the presence of a pair of complementary RSSs (10). Under these conditions, hairpin formation is believed to take place within a stable synaptic complex of complementary RSSs (11). Cleavage within the synaptic complex of complementary RSSs most likely reflects the conditions that govern cleavage *in vivo*, where greater than 90% of cleavage events follow the 12/23 rule (12). The requirement for a pair of complementary RSSs can be bypassed if cleavage is performed in Mn^{2+} (3, 13). Under these conditions, it is possible that both nicking and hairpin formation take place at a single RSS or, alternatively, that 12/12 or 23/23 RSS pairs are especially active.

After cleavage, the fate of coding and signal DNA ends is distinct both qualitatively and temporally. Coding ends are joined very rapidly (14, 15), whereas SEs persist long after cleavage (14, 16, 17) and are eventually joined at around the G_1/S transition. Signal joints are almost always perfect head-to-head fusions of the blunt ends, and examination of the broken ends suggests that no processing occurs before joining (14, 17, 18). Genetic evidence indicates that both coding and signal joint formation involve NHEJ factors. These include components of the DNA-dependent protein kinase (DNA-PK)—the Ku heterodimer (Ku70/86) and the large catalytic subunit (DNA-PK_{CS})—as well as DNA ligase IV and XRCC4 (6, 7, 19, 20). The properties of DNA ligase IV strongly suggest that it is the protein directly responsible for closing the double-strand break. XRCC4 binds tightly to DNA ligase IV (21, 22) and *in vitro* stimulates both its adenylation and double-stranded DNA-joining activity (22, 23). Formation of a higher order complex between XRCC4 and DNA ligase IV may allow for concerted ligation of two DNA strands as is necessary for repair of double-stranded DNA breaks (24). Ku70/86 binds with high affinity to DNA ends (25); it can translocate internally on linear DNA molecules and can move from one DNA molecule to another by bridging DNA ends (25–28). Some groups have found a stimulatory effect of Ku70/86 on ligase IV/XRCC4-mediated end joining (28, 29). Others have found an inhibitory effect that can be relieved by DNA-PK_{CS} (30). DNA-PK_{CS}-deficient cells can form signal joints on extrachromosomal V(D)J substrates and show a marked defect only in coding joint formation (31). The accumulation of hairpin-ended coding DNA in these cells indicates that DNA-PK_{CS} is required for the rapid opening of the hairpin (32).

The rapid formation of coding joints in normal cells indicates that the NHEJ machinery is present and active, and that it gains access to the coding ends almost immediately after cleavage. These NHEJ factors should be competent for the ligation of SEs as well. The persistence of SEs indicates that they are sequestered from the repair machinery. Here we describe a highly stable postcleavage complex that retains cleaved SEs. This complex sequesters the ends from NHEJ proteins that are otherwise competent for blunt-ended ligation, namely Ku70/86, XRCC4, and ligase IV.

Materials and Methods

Proteins and DNA. T4 ligase, T4 polynucleotide kinase, and all restriction enzymes were purchased from New England Biolabs.

Abbreviations: RSS, recombination signal sequence; RAG1/2, recombination activating gene products 1 and 2; DNA-PK, DNA-dependent protein kinase; DNA-PK_{CS}, DNA-PK large catalytic subunit; NHEJ, nonhomologous end joining; SE, signal end; SEC, SE complex.

*To whom correspondence should be addressed. E-mail: gellert@helix.nih.gov.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

DNA-PK was purchased from Promega. Murine RAG1 (amino acids 384-1008) and RAG2 (amino acids 1–387) proteins tagged on their amino termini with maltose-binding protein and on their carboxyl termini with polyhistidine and myc epitopes were coexpressed and purified as previously described (3). Polyhistidine-tagged human Ku70 and Ku86 were coexpressed and purified as previously described (28). HMG1, amino acids 1–163, was expressed from vector pDVG83 (33) in RJ1878 bacterial cells (gift of Reid C. Johnson, University of California Medical Center, Los Angeles), which do not express bacterial HU proteins. Purification was as for HU protein, Method A, as described previously (34).

Polyhistidine-tagged human DNA ligase IV, expressed from vector pDR119 (23), and full-length human XRCC4 protein, expressed from pACYC-based vector pWY1272 (gift of M. Junop and W. Yang, National Institutes of Health), were coexpressed in bacterial cells. The cell pellet was resuspended in Buffer A (50 mM Tris·HCl, pH 8.0/0.5 M KCl/20 mM β-mercaptoethanol/10% glycerol/0.2% Tween-20) plus 5 mM imidazole and 0.1 mg/ml of lysozyme and incubated for 30 min on ice. Cell debris were collected by centrifugation at 90,000 $\times$ *g* for 60 min. The supernatant (Fraction I) was loaded onto a Ni-NTA column (Qiagen, Chatsworth, CA) equilibrated in Buffer A plus 5 mM imidazole, and the column was washed with Buffer A plus 20 mM imidazole. Bound proteins were eluted with Buffer A plus 0.5 M imidazole. Peak fractions were pooled (Fraction II) and dialyzed overnight against Buffer B (20 M Tris·HCl, pH 8.0/0.2 M KCl/2 mM DTT/10% glycerol). After dialysis, Fraction II was loaded onto a Mono-Q column (Amersham Pharmacia Biotech) equilibrated in Buffer B, and bound proteins were eluted with Buffer B brought to 0.7 M KCl. Peak fractions were pooled (Fraction III) and loaded immediately onto a Superdex 200 h 10/30 column (Amersham Pharmacia Biotech) equilibrated in Buffer B. XRCC4 and ligase IV eluted in a single peak of ≈300 kDa. Peak fractions were pooled and frozen in small aliquots (Fraction IV, 2.4 ml, 0.25 mg/ml). Fraction IV was used for all experiments.

Plasmid substrate pDVG54 and oligonucleotide cleavage substrates DAR39/DAR40 (12 RSS) and DG61/DG62 (23 RSS) and precleaved substrates DG9/DG10 (12 RSS) and DG2/DG4 (23 RSS) have been described previously (3, 35). The 12 RSS 100-mer substrate was composed of oligonucleotides JMJ054 (5′-CAGTGCCAAGCTTGCATGCCTGCAGGTCGACTTACACAGTGCTACAGACTGGAACAAAAACCCTCGAGCATATGACGACCTTCGATATGGCCGCTGCTGT) and its complement, JMJ055. Oligonucleotides were gel-purified before use. DAR40, DG10, and JMJ055 were labeled by using T4 polynucleotide kinase and [γ^{32}P]-ATP (3,000 Ci/mmol; NEN Life Sciences). The 23 RSS 100-mer substrate was generated by PCR by using pDVG54 as a template and the primer pair JMJ047 (5′-AATTCGAGCTCGGTACCCGGGGATCC) and JMJ048 (5′-CGGCGGTGCACAATCTTCTCGCGCAACG). The 100-bp product was purified on an agarose gel before use. Southern blot probes specific for the SE fragment, or the coding end (CE) fragments resulting from cleavage at the 12 or 23 RSSs, were generated by PCR in the presence of [α^{32}P]-dCTP (3,000 Ci/mmol; NEN Life Sciences) by using pDVG54 as a template and primer pairs JMJ014 (5′-CTCGAGCATATGACGACCTTCGATATG) and JMJ015 (5′-ACGGCTTGCGCGAGAAGATTGTGCACC) (SE), JMJ016 (5′-GGTGTTGGCGGGTGTCGGGGCTGG) and JMJ017 (5′-GTTACCCAACTTAATCGCCTTGCAGC) (12 CE), or JMJ018 (5′-AATACGGTTTCCACAGAATCAGGGG) and JMJ019 (5′-CGTTCCACTGAGCGTCAGACCCCG) (23 CE).

Analysis of Pre- and Postcleavage Complexes. For cleavage of plasmid DNA by RAG1/2, plasmid pDVG54 (5 μg/ml), RAG1/2 (45 μg/ml), and HMG1 (5 μg/ml) were incubated together in "binding buffer" (25 mM 4-morpholinepropanesulfonic acid, pH 7.0/32 mM KCl/4 mM DTT/0.1 mg/ml BSA/1% glycerol/4 mM $CaCl_2$) for up to 10 min at room temperature. Cleavage was initiated by the addition of $MgCl_2$ (4 mM) and carried out for 60 min at 37°C. In some cases, pDVG54 was completely linearized with AatII before cleavage by RAG1/2. Aliquots from large-scale reactions were either deproteinized by treatment with 1 mg/ml of proteinase K and 0.5% SDS or treated with 0.1% glutaraldehyde (Sigma) for up to 30 min at 37°C and separated on 0.8% agarose gels in 1$\times$ TAE (40 mM Tris·acetate/(mM EDTA, pH 8.0) buffer. Gels were stained with SYBR green I (Molecular Probes) and blotted onto GeneScreen Plus membrane (NEN Life Sciences). Vacuum-dried Southern blots were hybridized overnight (37°C) then washed extensively in 0.5 $\times$ SSC/1% SDS (45°C). Unless otherwise indicated, Southern blots hybridized to the SE probe are shown.

For gel-shift analysis of precleavage complexes, labeled 12 RSS oligonucleotide substrates were combined with RAG1/2 (75 μg/ml) and HMG1 (5 μg/ml) in binding buffer. In some cases, reactions also included nonradioactive 23 RSS substrate. Reactions were incubated for up to 10 min at room temperature, then 1 μl of 100% glycerol was added and reactions were loaded onto 8% polyacrylamide gels (crosslinked at a ratio of 80:1) in 0.5$\times$ TBE buffer (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) ("shift gels"). Gels were electrophoresed at 140 V at room temperature. Gels were dried and exposed to phosphor-storage autoradiography. For gel-shift analysis of postcleavage complexes, reactions were assembled as for binding, then cleavage was initiated by the addition of 4 mM $MgCl_2$, and reactions were incubated for 120 min at 37°C before addition of glycerol and separation on shift gels. When pre- and postcleavage complexes were directly compared, binding reactions without $MgCl_2$ were also incubated at 37°C for 120 min. For two-dimensional electrophoresis, wet shift gels were exposed briefly to phosphor-storage autoradiography to identify bands. Lanes were excised and sandwiched between clean glass plates. A solution of 12% acrylamide/*N,N′*-methylene-bis-acrylamide/0.6% *N,N′*-methylene-bis-acrylamide/8 M urea/1% SDS/1$\times$ TBE was poured around the gel slice and allowed to polymerize. The glass plates were heated briefly on a hot plate, then electrophoresed at 40 W.

Kinetic Analysis of Cleavage. Labeled 12 RSS oligonucleotide substrate (1 nM) was combined with RAG1/2 (75 μg/ml) and HMG1 (5 μg/ml), as well as unlabeled 23 or 12 RSS as indicated, in binding buffer (100 μl) for 5 min at 37°C. Cleavage was initiated by the addition of 4 mM $MnCl_2$. Aliquots (5 μl) were removed at the times indicated and analyzed by separation on denaturing 15% polyacrylamide gels.

Joining Reactions. Cleavage of pDVG54 was carried out for 60 min as described above. Aliquots (10 μl) were combined with joining factors as indicated and 5 mM ATP in a total volume of 20 μl. All other buffer components were as for cleavage. Joining reactions were incubated for 30 min at room temperature, then diluted with 20 μl of 1% Tween-20. Signal joints were detected by PCR as described previously (35), by using 5-μl aliquots from the diluted joining reactions. PCR products were separated on precast NOVEX 10% TBE gels (Invitrogen), which were stained with ethidium bromide. In some cases, cleavage products were thoroughly deproteinized before joining. Large-scale cleavage reactions were treated with 1 mg/ml of proteinase K and 0.5% SDS for 60 min at 37°C, then extracted with phenol/chloroform and ethanol precipitated. DNA was resuspended in binding buffer plus HMG1 and 4 mM $MgCl_2$.

Other. All quantification of gel band intensities was by phosphor-storage autoradiography with Molecular Dynamics TYPHOON

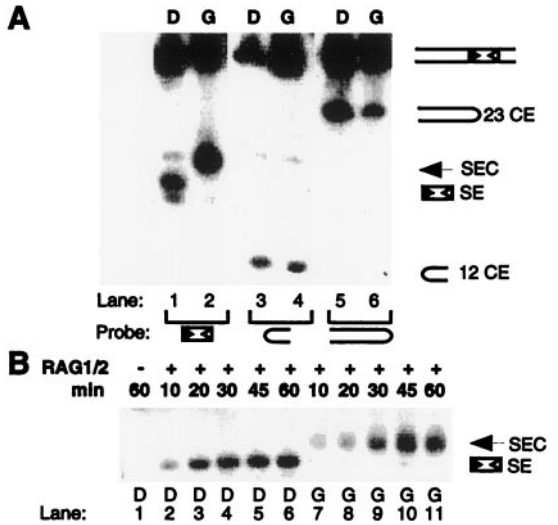

Fig. 1. Detection of the SE complex. (*A*) A large-scale cleavage reaction of a linear plasmid substrate was incubated for 10 min, then divided into aliquots (10 μl) and either deproteinized (D) or treated with glutaraldehyde (G). Southern blots were hybridized to probes specific for the SE fragment (SE, lanes 1-2), or the coding end (CE) fragments resulting from cleavage at the 12 RSS (lanes 3–4) or 23 RSS (lanes 5–6). The SE complex (SEC) is indicated by an arrow. (*B*) Cleavage reactions of supercoiled plasmid substrate were incubated for the time indicated then either deproteinized or treated with glutaraldehyde.

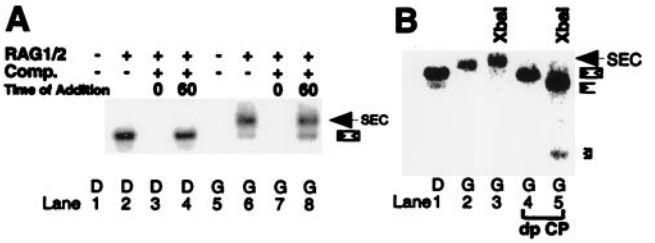

Fig. 2. Stability and structure of the SEC. (*A*) Cleavage reactions were conducted with plasmid substrate and 200-fold molar excess of an RSS-containing oligonucleotide competitor (DAR39/40), as indicated. Competitor was added either before substrate (0) or after 60 min of cleavage (60). After a total of 70 min, samples were deproteinized (D) or treated with glutaraldehyde (G). In lane 6, 84% of the SE fragment was retained in the SEC; in lane 8, 79% was retained. (*B*) Cleavage reactions of plasmid substrate were incubated for 60 min then deproteinized or treated with glutaraldehyde. After glutaraldehyde treatment, reactions were quenched with 0.1 M Tris·HCl [pH 8.0] for 5 min, and 100 units of *Xba*I was added. Digestion was carried out for 60 min, 37°C. To control for efficiency of digestion by *Xba*I under these conditions, cleavage products were thoroughly deproteinized (dp CP), as described in *Methods*, before glutaraldehyde treatment and digestion with *Xba*I (lane 5).

8600 and IMAGEQUANT 5.1 software. Gels stained with SybrGreen I or ethidium bromide were imaged with a Stratagene Eagle Eye II. DNA molar concentrations are given for the whole molecule.

Results

SEs Remain Bound to RAG1/2 After Cleavage. An agarose gel mobility assay was developed to separate and quantitatively analyze DNA ends bound in postcleavage complexes. When a linearized plasmid substrate was subjected to cleavage by RAG1/2 and treated with glutaraldehyde, SEs were associated with a distinct complex of altered mobility. Nearly 100% of the SE fragment was bound in this complex, on the basis of comparison with the SE band in the deproteinized lane (Fig. 1*A*, cf. lanes 1 and 2). Little or none of the coding end fragment resulting from cleavage at the 12 RSS migrated with altered mobility after glutaraldehyde treatment (Fig. 1*A*, cf. lanes 3 and 4). Most of the coding end fragment resulting from cleavage at the 23 RSS also migrated with unaltered mobility after glutaraldehyde treatment (Fig. 1*A*, cf. lanes 5 and 6). The SE complex (SEC) was examined in greater detail.

Several lines of evidence indicated that the SEC was a legitimate postcleavage complex in which RAG1/2 remain bound after cleavage. The SEC was not detected when RAG1/2 and HMG1 were combined with plasmid DNA that had previously been cleaved by RAG1/2 and then thoroughly deproteinized (data not shown), although a complex with the properties of the SEC could be assembled from precleaved ends by using a more sensitive band shift assay (ref. 11 and see below). A time course revealed that the SE cleavage product and SEC accumulated with very similar kinetics over the course of 60 min (Fig. 1*B*, cf. lanes 2–6 with lanes 7–11). Finally, the SEC was resistant to challenge with specific oligonucleotide competitor consisting of RSS sequence plus a 16-bp coding flank. Two-hundred-fold excess specific oligonucleotide competitor was sufficient to prevent coupled cleavage of the plasmid if it was present at the beginning of the reaction (Fig. 2*A*, cf. lanes 2 and 3). When competitor was added after 60 min of cleavage and reactions were continued for an additional 10 min, products were identical to those formed in the absence of competitor (Fig. 2*A*, cf. lanes 2 and 4), and most of the cleavage products were retained in the SEC (Fig. 2*A*, cf. lanes 6 and 8). Oligonucleotide competitor consisting of blunt RSS sequence, similar to the cleaved SE, failed to inhibit cleavage if added at the beginning of the reaction and also did not disrupt the SEC (data not shown). This strongly suggests that the SEC observed in this assay does not result from reassociation of RAG1/2 with cleaved RSS ends.

The SEC maintained the cleaved RSS ends in a synaptic complex. Digestion of the SE fragment bound in the SEC with an enzyme that cuts between the RSSs (*Xba*I) caused the SEC to migrate slightly more slowly, indicating that the digested fragments remain associated with each other via the SEC (Fig. 2*B*, cf. lanes 2 and 3). In multiple trials, 60 to greater than 95% of the fragments remained associated with the SEC band after digestion with *Xba*I. Digestion of a thoroughly deproteinized SE fragment under identical conditions created the two smaller bands expected if the RSSs within the SEC were not maintained in a synaptic complex (Fig. 2*B*, lane 5). These data demonstrated that the proteins necessary for efficient cleavage, RAG1/2 and HMG1, were sufficient to maintain the synaptic complex after cleavage.

The SEC Is More Stable than Precleavage RAG1/2–RSS Complexes. The SEC was stable to challenge with 1 mg/ml of heparin or 0.5 M NaCl (data not shown), agents expected to dissociate loosely bound proteins from DNA. Mobility-shift assays on polyacrylamide gels in which RSSs were provided on oligonucleotide substrates were performed to directly compare the stability of pre- and postcleavage complexes. In the presence of HMG1 and Ca^{2+}, RAG1/2 can form a single precleavage complex when either 12 or 23 RSS is present or a distinct complex when a pair of complementary RSSs are provided (8, 11, 13). The stoichiometry of these complexes with respect to DNA was confirmed by using RSSs provided on oligonucleotides of different lengths. RAG1/2 formed a single shifted complex when the 12 RSS was provided on an oligonucleotide of either 50 or 100 bp (Fig. 3, lanes 1–4; R + 50 mer and R + 100 mer). When RAG1/2 was incubated with the 50- and 100-mer simultaneously, the same two bands were observed, indicating that the shifted complexes included only a single RSS (Fig. 3, lane 6). A second more slowly migrating complex was present when the labeled 12 RSS sub-

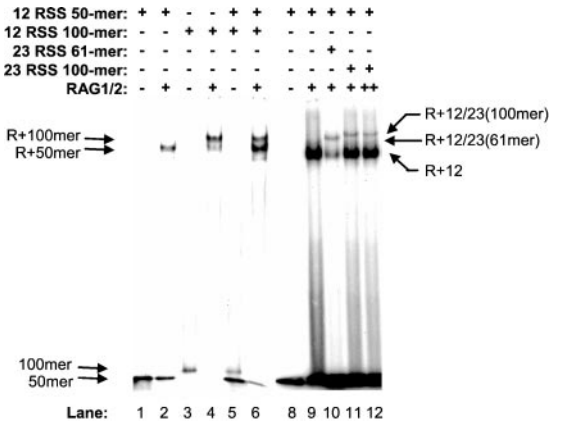

Fig. 3. Gel-shift analysis of precleavage complexes. Binding reactions (5 μl of total volume) were assembled with labeled 12 RSS (1 nM) provided on oligonucleotides of 50 or 100 bp as well as HMG1 and RAG1/2 (R) as indicated. (The specific activity of the 50 mer is about one-half that of the 100 mer.) To form the synaptic complex of two RSSs, excess unlabeled 23 RSS provided on oligonucleotides of 61 (100 nM, lane 10) or 100 bp (10 nM, lane 11, or 20 nM, lane 12) was included. In these reactions 12 RSS, 23 RSS, and HMG1 were combined before the addition of RAG1/2.

strate was complemented with a nonradioactive 23 RSS substrate provided on a 61 mer [Fig. 3, lane 10; R + 12/23 (61 mer)]. The mobility of this complex was further decreased when the 23 RSS was provided on a 100 mer [Fig. 3, lanes 11–12; R + 12/23 (100 mer)], indicating that the more slowly migrating complex incorporated the complementary RSS.

Only the complex of RAG1/2 with cleaved SEs demonstrated resistance to heparin treatment. RAG1/2 was incubated with labeled 12 RSS substrate and unlabeled 23 RSS, as indicated, in reactions that included either Ca^{2+} alone (C) to support binding or both Ca^{2+} and Mg^{2+} to support cleavage (M). Regardless of the cation, the complex of RAG1/2 with the single RSS and with the synaptic complex of two RSSs was observed (Fig. 4*A*, lanes 2–5). When cleavage reactions were challenged with heparin, only a single band migrating slightly faster than the synaptic complex was present (Fig. 4*A*, lane 7, block arrow). To confirm that this band represented the cleaved SE product, lanes 5 and 7 were excised and run in a second dimension on a denaturing polyacrylamide gel. This procedure revealed that in the absence of heparin, both cleaved product and a small amount of full-length substrate were present in the band of shifted mobility (Fig. 4*B*). When heparin was added, only the cleaved product remained in the shifted position (Fig. 4*C*). A band of similar mobility to the heparin-resistant postcleavage complex could be formed by using blunt-cut RSS ends (Fig. 4*A*, lane 9), and this complex was also resistant to challenge with heparin (Fig. 4*A*, lane 11). These data highlight the relative stability of the complex of RAG1/2 with cleaved SEs, and they strongly suggest that the synaptic complex identified here and previously (11) is competent for cleavage in the presence of Mg^{2+}.

The SEC Is Not Detected if Cleavage Between Complementary RSSs Is Not Coupled. Cleavage in Mn^{2+} does not require assembly of a synaptic complex of complementary RSSs (3, 13). Cleavage of the plasmid substrate in the presence of Mn^{2+} decreased the percentage of SE fragment retained in the SEC, but Mn^{2+} did not disrupt an SEC resulting from cleavage in Mg^{2+}. Cleavage was carried out for 70 min in 4 mM Mg^{2+}, with 0.4 mM Mn^{2+} being added either at the same time as Mg^{2+} (time 0) or 60 min later. When Mn^{2+} was added at the same time as Mg^{2+}, no SEC could be detected (Fig. 5*A*, cf. lanes 3 and 7). When Mn^{2+} was added 60 min after Mg^{2+}, the quantity of SEC was indistinguish-

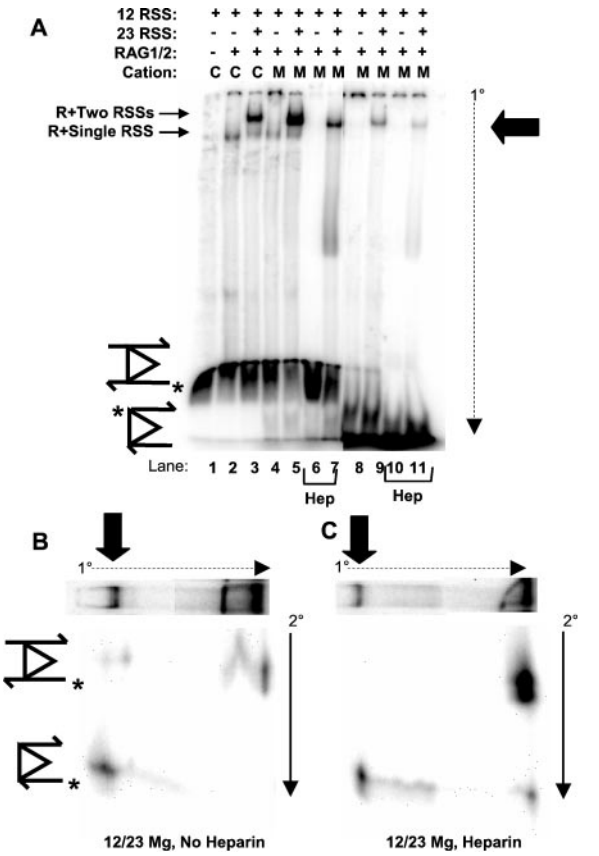

Fig. 4. Side-by-side gel-shift analysis of pre- and postcleavage complexes. (*A*) Gel shifts were performed with labeled cleavage substrate containing a 12 RSS (2 nM; 50 mer) and unlabeled 23 RSS (12.5 nM; 61 mer) as indicated (lanes 1–7) or the equivalent concentrations of precleaved 12 and 23 RSS substrate (lanes 8–11) under binding (C) or cleavage (M) conditions, as described in *Methods* (10 μl of total volume). 12 RSS was combined with HMG1 and RAG1/2 (R) before the addition of 23 RSS. Where indicated, heparin (1 mg/ml) was added after cleavage. The direction of electrophoresis is indicated by a dotted arrow; this corresponds to the first (1°) direction for electrophoresis in the two-dimensional electrophoresis experiments. The position of the radioactive label on the 12 RSS substrate(s) is indicated by an asterisk (*). (*B* and *C*) Reactions were performed as in *A* lanes 5 and 7, respectively, and separated on a shift gel (1°). Lanes from this gel were excised, rotated 90° counterclockwise and subjected to denaturing gel electrophoresis (2°; solid arrow). The positions of intact substrate and cleaved SE product on the denaturing gel are indicated.

able from that produced in the absence of Mn^{2+}. Despite the presence of complementary RSSs on a single plasmid, it is possible that in the presence of Mn^{2+}, only a very small proportion of the cleavage products detected are the result of coupled cleavage.

We performed kinetic analysis of cleavage on oligonucleotide substrates to define conditions that promote coupled cleavage in the presence of Mn^{2+}. The initial rate of cleavage of labeled 12 RSS was stimulated by the addition of 20- to 100-fold molar excess of unlabeled 23 RSS (Fig. 5*B*) but not by the addition of unlabeled 12 RSS (Fig. 5*C*). The unlabeled 23 or 12 RSS did not affect the rate of nicking (data not shown). These data suggested that both cleavage at a single RSS and coupled cleavage between complementary RSSs occurred in Mn^{2+}, but that the latter was observed only when the complementary RSS was provided in molar excess over the labeled RSS. By using the two-dimensional gel analysis technique described above, we found that under these conditions, 40–60% of the cleaved SEs were retained in a complex with RAG1/2 (data not shown). Taken together, these

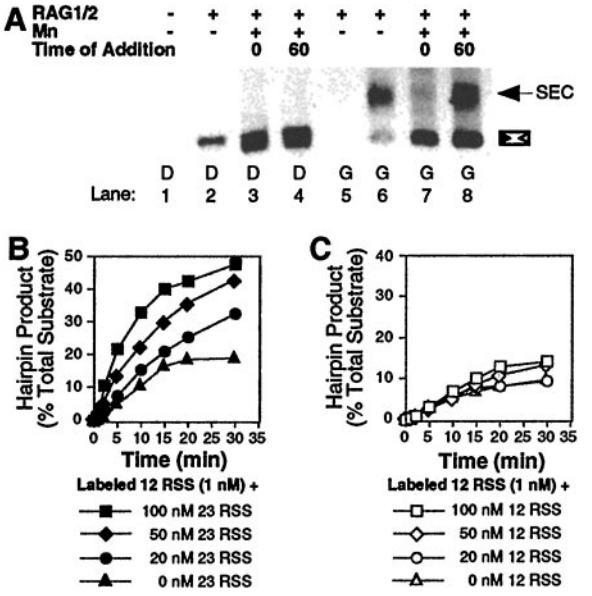

Fig. 5. The effect of Mn^{2+} on formation of the SEC. (*A*) Cleavage reactions of plasmid substrate were conducted in the presence of $MgCl_2$ (4 mM) with $MnCl_2$ (0.4 mM) being added either at the same time as $MgCl_2$ (0) or added 60 min after $MgCl_2$ (60), as indicated. The agarose gel stained with SYBR green I is shown. (*B* and *C*) Kinetic analyses were performed as described in *Methods* by using labeled 12 RSS plus unlabeled 23 RSS (*B*) or 12 RSS (*C*) at the concentrations indicated. Percent of labeled substrate converted into hairpin cleavage product at each time point was determined.

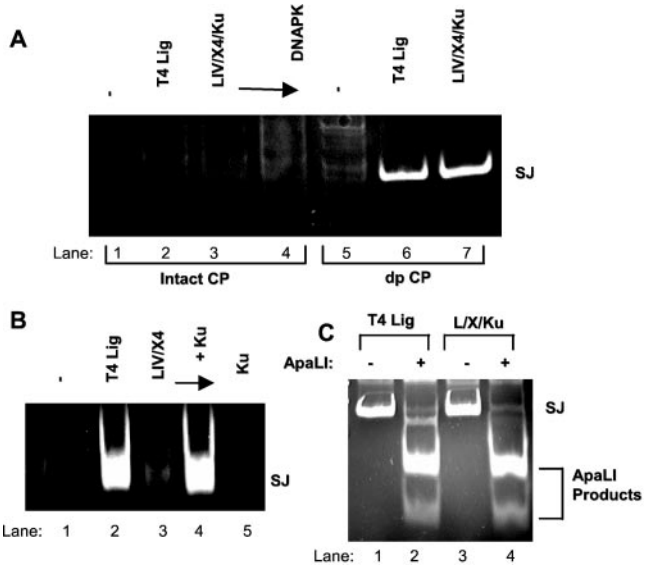

Fig. 6. Joining reactions using mammalian DNA repair proteins. (*A*) Joining reactions using T4 ligase (40 units), or DNA ligase IV and XRCC4 (LIV/X4, 250 ng) and Ku heterodimer (Ku, 200 ng), as indicated, were conducted on either intact cleavage products from reactions including $MgCl_2$ (CP; lanes 1–4) or thoroughly deproteinized cleavage products (dp CP; lanes 5–7). Where indicated, DNA-PK (100 units) was included in the joining reaction. Signal joints (SJ) were detected by PCR. Ethidium bromide-stained gels are shown. (*B*) Joining reactions using thoroughly deproteinized cleavage products were conducted per *A* with T4 ligase, DNA ligase IV and XRCC4, and/or the Ku heterodimer, as indicated. (*C*) Joining reactions conducted per *B* were subjected to digestion by *Apa*LI (5 units) for 60 min at 37°C.

data indicate that SEs are retained in the SEC when cleavage takes place in the context of a synaptic complex, regardless of the divalent cation present.

The SEC Sequesters SEs from Joining Factors Including Ku, XRCC4, and Ligase IV. It has been demonstrated that T4 ligase can join SEs *in vitro* to form signal joints (35). In those experiments, cleavage products were combined with T4 ligase, and signal joints were detected by PCR. However, cleavage was conducted in the presence of 4 mM Mg^{2+} plus 0.4 mM Mn^{2+}, conditions that do not favor production of the SEC. We confirmed that T4 ligase could join SEs generated under these conditions (data not shown), but when cleavage was conducted in the presence of 4 mM Mg^{2+} alone, T4 ligase was unable to join the cleaved ends (Fig. 6*A*, lane 2). These data indicate that SEs retained in the SEC are not accessible to T4 ligase.

We assayed for signal joints using cleavage products that had been deproteinized before joining to demonstrate that a mammalian joining apparatus consisting of DNA ligase IV, XRCC4, and the Ku heterodimer was competent for blunt-ended ligation of naked DNA ends. The DNA ligase IV–XRCC4 complex was competent for high levels of ''sticky-ended'' ligation (data not shown) but was not able to efficiently join the blunt-ended substrate in the absence of Ku (Fig. 6*B*, lane 3). Blunt-ended ligation product was observed when DNA ligase IV–XRCC4 was complemented with the Ku heterodimer (Fig. 6*B*, lane 4); the Ku preparation by itself had no ligation activity on blunt or sticky-ended substrates (Fig. 6*B*, lane 5, and data not shown). Perfect head-to-head joining of two RSSs forms an *Apa*LI site. Products joined by Ku, XRCC4, and ligase IV could be digested nearly to completion with *Apa*LI (Fig. 6*C*, cf. lanes 3 and 4). This indicates that the mammalian factors joined the ends without gain or loss of oligonucleotides. Unlike naked SEs, SEs sequestered in the SEC were not accessible to the mammalian joining apparatus (Fig. 6*A*, cf. lanes 3 and 7), and the addition of DNA-PK_{CS} was not sufficient to restore joining activity to Ku/XRCC4/Ligase IV (Fig. 6*A*, lane 4).

Discussion

Although V(D)J recombination results in high levels of variability and diversity in antigen-coding regions, the process is subject to careful control at many levels. Recombination loci are made accessible in a developmentally regulated manner (36–38), and recombination is normally restricted to the G_1 phase of the cell cycle in developing B and T cells (39). The RAG1/2 recombinase displays an inherent preference for coupled cleavage of complementary RSS pairs (10). Our data corroborate an important regulatory role for RAG1/2 after normal cleavage. RAG1/2 remains bound to the blunt RSS ends and maintains these ends in a synaptic complex. Ends sequestered in this complex cannot be ligated by an active joining apparatus composed of Ku70/86, XRCC4, and ligase IV, accurately reflecting these ends' sequestration during V(D)J recombination in the cell.

It has long been postulated that a stable postcleavage complex may account for the persistence of SEs during V(D)J recombination. We demonstrate that the formation of a stable postcleavage complex strictly requires synapsis of a pair of RSSs before cleavage. Under these conditions, virtually all cleaved RSS ends are retained by RAG1/2 in the SEC. The SEC is resistant to challenge with competitor, indicating that it is the direct product of cleavage and not reassembled from precleaved ends. Although it is possible to reassemble a complex of RAG1/2 with precleaved RSS ends (11, 40), the persistence of SEs *in vivo* without modification suggests that they are never exposed to the general cellular milieu.

The mammalian repair proteins Ku70/86, XRCC4, and DNA ligase IV can join naked blunt-ended DNA (Fig. 6). We find that these proteins do not have access to the ends within the SEC

inasmuch as they cannot join the ends, but we cannot rule out the possibility of a nonproductive (i.e., ligation-incompetent) association. Previous work has suggested that factors such as Ku70/86 and DNA-PK_{CS} are present with the recombinase on cleaved DNA (40) but did not demonstrate a direct interaction of these factors with RAG1/2 on the SEs. Under the conditions used in those experiments, it could not be ruled out that Ku70/86 might have translocated to the interior of the DNA (25, 41) before cleavage by RAG1/2 and was not directly associated with RAG1/2 or the cleaved DNA ends.

The SEC is the most stable of all RAG–DNA complexes examined. The persistence of cleaved RSS ends in the cell during normal recombination at the chromosomal loci suggests that the recombinase in cells likewise remains tightly bound to the RSS ends and releases the ends only in response to specific physiologic stimuli. Evidence suggests that regions outside the core domains used in these experiments contribute to efficient signal joint formation in the cell (42). Phosphorylation of RAG2 at threonine 490, which is outside the core domain, by the cyclinA–cdk2 complex targets RAG2 for degradation at the G_1/S transition (43, 44). This timing coincides with resolution of SEs into signal joints. Disassembly of the stable SEC may, therefore, simply be a matter of degradation of RAG2. Alternatively, phosphorylation or other events preceding degradation may be sufficient to remodel the complex and allow access of the joining machinery to the RSS ends.

What purpose is served by such a stable complex of RAG proteins with SEs? V(D)J recombination in wild-type cells does not appear to activate the G_1/S checkpoint even though the broken RSS ends persist throughout G_1. This observation is even more puzzling in light of the increasing body of evidence indicating that double-strand DNA breaks during V(D)J recombination are initially recognized in a manner similar to those that occur after irradiation or other modes of DNA damage. In both cases, breaks induce rapid phosphorylation of histone H2AX across a large expanse of chromatin and its concentration into nuclear foci (45–47), followed by localization of other repair factors to these foci (45, 48). Little is known about how broken DNA ends are first sensed in the cell. Most hypotheses propose that damage-sensing surveillance proteins are activated by binding directly or indirectly to broken DNA ends, but other possibilities exist. For example, these factors could sense the loss of tension within a chromatin loop that would result from a double-strand break anywhere within that loop. Repair could then be monitored by testing the ability of the chromatin loop to accept and maintain tension, and only persistent DNA breaks would be expected to prevent passage into S phase. Models of cyclic chromatin stress and relaxation are currently being explored with regard to meiotic recombination mechanisms (49). Although broken RSS ends associated with V(D)J recombination are initially detected by the cell, they do not appear to constitute a persistent signal. The tightly bound recombinase may be responsible for masking this signal, either by directly blocking access to the ends or possibly by maintaining the region of chromatin between the cleaved RSS ends in a stable loop.

We thank Dr. R. C. Johnson for the kind gift of bacterial strain RJ1878 and Drs. W. Yang and M. Junop for the kind gift of pWY1272. We also thank Drs. M. Melek and I. Shih of this laboratory for critical reading of this manuscript. J. M. Jones is supported by the Cancer Research Fund of the Damon Runyon–Walter Winchell Foundation, Fellowship DRG1582.

1. Gellert, M. (1997) *Adv. Immunol.* **64,** 39–64.
2. Tonegawa, S. (1983) *Nature (London)* **302,** 575–581.
3. McBlane, J. F., van Gent, D. C., Ramsden, D. A., Romeo, C., Cuomo, C. A., Gellert, M. & Oettinger, M. A. (1995) *Cell* **83,** 387–395.
4. Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. (1990) *Science* **248,** 1517–1523.
5. Schatz, D. G. & Baltimore, D. (1988) *Cell* **53,** 107–115.
6. Blunt, T., Finnie, N. J., Taccioli, G. E., Smith, G. C., Demengeot, J., Gottlieb, T. M., Mizuta, R., Varghese, A. J., Alt, F. W., Jeggo, P. A., *et al*. (1995) *Cell* **80,** 813–823.
7. Taccioli, G. E., Rathbun, G. A., Oltz, E. M., Stamato, T. D., Jeggo, P. A. & Alt, F. (1993) *Science* **260,** 207–210.
8. van Gent, D. C., Hiom, K., Paull, T. T. & Gellert, M. (1997) *EMBO J.* **16,** 2665–2670.
9. van Gent, D. C., Mizuuchi, K. & Gellert, M. (1996) *Science* **271,** 1592–1594.
10. van Gent, D. C., Ramsden, D. A. & Gellert, M. (1996) *Cell* **85,** 107–113.
11. Hiom, K. & Gellert, M. (1998) *Mol. Cell* **1,** 1011–1019.
12. Steen, S. B., Gomelsky, L., Speidel, S. L. & Roth, D. B. (1997) *EMBO J.* **16,** 2656–2664.
13. Hiom, K. & Gellert, M. (1997) *Cell* **88,** 65–72.
14. Ramsden, D. A. & Gellert, M. (1995) *Genes Dev.* **9,** 2409–2420.
15. Zhu, C. & Roth, D. B. (1995) *Immunity* **2,** 101–112.
16. Roth, D. B., Nakajima, P. B., Menetski, J. P., Bosma, M. J. & Gellert, M. (1992) *Curr. Top. Microbiol. Immunol.* **182,** 115–124.
17. Schlissel, M., Constantinescu, A., Morrow, T., Baxter, M. & Peng, A. (1993) *Genes Dev.* **7,** 2520–2532.
18. Roth, D. B., Zhu, C. & Gellert, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10788–10792.
19. Frank, K. M., Sekiguchi, J. M., Seidl, K. J., Swat, W., Rathbun, G. A., Cheng, H. L., Davidson, L., Kangaloo, L. & Alt, F. W. (1998) *Nature (London)* **396,** 173–177.
20. Grawunder, U., Zimmer, D., Fugmann, S., Schwarz, K. & Lieber, M. R. (1998) *Mol. Cell* **2,** 477–484.
21. Critchlow, S. E., Bowater, R. P. & Jackson, S. P. (1997) *Curr. Biol.* **7,** 588–598.
22. Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T. E., Mann, M. & Lieber, M. R. (1997) *Nature (London)* **388,** 492–495.
23. Modesti, M., Hesse, J. E. & Gellert, M. (1999) *EMBO J.* **18,** 2008–2018.
24. Lee, K. J., Huang, J., Takeda, Y. & Dynan, W. S. (2000) *J. Biol. Chem.* **275,** 34787–34796.
25. Paillard, S. & Strauss, F. (1991) *Nucleic Acids Res.* **19,** 5619–5624.
26. Bliss, T. M. & Lane, D. P. (1997) *J. Biol. Chem.* **272,** 5765–5773.
27. Cary, R. B., Peterson, S. R., Wang, J., Bear, D. G., Bradbury, E. M. & Chen, D. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4267–4272.
28. Ramsden, D. A. & Gellert, M. (1998) *EMBO J.* **17,** 609–614.
29. Nick McElhinny, S. A., Snowden, C. M., McCarville, J. & Ramsden, D. A. (2000) *Mol. Cell. Biol.* **20,** 2996–3003.
30. Chen, L., Trujillo, K., Sung, P. & Tomkinson, A. E. (2000) *J. Biol. Chem.* **275,** 26196–26205.
31. Lieber, M. R., Hesse, J. E., Lewis, S., Bosma, G. C., Rosenberg, N., Mizuuchi, K., Bosma, M. J. & Gellert, M. (1988) *Cell* **55,** 7–16.
32. Roth, D. B., Menetski, J. P., Nakajima, P. B., Bosma, M. J. & Gellert, M. (1992) *Cell* **70,** 983–991.
33. Mo, X., Bailin, T., Noggle, S. & Sadofsky, M. J. (2000) *Nucleic Acids Res.* **28,** 1228–1236.
34. Johnson, R. C., Bruist, M. F. & Simon, M. I. (1986) *Cell* **46,** 531–539.
35. Ramsden, D. A., Paull, T. T. & Gellert, M. (1997) *Nature (London)* **388,** 488–491.
36. Krangel, M. S., Hernandez-Munain, C., Lauzurica, P., McMurry, M., Roberts, J. L. & Zhong, X. P. (1998) *Immunol. Rev.* **165,** 131–147.
37. Krangel, M. S. (2001) *J. Exp. Med.* **193,** F27–30.
38. Sleckman, B. P., Bassing, C. H., Hughes, M. M., Okada, A., D'Auteuil, M., Wehrly, T. D., Woodman, B. B., Davidson, L., Chen, J. & Alt, F. W. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 7975–7980. (First Published June 27, 2000; 10.1073/pnas.130190597)
39. Lin, W. C. & Desiderio, S. (1995) *Immunol. Today* **16,** 279–289.
40. Agrawal, A. & Schatz, D. G. (1997) *Cell* **89,** 43–53.
41. Yaneva, M., Kowalewski, T. & Lieber, M. R. (1997) *EMBO J.* **16,** 5098–5112.
42. Steen, S. B., Han, J. O., Mundy, C., Oettinger, M. A. & Roth, D. B. (1999) *Mol. Cell. Biol.* **19,** 3010–3017.
43. Lee, J. & Desiderio, S. (1999) *Immunity* **11,** 771–781.
44. Li, Z., Dordai, D. I., Lee, J. & Desiderio, S. (1996) *Immunity* **5,** 575–589.
45. Chen, H. T., Bhandoola, A., Difilippantonio, M. J., Zhu, J., Brown, M. J., Tai, X., Rogakou, E. P., Brotz, T. M., Bonner, W. M., Ried, T. & Nussenzweig, A. (2000) *Science* **290,** 1962–1965.
46. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S. & Bonner, W. M. (1998) *J. Biol. Chem.* **273,** 5858–5868.
47. Rogakou, E. P., Boon, C., Redon, C. & Bonner, W. M. (1999) *J. Cell Biol.* **146,** 905–916.
48. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M. & Bonner, W. M. (2000) *Curr. Biol.* **10,** 886–895.
49. Zickler, D. & Kleckner, N. (1999) *Annu. Rev. Genet.* **33,** 603–754.