MOLECULAR AND CELLULAR BIOLOGY, Dec. 1997, p. 6932–6939 Vol. 17, No. 12
0270-7306/97/$04.00+0


# RAG-1- and RAG-2-Dependent Assembly of Functional Complexes with V(D)J Recombination Substrates in Solution

WENHUI LI, PATRICK SWANSON, AND STEPHEN DESIDERIO*

*Department of Molecular Biology and Genetics and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

Received 7 July 1997/Returned for modification 15 August 1997/Accepted 3 September 1997

**V(D)J recombination is initiated by RAG-1 and RAG-2, which introduce double-strand DNA breaks at recombination signal sequences (RSSs) of antigen receptor gene segments to produce signal ends, terminating in blunt, double-strand breaks, and coding ends, terminating in DNA hairpins. While the formation of RAG-RSS complexes has been documented, observations regarding the individual contributions of RAG-1 and RAG-2 to RSS recognition are in conflict. Here we describe an assay for formation and maintenance of functional RAG-RSS complexes in the course of the DNA cleavage reaction. Under conditions of in vitro cleavage, the RAG proteins sequester intact substrate DNA in a stable complex which is formed prior to strand scission. The cleavage reaction subsequently proceeds through nicking and hairpin formation without dissociation of substrate. Notably, the presence of both RAG-1 and RAG-2 is essential for formation of stable, functional complexes with substrate DNA under conditions of the sequestration assay. Two classes of substrate mutation are distinguished by their effects on RAG-mediated DNA cleavage in vitro. A mutation of the first class, residing within the RSS nonamer and associated with coordinate impairment of nicking and hairpin formation, greatly reduces the stability of RAG association with intact substrate DNA. In contrast, a mutation of the second class, lying within the RSS heptamer and associated with selective abolition of hairpin formation, has little or no effect on the half-life of the RAG-substrate complex.**

The antigen receptors of B and T cells are encoded in the germ line by discrete DNA segments, V, D, and J, that are joined during lymphocyte development. This process, termed V(D)J recombination, is mediated by conserved heptamer and nonamer signal sequences, separated by less highly conserved spacer regions of 12 or 23 bp (12); recombination normally occurs between gene segments carrying spacers of different length (the 12/23 rule). V(D)J recombination is initiated by the recombination activating proteins RAG-1 and RAG-2 (15, 22), which act together at the junctions between coding segments and recombination signal sequences (RSSs) to produce two types of DNA end: a signal end, terminating in a blunt, double-strand break, and a coding end, terminating in a DNA hairpin (14, 20, 21, 23, 28, 29). The cleaved coding and signal ends are subsequently joined in what appear to be distinct reactions, requiring the activity of at least four genes that also function in general double-strand DNA break repair (1, 2, 6, 7, 10, 11, 13, 16, 17, 19, 24, 26, 27, 31).

RAG-1 and RAG-2 are necessary and sufficient for the production of signal and coding ends, which occurs in two kinetically distinguishable steps (14). The first step involves nicking of the upper DNA strand at the junction between the RSS heptamer and the coding sequence. This is followed by a transesterification reaction in which the free hydroxyl group at the 3′ end of the coding sequence attacks a phosphate group on the intact opposite strand, forming a DNA hairpin (29). The substrate sequence requirements for nicking and hairpin formation apparently differ, because certain mutations within the RSS heptamer impair hairpin formation without significantly affecting the efficiency of site-specific nicking by the RAG proteins (references 3 and 18 and this communication). RSS recognition, enforcement of the 12/23 rule (5, 30), and DNA cleavage are intrinsic properties of RAG-1 and RAG-2, indicating that the RAG proteins form one or more specific complexes with substrate DNA.

Nonetheless, until recently it has been difficult to demonstrate the existence of such complexes directly. By assessing the ability of various mutant oligonucleotides to compete with a wild-type substrate in the cleavage reaction, the RSS heptamer and nonamer elements have both been implicated as important in the initial interaction between the RAG proteins and DNA, with the nonamer apparently playing a more prominent role (18). Two recent studies, employing a one-hybrid transfection assay (4) or surface plasmon resonance (25), suggest that RAG-1, acting alone, can interact specifically with the nonamer. While the affinity of this interaction is as yet undetermined, the ability of certain RAG-1 mutations to impair both V(D)J recombination and nonamer binding has suggested that the interaction detected by these assays may be of physiologic significance (4, 25). These interpretations, however, are in apparent conflict with the results of a third study, employing an electrophoretic mobility shift assay, in which formation of RSS-dependent complexes with substrate DNA required the presence of both RAG-1 and RAG-2 (9).

A means of assessing formation and maintenance of RAG-RSS complexes in the course of RAG-mediated DNA cleavage is suggested by the protracted kinetics of the in vitro reaction. Prior formation of a stable complex between RAG proteins and substrate DNA is expected to render a radiolabeled substrate refractory to subsequent challenge by an excess amount of identical, unlabeled DNA. We have used this prediction to develop an assay for formation of stable RAG-substrate complexes in vitro; the assay differs from previous approaches in its ability to monitor the persistence of RAG-DNA association over the entire course of the cleavage reaction.

Here we demonstrate that under conditions of in vitro cleavage, the RAG proteins sequester substrate DNA in one or more stable complexes; following nicking, the reaction pro-

* Corresponding author. Phone: (410) 955-4735. Fax: (410) 955-9124.

ceeds to the transesterification stage of the reaction without dissociation of substrate. Notably, under these conditions formation of a stable, functional complex with substrate DNA requires the presence of both RAG-1 and RAG-2; radiolabeled substrate is not sequestered from exchange with unlabeled competitor upon preincubation with either RAG-1 or RAG-2 alone. Substitution of $Ca^{2+}$ for $Mn^{2+}$ in the in vitro reaction supports formation of a stable complex between RAG proteins and intact substrate DNA. These complexes, however, are trapped at a stage prior to nicking and do not proceed to cleavage and hairpin formation until $Mn^{2+}$ is added. Formation of a stable RAG-substrate complex is dependent on an intact nonamer signal sequence: a point mutation in the nonamer that impairs nicking and hairpin formation also impairs sequestration of radiolabeled substrate DNA by the RAG proteins. In contrast, a point mutation in the heptamer that permits nicking but abolishes hairpin formation has no effect on the half-life of the RAG-substrate complex. Taken together, our observations support the idea that RAG-1 and RAG-2 cooperate in the assembly of a stable, RSS-dependent complex with substrate DNA and suggest that under the reaction conditions employed here, any interaction that may exist between RAG-1 alone and the RSS is unproductive or short lived.

## MATERIALS AND METHODS

**Proteins.** RAG-1 and RAG-2 were coexpressed as truncated fusion proteins in baculovirus and purified as described elsewhere (14). For experiments involving reconstitution of cleavage activity from individually expressed proteins, RAG-1 and RAG-2 fusion constructs were transferred from baculovirus expression vectors to the vector pcDNA-1 (Invitrogen). The resulting constructs were expressed individually by cotransfection with pRSV-T in 293 cells. Cells were harvested at 48 h after transfection, washed in phosphate-buffered saline–EDTA, and stored as a pellet at −80°C. RAG-1 protein was purified by amylose affinity chromatography as described elsewhere (14). For purification of RAG-2, cells were lysed by an alternative procedure (25). Briefly, cell pellets were resuspended in 1.5 ml of cold RSB buffer (10 mM Tris Cl [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) and allowed to swell for 5 min on ice, after which time 1.5 volumes of LSB (20 mM Tris Cl [pH 7.4], 1 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.2 mM $MgCl_2$, 0.2% Nonidet P-40 was added. After rocking for 1 h at 4°C, the lysate was clarified by centrifugation in a SW50.1 rotor at 30,000 rpm for 45 min at 4°C and the resulting supernatant was passed over amylose resin equilibrated in a 1:1.5 ratio of RSB to LSB. The resin was washed with 5 volumes of a 1:1.5 ratio of RSB to LSB and 4 volumes of WB (20 mM Tris Cl [pH 7.4], 0.5 M NaCl, 5 mM $MgCl_2$). Fusion protein was eluted in WB containing 10 mM maltose and dialyzed as described in reference 14.

**Oligonucleotide cleavage assay.** For mutational studies, standard cleavage assays involved incubation of ≈50 ng of coexpressed RAG-1 and RAG-2 with 0.02 pmol of substrate (specific activity, $1 \times 10^7$ to $5 \times 10^7$ cpm/pmol) in a 10-μl reaction volume containing 25 mM MOPS (morpholinepropanesulfonic acid)-KOH (pH 7.0), 60 mM potassium glutamate, 60 mM KCl, 10 mM Tris Cl (pH 8.0), 1 mM $MnCl_2$, and 0.8 mM dithiothreitol. After 2 h at 37°C, the reaction was terminated by addition of an equal volume of 95% formamide–20 mM EDTA and then heated for 2 min at 95°C. For substrate challenge assays, coexpressed RAG-1 and RAG-2 were preincubated with either intact or prenicked duplex substrates under standard cleavage conditions for various times prior to addition of excess unlabeled, intact substrate DNA as indicated. Fractional conversion of substrate to cleavage product (nicked and hairpin) ranged from 9 to 19%, as indicated in figure legends. In some experiments, 1 mM $CaCl_2$ was substituted for $MnCl_2$ as described in Results. Cleavage products were fractionated on a denaturing 15% polyacrylamide (19:1 ratio of acrylamide to methylene [bis]acrylamide) sequencing gel and detected by autoradiography. Autoradiographic data were quantified by densitometry or by digital scanning. Image data, obtained by digital scanning or by phosphorimager, were analyzed with the programs NIH Image or ImageQuant (Molecular Dynamics).

**Substrates.** Oligonucleotides were synthesized with an ABI 394 DNA/RNA synthesizer and purified by reverse-phase high-pressure liquid chromatography. The standard intact duplex substrate was constructed as described elsewhere (14) from two oligonucleotides, DAR39 (5′-GATCTGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCTGCAG; heptamer and nonamer sequences are underlined) and its complement, DAR40; after annealing, these oligonucleotides form a 50-bp duplex substrate containing a single 12-bp RSS. Single-base-pair substitutions were introduced into this standard duplex as noted in the text. DAR39 was labeled with $^{32}P$ at its 5′ end with T4 polynucleotide kinase and annealed to a fivefold excess of unlabeled DAR40; the resulting duplex DNA was purified on a 10% nondenaturing polyacrylamide gel, removed from the gel by electroelution (GE200; Pharmacia), and exchanged into 1 mM Tris (pH 8.0) by gel filtration over Sephadex G-25 (NAP-5; Pharmacia).

For use in competition experiments, substrate analogs containing mutated heptamer and/or nonamer motifs were constructed from complementary oligonucleotides and purified by gel electrophoresis as described above. The upper strand sequences of the resulting duplex substrates are as follows: 5′-GATCTGGCCTGTCTTAatCAGctCTACAGACTGGAACAAAAACCCTGCAG; 5′-GATCTGGCCTGTCTTACACAGTGCTACAGACTGGAAacAAccaaCTGCAG; and 5′-GATCTGGCCTGTCTTAatCAGctCTACAGACTGGAAacAAccaaCTGCAG (positions of heptamer and nonamer elements are underlined, and mutated residues are denoted by lowercase letters).

## RESULTS

**Distinct substrate sequence requirements for nicking and hairpin formation by RAG proteins.** Strand scission and transesterification represent discrete steps in the overall process of RAG-mediated DNA cleavage (14). Mutational analysis revealed that these two stages in the overall cleavage reaction depend on distinct features of the RSS. Previous work by others has shown that the three heptamer residues abutting the coding sequence and residues 6 and 7 of the nonamer (counting from the heptamer-proximal to heptamer-distal position) are most critical for recombination of an extrachromosomal substrate in transfected cells (8). We assessed the effects of mutations at the corresponding positions (C17, A18, C19, A41, and A42) on cleavage of an oligonucleotide substrate by purified RAG proteins in vitro (Fig. 1). The RAG preparations used in these experiments were obtained by baculovirus-mediated coexpression of RAG-1 and RAG-2 core fragments, followed by copurification as described elsewhere (14). Accumulation of hairpins, but not nicked intermediates, was profoundly impaired by mutation of C17 or A18, the two heptamer residues closest to the coding flank; mutation of the third heptamer residue, C19, had relatively little effect on the yield of hairpins as assayed at 2 h, although the ratio of hairpins to nicked intermediates was reduced relative to that of wild type. All substitutions at C17, A18, or C19 were associated with the appearance of adventitious nicks, although the distribution of these nicks varied with the identity of the mutated base pair (Fig. 1). Mutations at nonamer positions A41 and A42, in contrast, reduced the absolute yield of nicks and hairpins coordinately but did not greatly affect the relative amounts of hairpins and nicked intermediates (Fig. 1). While a modest decrease in the precision of nicking was observed for all mutations at A41 and A42, this effect was small compared to the effects of mutations at C17, A18, and C19.

Suppression of hairpin production by heptamer mutations at C17 and A18 could, in principle, result from a delay in the conversion of nicked intermediates or from selective impairment of the transesterification reaction. To distinguish between these possibilities, we examined the kinetics of strand scission and hairpin formation for substrates carrying mutations at C17 and A18, relative to wild-type substrates. In a reaction containing the wild-type substrate (Fig. 2, upper panel), accumulation of nicked intermediates was distinguishable kinetically from accumulation of hairpin products, as previously documented (14); nicked products accumulated to a maximum by about 15 min, declining somewhat with increased accumulation of hairpin product. In contrast, accumulation of hairpins was undetectable in reactions containing the C17G mutant, even at 8 h, despite accumulation of nicked products to a level exceeding that observed for the wild-type reaction (Fig. 2, lower panel). As indicated in Fig. 1, the C17G mutation is associated with the appearance of a prominent, adventitious nick at A18, the second heptamer position. Products terminat-

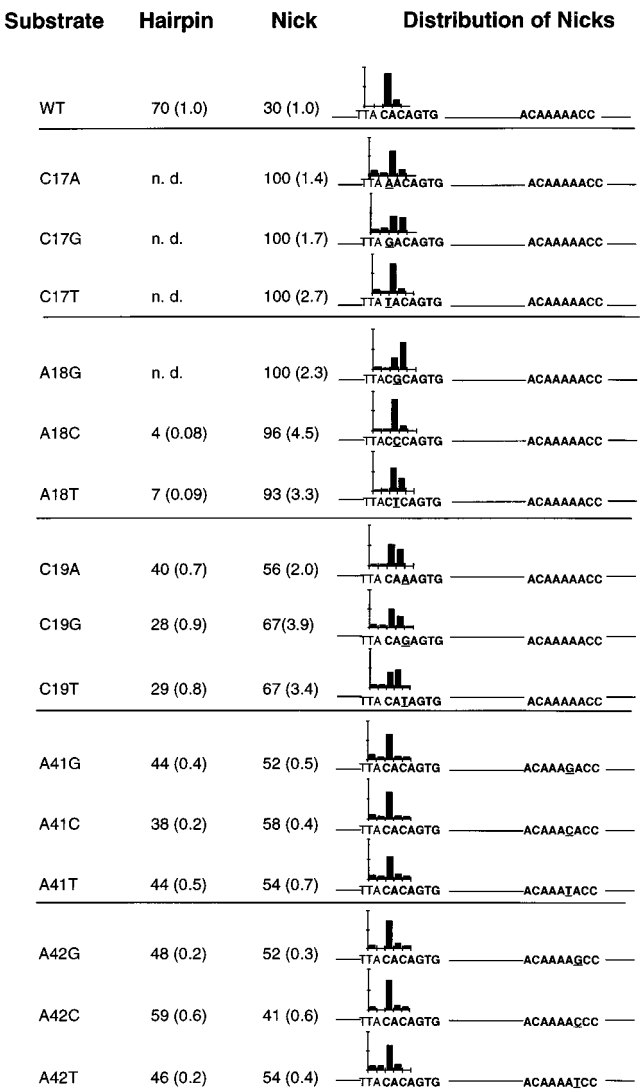


FIG. 1. Frequency and distribution of nicking and hairpin products obtained from wild-type (WT) and mutant oligonucleotide substrates. Substrates containing the indicated RSS point mutations were tested in parallel with a wild-type substrate for their ability to undergo nicking and hairpin formation when incubated with RAG-1 and RAG-2 in the presence of $Mn^{2+}$. Accumulation of nicked or hairpin product after 2 h of incubation is expressed as a percentage of total cleaved substrate (nicked and hairpin). The yield of nicked or hairpin product, relative to the amount obtained from a wild-type substrate assayed in parallel, is given in parentheses. Species undetectable under conditions of the assay are indicated (n.d.). For each substrate, the distribution of nicked products is given at right. The abscissa is aligned with the corresponding substrate residues below; the ordinate indicates the percentage of total nicked product occurring at each position. Heptamer and nonamer motifs are indicated by boldface; mutations are underlined.

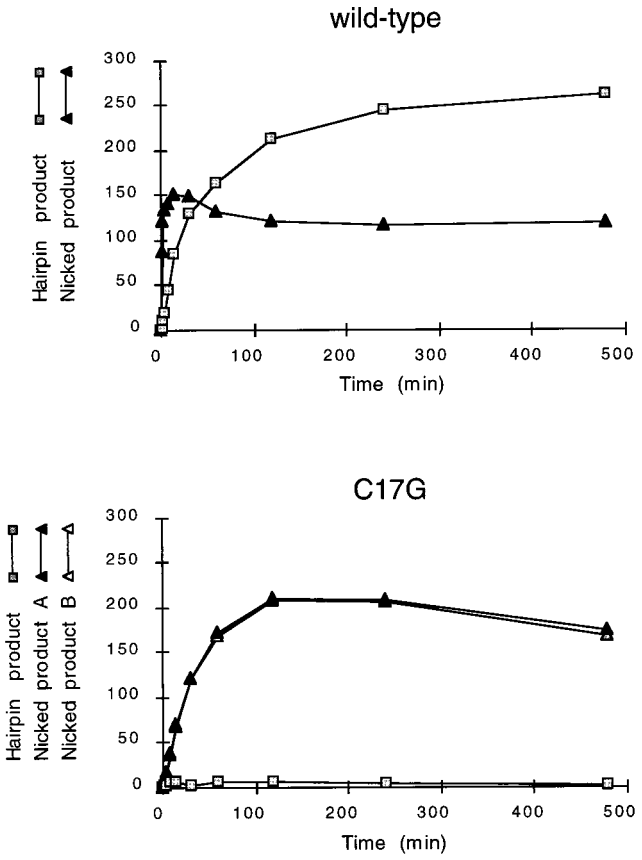


FIG. 2. Kinetic analysis of nicking and hairpin formation from wild-type and C17G mutant substrates. RAG-1 and RAG-2 were incubated with a wild-type substrate or with the C17G mutant. Reactions were sampled at the times indicated, and products were resolved by electrophoresis through a polyacrylamide-urea gel. Nicked and hairpin products were detected by autoradiography and quantified by densitometry. Arbitrary absorbance units are plotted as a function of time.

ing at the first heptamer position, G17 (Fig. 2, lower panel, nicked product A), and at A18 (Fig. 2, lower panel, nicked product B) accumulated with identical kinetics, indicating that the product terminating at A18 arises from imprecise cleavage rather than subsequent modification of a precisely nicked initial product. Similar kinetics of nicking in the absence of hairpin formation were observed for the A18T mutant substrate (data not shown). In agreement with observations recently published by others (3, 18), these results indicate that heptamer mutations at C17 and A18 selectively impair transesterification by the RAG proteins, effectively uncoupling nicking from hairpin formation.

**A stable complex between substrate DNA and RAG protein persists through the transition from strand scission to hairpin formation.** The differential effects of mutations at C17 and A41 on the cleavage reaction suggested that base pairs at these positions make distinct contributions to RAG-RSS recognition. As one approach to the detection of putative RAG-DNA complexes, we exploited the protracted kinetics of strand scission and hairpin formation. Incubation of copurified RAG-1 and RAG-2 with radiolabeled substrate DNA under standard reaction conditions (Fig. 2 and Fig. 3A) results in formation of nicked intermediates, detectable by about 30 s, followed by the appearance of hairpin products, first evident at about 4 min. Addition of a 50-fold excess of unlabeled substrate DNA at the initial reaction time results in a large diminution in the yield of radiolabeled products detectable at 2 h (Fig. 3B, lane 1), as expected by free exchange of labeled and unlabeled substrate, with concomitant dilution of specific radioactivity. In contrast, if excess unlabeled substrate were to be added after formation of a stable complex between radiolabeled substrate DNA and RAG protein, then one would expect any RAG-associated radiolabeled substrate to be sequestered from free exchange with unlabeled substrate. To test this, we allowed the reaction to proceed for times ranging from 15 s to 4 min before adding

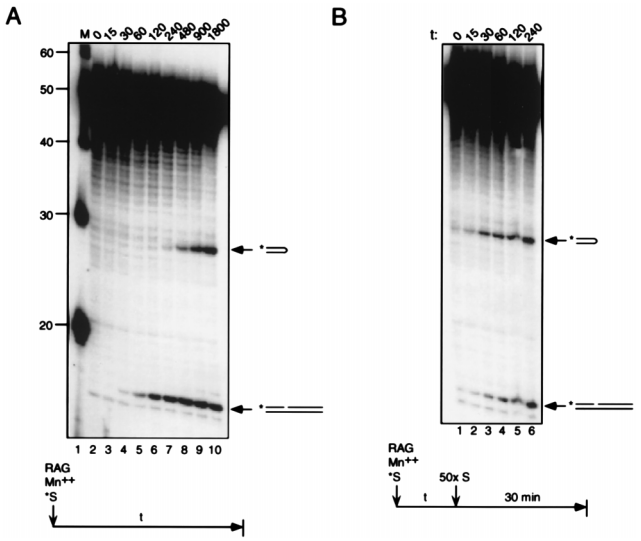


FIG. 3. Preincubation with RAG proteins sequesters radiolabeled substrate DNA from unlabeled competitor. (A) Kinetics of RAG-mediated DNA cleavage. Lane 1: marker DNA; lanes 2 to 10: a reaction containing RAG-1, RAG-2, $Mn^{2+}$, and radiolabeled wild-type substrate was incubated at 37°C and sampled at times indicated at top, in seconds. The reaction scheme is diagrammed below; *S denotes radiolabeled substrate. Positions of radiolabeled nicked and hairpin products are indicated at right. Lengths of size standards, in nucleotides, are indicated at left. By 30 min (lane 10), 9% of input substrate was converted to nicked or hairpin product, as assessed by densitometry. (B) Radiolabeled wild-type substrate DNA was incubated in the presence of RAG-1, RAG-2, and $Mn^{2+}$ at 37°C. At various times, a 50-fold excess of unlabeled substrate DNA was added and the reaction was allowed to proceed for 30 min thereafter, at which time products were analyzed by gel electrophoresis and autoradiography as described above. The reaction scheme is diagrammed below. *S denotes radiolabeled substrate; S indicates unlabeled competitor. The time of addition of unlabeled substrate, in seconds, is indicated above each lane. Positions of radiolabeled nicked and hairpin products are indicated at right.

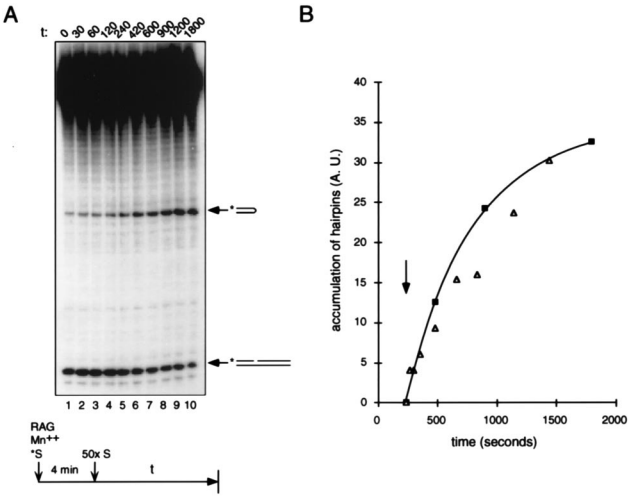


FIG. 4. Sequestration of labeled substrate DNA by RAG proteins permits continued accumulation of hairpin products following challenge with unlabeled competitor. (A) Radiolabeled substrate DNA was incubated with RAG-1 and RAG-2 in the presence of $Mn^{2+}$ at 37°C for 4 min, at which time a 50-fold excess of unlabeled substrate was added. The reaction was allowed to proceed for various times thereafter, and products were analyzed as described for Fig. 2. The reaction scheme is diagrammed below. Time of incubation following addition of competitor, in seconds, is indicated above each lane. Positions of radiolabeled nicked and hairpin products are indicated at right. (B) The time course of hairpin formation in a standard reaction (filled squares) is compared to that of hairpin formation under conditions in which unlabeled competitor was added after a 4-min preincubation of labeled substrate with RAG proteins (open triangles). Data were quantitated by digital scanning of autoradiographs presented in this figure and in Fig. 3A, as described in Materials and Methods. The experiments of Fig. 3A and 4A were performed in parallel with substrates of the same specific activity, and autoradiographs were exposed for identical times. For each series, the hairpin signal intensity at 4 min, in arbitrary units, was subtracted. The vertical arrow indicates the time of addition of unlabeled competitor.

excess unlabeled substrate and then continued incubation for 30 min thereafter. As the time of addition of unlabeled substrate increased, so did the subsequent accumulation of radiolabeled products (Fig. 3B, lanes 2 to 6). Moreover, in the reactions to which unlabeled substrate was added at 30 s, 1 min, 2 min, or 4 min, radiolabeled product continued to accumulate after addition of unlabeled competitor (compare Fig. 3B, lanes 3 to 6, to Fig. 3A, lanes 4 to 7).

Continued accumulation of radiolabeled products after addition of excess unlabeled substrate was evident upon kinetic analysis. The reaction examined in Fig. 4A was identical to that of Fig. 3A, except that a 50-fold excess of unlabeled substrate oligonucleotide was added to a standard cleavage reaction after 4 min at 37°C. As expected, the amount of nicked intermediate continued to increase transiently after addition of competitor and then declined as hairpin products accumulated (Fig. 4A). Notably, neither the amount of radiolabeled product nor the kinetics of accumulation were perturbed by addition of unlabeled substrate; this is seen most clearly by comparing the time course of hairpin accumulation in the presence or absence of unlabeled competitor (Fig. 4B). Thus, within 4 min after the start of the standard cleavage reaction, most or all of the radiolabeled substrate that is destined for subsequent conversion into nicked and hairpin product is sequestered from free exchange with unlabeled substrate DNA. From these observations we infer (i) that radiolabeled substrate DNA forms a stable complex with one or both RAG proteins and (ii) that this association is maintained through the transition from the nicking to the transesterification phase of the overall cleavage reaction. Consistent with this result, nicked substrates were also found to form stable, de novo complexes with RAG proteins under these conditions (data not shown).

**Identification of a prenicking complex between intact substrate DNA and RAG protein.** From the continued accumulation of radiolabeled, nicked intermediates following addition of excess unlabeled substrate, we inferred that a stable complex between substrate DNA and one or both RAG proteins is formed prior to strand scission. To obtain more direct evidence for this reaction intermediate, we sought conditions under which radiolabeled substrate DNA might be sequestered by RAG protein in the absence of DNA cleavage. When $Ca^{2+}$ was substituted for $Mn^{2+}$ in the in vitro reaction, neither nicking nor hairpin formation was observed (Fig. 5, compare lanes 1 and 3). $Ca^{2+}$ does, however, support formation of a stable substrate-RAG complex, as indicated by the following experiment. Radiolabeled substrate DNA was incubated with RAG proteins under standard reaction conditions, except that $Ca^{2+}$ was substituted for $Mn^{2+}$. At 4 min, $Mn^{2+}$ and 1,000-fold excess unlabeled substrate were added; incubation was continued for 2 h, at which time radiolabeled products were readily detected (Fig. 5, lane 6). If, however, $Mn^{2+}$ and competitor DNA were added at 0 min, radiolabeled products were not observed (Fig. 5, lane 7). Thus, radiolabeled substrate DNA is sequestered from subsequent challenge by unlabeled competitor by preincubation with RAG proteins in the presence of $Ca^{2+}$. Furthermore, the extent of protection from competition is comparable to that observed upon preincubation in the presence of $Mn^{2+}$ (Fig. 5, lanes 2 and 8). We conclude that $Ca^{2+}$ supports formation of a functional RAG-substrate complex

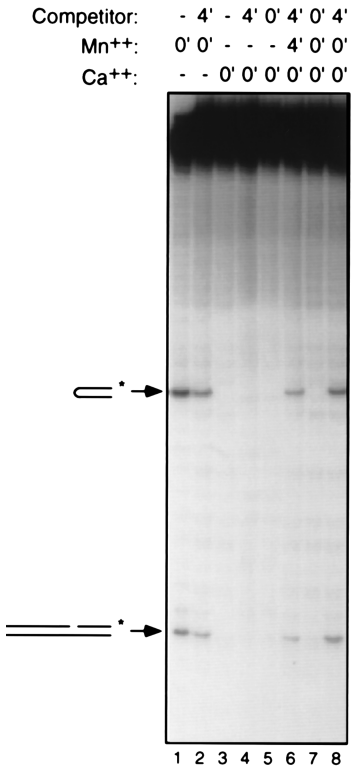


FIG. 5. A stable, functional complex between RAG proteins and substrate DNA is formed in the presence of $Ca^{2+}$. Radiolabeled, wild-type substrate DNA was incubated with RAG-1 and RAG-2 at 37°C. Reaction mixtures were supplemented with 1,000-fold excess unlabeled competitor, $Mn^{2+}$, or $Ca^{2+}$ as indicated at top. Supplements were added at initial time (0′), added at 4 min (4′), or omitted (−). All reactions were allowed to proceed for 2 h; products were resolved by electrophoresis and detected by autoradiography. Positions of radiolabeled nicked and hairpin products are indicated at left.

and that upon addition of $Mn^{2+}$ this complex undergoes cleavage without dissociation of RAG proteins from the DNA.

**Stability of the prenicking complex is impaired by the A41C nonamer mutation but not by the C17A heptamer mutation.** Consistent with previous observations (18) and confirming the RSS dependence of the RAG-DNA interaction assessed here, the ability of unlabeled competitor DNA to interfere with cleavage of a radiolabeled, wild-type substrate was greatly reduced by mutation of the nonamer, the heptamer, or both (data not shown). RAG-mediated DNA nicking and transesterification have distinct RSS requirements (3, 18; see also above). A subset of mutations in the nonamer, typified by the A41C substitution, was associated with coordinate impairment of nicking and transesterification, while a subset of heptamer mutations, typified by C17A, resulted in selective impairment of transesterification. The ability of $Ca^{2+}$ to support formation of a stable complex in the absence of nicking and double-strand cleavage allowed us to assess the stability of complexes between RAG proteins and wild-type or mutant substrate DNA.

To measure the stability of specific RAG-DNA complexes, radiolabeled substrate DNA was incubated with RAG-1 and RAG-2 in the presence of $Ca^{2+}$. After 20 min, 1,000-fold excess unlabeled wild-type competitor DNA was introduced. $Mn^{2+}$ was added at the same time as unlabeled competitor DNA or at various times thereafter, and the reaction was permitted to proceed for 2 h. As the time between addition of competitor DNA and $Mn^{2+}$ increased, the yield of nicked and hairpin product was observed to decline (Fig. 6A, lanes 5 to 12, and D). To exclude the possibility that this decrease resulted from loss of RAG activity over time, RAG proteins were preincubated with substrate DNA in $Ca^{2+}$ for various times ranging from 20 to 50 min before simultaneous addition of competitor and $Mn^{2+}$, after which the reaction was allowed to proceed for 2 h more. No decline in the amount of cleavage product was observed (Fig. 6A, lanes 13 to 20), indicating that RAG activity is stable for the period examined under the reaction conditions used here. We infer that the decline in the yield of cleavage products that occurs with increasing time between addition of competitor DNA and $Mn^{2+}$ reflects the kinetics of RAG dissociation from the wild-type substrate. Under the present experimental conditions, this proceeds with a half time of 2 to 3 min (Fig. 6D). This experiment was performed three times with similar results. Consistent with data presented above, the product yield was reduced somewhat in the presence of unlabeled competitor, presumably because of interference with continued cleavage of labeled substrate. The magnitude of this decrease, ranging in various experiments from about two- to fourfold as assessed by densitometry or phosphorimager analysis, was similar in reactions containing the wild-type or C17A mutant substrate, indicating that there is little or no difference between these substrates with respect to the relative abundance of labile versus stable complexes.

In agreement with results described above, incubation of the radiolabeled C17A mutant substrate with RAG proteins for 2 h under standard reaction conditions resulted in exclusive accumulation of nicked products (Fig. 6B, lane 3); a similar reaction with the A41C mutant substrate yielded a mixture of nicked and hairpin-containing products (Fig. 6C, lane 3). When $Ca^{2+}$ was substituted for $Mn^{2+}$, no cleavage or hairpin formation was observed for either mutant substrate (Fig. 6B and C, lanes 4), consistent with results obtained with wild-type DNA (Fig. 6A, lane 4). Preincubation of the radiolabeled C17A substrate and the RAG proteins for 20 min in the presence of $Ca^{2+}$ resulted in formation of a stable complex that decayed with kinetics similar to those observed for the wild-type substrate (Fig. 6B, lanes 5 to 12, and D). Assuming first-order kinetics, the wild-type and C17A mutant sequestration complexes decay with apparent half-lives of approximately 3 min (Fig. 6D). We conclude that the stability of RAG association with substrate is unimpaired by the C17A mutation. In contrast, under the same conditions the A41C substrate exchanged rapidly with competitor DNA, as evidenced by the nearly complete suppression of radiolabeled product formation at even the earliest times of addition of $Mn^{2+}$ (Fig. 6C, lanes 5 to 12). Thus, the A41C mutation, unlike the C17A mutation, greatly reduces the stability of interaction between substrate DNA and the RAG proteins. The destabilizing effect of the A41C mutation is consistent with its coordinate impairment of nicking and hairpin formation.

**RAG-1 and RAG-2 are both required for assembly of stable, functional complexes with substrate DNA.** The preceding experiments provided evidence for stable association of substrate DNA with RAG proteins and the persistence of this association through strand scission and transesterification. To examine the roles of the individual RAG proteins in formation of functional DNA complexes, RAG-1 and RAG-2 were expressed independently in 293 cells and purified separately. Cleavage was reconstituted by combining RAG-1 and RAG-2 (Fig. 7, lane 12); as expected, neither protein alone exhibited cleavage activity (Fig. 7, lanes 2 and 3). We next asked whether the RAG proteins, expressed separately, retained the ability to form a stable sequestration complex with substrate DNA. RAG-1 and RAG-2 were combined and incubated with labeled

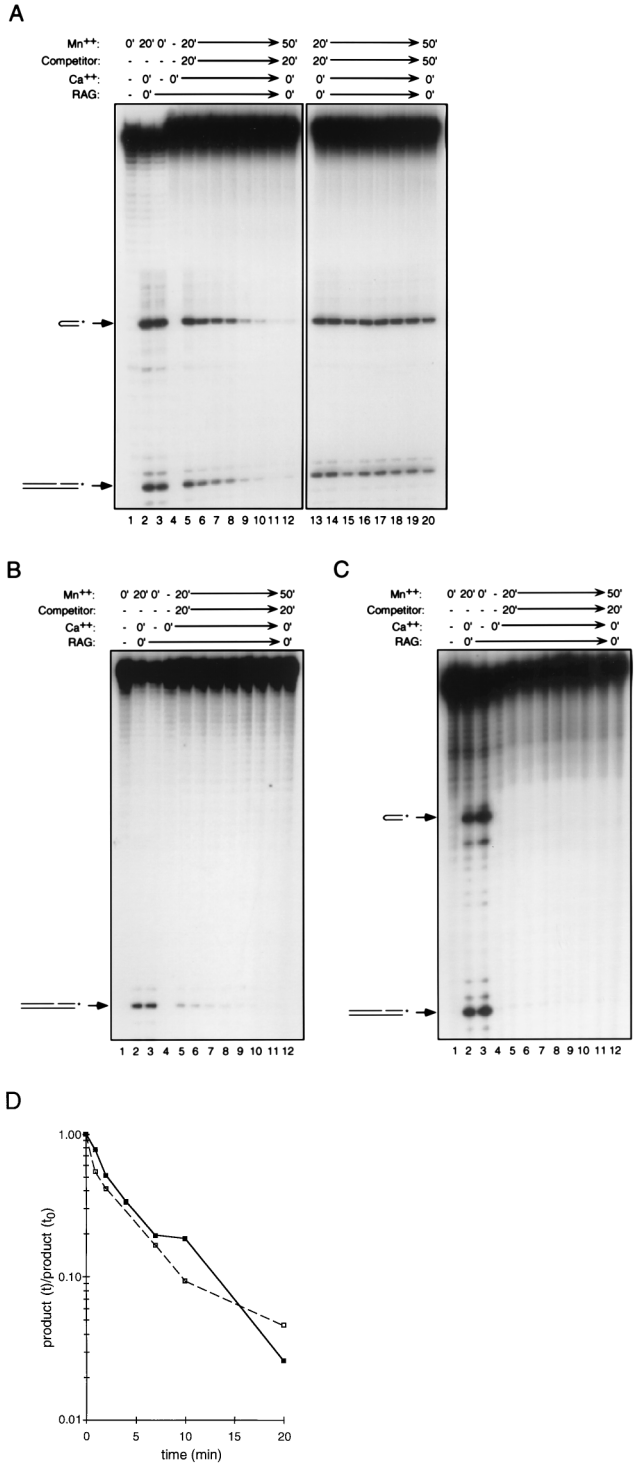


FIG. 6. Stability of complexes between RAG proteins and wild-type or mutant substrates. (A) Radiolabeled wild-type substrate was incubated in the absence (lane 1) or presence (lanes 2 to 20) of RAG-1 and RAG-2 at 37°C. Incubation was carried out in the presence (lanes 2 and 4 to 20) or absence (lanes 1 and 3) of $Ca^{2+}$. $Mn^{2+}$ was added at initial time (lanes 1 and 3), 20 min (lanes 2, 5, and 13), 21 min (lanes 6 and 14), 22 min (lanes 7 and 15), 24 min (lanes 8 and 16), 27 min (lanes 9 and 17), 30 min (lanes 10 and 18), 40 min (lanes 11 and 19), or 50 min (lanes 12 and 20). $Mn^{2+}$ was omitted from the reaction in lane 4. Reactions were supplemented with 1,000-fold excess unlabeled wild-type competitor at 20 (lanes 5 to 13), 21 (lane 14), 22 (lane 15), 24 (lane 16), 27 (lane 17), 30 (lane 18), 40 (lane 19), or 50 (lane 20) min. The reaction mixture in lane 4 was incubated for 2 h; all other reactions were allowed to proceed for 2 h after addition of $Mn^{2+}$. Products were resolved by electrophoresis and detected by autoradiography. Positions of radiolabeled nicked and hairpin products are indicated at left. In this experiment, 19% of input substrate was converted to nicked or hairpin product under standard conditions (lane 3), as assessed by phosphorimager analysis. (B and C) Radiolabeled mutant substrate C17A (B) or A41C (C) was incubated in the absence (lanes 1) or presence (lanes 2 to 12) of RAG-1 and RAG-2 at 37°C. Incubation was carried out in the presence (lanes 2 and 4 to 12) or absence (lanes 1 and 3) of $Ca^{2+}$. $Mn^{2+}$ was added at initial time (lanes 1 and 3), 20 min (lanes 2 and 5), 21 min (lanes 6), 22 min (lanes 7), 24 min (lanes 8), 27 min (lanes 9), 30 min (lanes 10), 40 min (lanes 11), or 50 min (lanes 12). $Mn^{2+}$ was omitted from the reactions in lanes 4. Reactions were supplemented with 1,000-fold excess unlabeled wild-type competitor at 20 min (lanes 5 to 12). The reaction mixture in lanes 4 was incubated for 2 h; all other reactions were allowed to proceed for 2 h after addition of $Mn^{2+}$. Products were analyzed as for panel A. Positions of radiolabeled nicked and hairpin products are indicated at left. (D) Kinetics of RAG-DNA dissociation. The total amount of radiolabeled wild-type (nicked and hairpin [open squares]) or C17A mutant (nicked [filled squares]) product obtained after incubation with competitor DNA for various times was quantitated by densitometry from data in panels A and B. *x* axis, lag time between addition of competitor DNA and addition of $Mn^{2+}$; *y* axis, product obtained from reactions with lag time *t*, normalized to the amount of product obtained from the reaction with zero lag time.

substrate. At 2 min, an aliquot was removed for assay; 1,000-fold excess unlabeled substrate was added to the remainder of the reaction mixture and incubation was continued for 2 h. As expected, the nicked product predominated at 2 min (Fig. 7, lane 13) and the bulk of this material was converted to hairpin product upon further incubation in the presence of excess unlabeled competitor (Fig. 7, lane 14). No cleavage product was detectable if unlabeled competitor was added at 0 min (Fig. 7, lane 15). We conclude that the separately purified RAG proteins are capable of forming a stable complex with the labeled DNA substrate.

Results of surface plasmon resonance and one-hybrid assays have suggested that RAG-1 and substrate DNA form a functional complex, to which RAG-2 is subsequently recruited (4, 25). The sequestration assay was used to test this model. Labeled substrate DNA was incubated with RAG-1 for 2 min, followed by simultaneous addition of RAG-2 and 1,000-fold excess unlabeled competitor. No cleavage product was detectable upon further incubation for 2 h (Fig. 7, lane 4; compare to Fig. 7, lane 14). Similarly, when RAG-2 was incubated alone with labeled substrate for 2 min, followed by addition of RAG-1 and unlabeled competitor, no cleavage product was detectable at 2 h (Fig. 7, lane 8). When competitor was omitted, cleavage products were readily detected, indicating that RAG-1 and RAG-2 remain active during the preincubation period (Fig. 7, lanes 5 and 9). Taken together, these results are consistent with a requirement for both RAG-1 and RAG-2 in formation of a functional complex with RSS-containing DNA and argue against the proposal that RAG-1 alone associates with substrate DNA prior to recruitment of RAG-2.

## DISCUSSION

Sequence-specific recognition of substrate DNA by the RAG proteins is implicit in their ability to catalyze RSS-dependent DNA cleavage. By exploiting the protracted kinetics of RAG-mediated transesterification and the divalent cation dependence of DNA cleavage by the RAG proteins, we have examined the association of RAG proteins with substrate DNA during the course of the two-step cleavage reaction. These observations permit us to draw several inferences. First, a substrate DNA fragment bearing a single RSS is capable of forming a stable complex with RAG proteins in solution, as revealed by sequestration of radiolabeled substrate DNA from an unlabeled competitor under conditions that support nicking and transesterification. Second, when $Ca^{2+}$ is used in place of

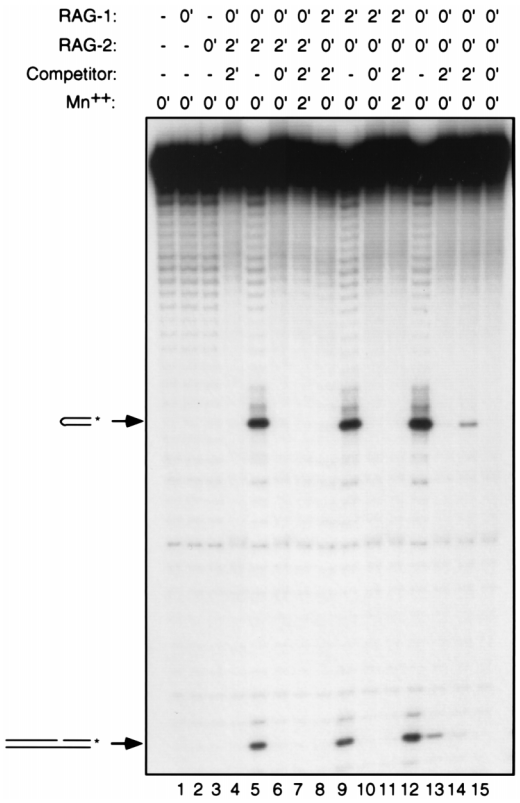


FIG. 7. RAG-1 and RAG-2 dependence of complex formation with substrate DNA. Radiolabeled wild-type substrate was incubated at 37°C in the absence of RAG-1 (lanes 1 and 3), in the absence of RAG-2 (lanes 1 and 2), or with combinations of RAG-1 and RAG-2 (lanes 4 to 15), added at initial time (0′) or 2 min (2′) as indicated. Reactions were supplemented with 1,000-fold excess unlabeled competitor DNA and $Mn^{2+}$ at initial time (0′) or 2 min (2′) as indicated above. Competitor DNA was omitted from reactions marked with a minus sign. All reactions were allowed to proceed for 2 h; products were resolved by electrophoresis and detected by autoradiography. Positions of radiolabeled nicked and hairpin products are indicated at left. In this experiment, 10% of substrate DNA was converted to product (nicked plus hairpin) under standard conditions (lane 12), as assessed by phosphorimager analysis.

$Mn^{2+}$, a stable RAG-DNA complex is formed in the absence of nicking. This result indicates that intact substrate DNA associates stably with one or both RAG proteins prior to strand scission and identifies complex formation as a step in the RAG-mediated cleavage reaction distinct from nicking and transesterification. Third, radiolabeled substrate DNA fails to exchange with unlabeled competitor at any point in the reaction subsequent to formation of this initial complex; we conclude, therefore, that substrate DNA remains associated with RAG during strand scission and transesterification.

Mutations at specific positions within the RSS heptamer and nonamer motifs impair V(D)J recombination in vivo (8). Two classes of RSS mutations have been recognized on the basis of their effects on cleavage in vitro (references 3 and 18 and this communication). One class, which includes the A41C nonamer mutation, is associated with coordinate impairment of RAG-mediated nicking and hairpin formation in vitro. In principle, such mutations could exert their effects by decreasing the affinity of substrate DNA for RAG proteins or through direct interference with catalysis. A second class, typified by the C17A heptamer mutation, is associated with selective impairment of transesterification in vitro. Using the sequestration assay, we assessed the effects of these mutations on the stability of RAG-substrate complexes. The complex between RAG proteins and wild-type substrate was observed to decay with a half-life of about 3 min. Similar dissociation kinetics were observed for a complex between RAG proteins and a substrate bearing the C17A heptamer mutation. In contrast, complexes between the RAG proteins and the A41C mutant substrate were highly unstable, as evidenced by rapid exchange of substrate in the sequestration assay. The effect of the A41C nonamer mutation on DNA cleavage in vitro and V(D)J recombination in vivo is therefore likely to reflect impaired binding of substrate DNA to RAG proteins.

An electrophoretic mobility shift assay for RAG-DNA complexes has been recently described (9). Formation of the mobility shift complex was found to require intact heptamer and nonamer elements, consistent with the requirements for V(D)J recombination and RAG-mediated DNA cleavage. In the electrophoretic assay, formation of a RAG-DNA complex was abolished by complete mutation of the heptamer but only modestly reduced by mutation of the first three or last four heptamer positions (corresponding to residues 17 to 19 and 20 to 23 of the substrate used in the present study). We have shown here that a heptamer point mutation at residue C17, sufficient to abrogate transesterification, has little or no effect on the stability of RAG association with intact DNA. This result is consistent with the relatively modest effect of partial heptamer mutation as detected by mobility shift; the insensitivity of substrate sequestration to mutation of C17 may simply reflect a difference in the extent of heptamer mutation in the two experiments.

From the results of one-hybrid (4) and surface plasmon resonance (25) assays, it has been inferred that RAG-1 interacts with the RSS nonamer element in the absence of RAG-2 but has been unclear whether this interaction represents formation of a functional reaction intermediate. The sequestration assay differs substantially from the one-hybrid and surface plasmon resonance assays in that it monitors continuing RAG-DNA association in the course of the in vitro cleavage reaction. By employing active RAG-1 and RAG-2 proteins, expressed and purified separately, it was possible to test the ability of RAG-1 or RAG-2 to interact independently with substrate DNA. RAG-1 or RAG-2 alone was unable to sequester substrate DNA from exchange with competitor. These observations are consistent with the requirement for both RAG-1 and RAG-2 in the electrophoretic mobility shift assay for RSS-dependent DNA binding (9) and are in conflict with the hypothesis that formation of a stable RSS–RAG-1 complex precedes recruitment of RAG-2. Another formal possibility, that RAG-1 and RAG-2 are each able to bind substrate DNA independently, is also consistent with the results of the sequestration assay, but we consider this unlikely, given that interactions between DNA and RAG-2 alone have not been observed in any assay system.

It has been possible to detect an association between DNA and RAG-1 alone by electrophoretic mobility shift assays performed under conditions of lowered temperature; although this interaction is insensitive to mutation of the RSS heptamer or nonamer, it may correspond to the DNA–RAG-1 interaction observed in one-hybrid and surface plasmon resonance assays (25a). The sequestration assay and the mobility shift assay of Hiom and Gellert (9) remain at variance with results obtained by surface plasmon resonance and one-hybrid analysis. This discrepancy may be explained if the interaction between RAG-1 alone and substrate DNA is not sufficiently stable to be detected by sequestration or by mobility shift at room temperature. Alternatively or in addition, formation of RAG-1–DNA complexes may represent an unproductive side

reaction, which would remain undetected in the sequestration assay. Taken together, our results and those obtained by mobility shift analysis are most consistent with the interpretation that RAG-1 and RAG-2 cooperate in recognition of the RSS to form a long-lived, functional DNA-protein complex that is maintained through strand scission.

## ACKNOWLEDGMENTS

We thank Dik van Gent, Fraser McBlane, Martin Gellert, and Joanne Hesse for reagents and advice; Dominic Dordai for technical assistance; and Clark Riley and the staff of the HHMI Biopolymers Facility at Johns Hopkins for synthesis of oligonucleotides.

This work was supported by the Howard Hughes Medical Institute and by grant CA16519 from the National Cancer Institute.

## REFERENCES

1. **Blunt, T., N. Finnie, G. Taccioli, G. Smith, J. Demengeot, T. Gottlieb, R. Mizuta, A. Varghese, F. Alt, P. Jeggo, and S. Jackson.** 1995. Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. Cell **80:**813–823.
2. **Boubnov, N. V., and D. T. Weaver.** 1995. *scid* cells are deficient in Ku and replication protein A phosphorylation by the DNA-dependent protein kinase. Mol. Cell. Biol. **15:**5700–5706.
3. **Cuomo, C. A., C. L. Mundy, and M. A. Oettinger.** 1996. DNA sequence and structure requirements for cleavage of V(D)J recombination signal sequences. Mol. Cell. Biol. **16:**5683–5690.
4. **Difilippantonio, M. J., C. J. McMahan, Q. M. Eastman, E. Spanopoulou, and D. G. Schatz.** 1996. RAG1 mediates signal sequence recognition and recruitment of RAG2 in V(D)J recombination. Cell **87:**253–262.
5. **Eastman, Q. M., T. M. J. Leu, and D. G. Schatz.** 1996. Initiation of V(D)J recombination *in vitro* obeying the 12/23 rule. Nature **380:**85–88.
6. **Getts, R. C., and T. D. Stamato.** 1994. Absence of a Ku-like DNA end binding activity in the xrs double-strand DNA repair-deficient mutant. J. Biol. Chem. **269:**15981–15984.
7. **Hartley, K. O., D. Gall, G. C. M. Smith, H. Zhang, N. Divecha, M. A. Connelly, A. Admon, S. P. Lees-Miller, C. W. Anderson, and S. P. Jackson.** 1995. DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. Cell **82:**849–856.
8. **Hesse, J. E., M. R. Lieber, K. Mizuuchi, and M. Gellert.** 1989. V(D)J recombination: a functional definition of the joining signals. Genes Dev. **3:**1053–1061.
9. **Hiom, K., and M. Gellert.** 1997. A stable RAG1-RAG2-DNA complex that is active in V(D)J cleavage. Cell **88:**65–72.
10. **Kirchgessner, C., C. Patil, J. Evans, C. Cuomo, L. Fried, T. Carter, M. Oettinger, and J. Brown.** 1995. DNA-dependent kinase (p350) as a candidate gene for the murine scid defect. Science **267:**1178–1183.
11. **Lee, S. E., C. R. Pulaski, D. M. He, D. M. Benjamin, M. Voss, J. Um, and E. A. Hendrickson.** 1995. Isolation of mammalian cell mutants that are X-ray sensitive, impaired in DNA double-strand break repair and defective for V(D)J recombination. Mutat. Res. **336:**279–291.
12. **Lewis, S.** 1994. The mechanism of V(D)J joining: lessons from molecular, immunological and comparative analyses. Adv. Immunol. **56:**27–150.
13. **Li, Z. Y., T. Otevrel, Y. J. Gao, H. L. Cheng, B. Seed, T. D. Stamato, G. E. Taccioli, and F. W. Alt.** 1995. The XRCC4 gene encodes a novel protein involved in DNA double-strand break repair and V(D)J recombination. Cell **83:**1079–1089.
14. **McBlane, J. F., D. C. van Gent, D. A. Ramsden, C. Romeo, C. A. Cuomo, M. Gellert, and M. A. Oettinger.** 1995. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell **83:**387–395.
15. **Oettinger, M. A., D. G. Schatz, C. Gorka, and D. Baltimore.** 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science **248:**1517–1523.
16. **Pergola, F., M. Z. Zdzienicka, and M. R. Lieber.** 1993. V(D)J recombination in mammalian cell mutants defective in DNA double-strand break repair. Mol. Cell. Biol. **13:**3464–3471.
17. **Peterson, S. R., A. Kurimasa, M. Oshimura, W. S. Dynan, E. M. Bradbury, and D. J. Chen.** 1995. Loss of the catalytic subunit of the DNA-dependent protein kinase in DNA double-strand-break-repair mutant mammalian cells. Proc. Natl. Acad. Sci. USA **92:**3171–3174.
18. **Ramsden, D. A., J. F. McBlane, D. C. van Gent, and M. Gellert.** 1996. Distinct DNA sequence and structure requirements for the two steps of V(D)J recombination signal cleavage. EMBO J. **15:**3197–3206.
19. **Rathmell, W. K., and G. Chu.** 1994. Involvement of the Ku autoantigen in the cellular response to DNA double-strand breaks. Proc. Natl. Acad. Sci. USA **91:**7623–7627.
20. **Roth, D., C. Zhu, and M. Gellert.** 1993. Characterization of broken DNA molecules associated with V(D)J recombination. Proc. Natl. Acad. Sci. USA **90:**10788–10792.
21. **Roth, D. B., J. P. Menetski, P. B. Nakajima, M. J. Bosma, and M. Gellert.** 1992. V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes. Cell **70:**983–991.
22. **Schatz, D. G., M. A. Oettinger, and D. Baltimore.** 1989. The V(D)J recombination activating gene, RAG-1. Cell **59:**1035–1048.
23. **Schlissel, M., A. Constantinescu, T. Morrow, M. Baxter, and A. Peng.** 1993. Double-strand signal sequence breaks in V(D)J recombination are blunt, 5′-phosphorylated, RAG-dependent, and cell cycle regulated. Genes Dev. **7:**2520–2532.
24. **Smider, V., W. K. Rathmell, M. R. Lieber, and G. Chu.** 1994. Restoration of x-ray resistance and V(D)J recombination in mutant cells by ku cdna. Science **14:**288–291.
25. **Spanopoulou, E., F. Zaitseva, F.-H. Wang, S. Santagata, D. Baltimore, and G. Panayotou.** 1996. The homeodomain region of Rag-1 reveals the parallel mechanisms of bacterial and V(D)J recombination. Cell **87:**263–276.

25a. **Swanson, P., and S. Desiderio.** Unpublished data.

26. **Taccioli, G. E., T. M. Gottlieb, T. Blunt, A. Priestley, J. Demengeot, R. Mizuta, A. R. Lehmann, F. W. Alt, S. P. Jackson, and P. A. Jeggo.** 1994. Ku80—product of the XRCC5 gene and its role in DNA repair and V(D)J recombination. Science **265:**1442–1445.
27. **Taccioli, G. E., G. Rathbun, E. Oltz, T. Stamato, P. A. Jeggo, and F. W. Alt.** 1993. Impairment of V(D)J recombination in double-strand break repair mutants. Science **260:**207–210.
28. **van Gent, D. C., J. F. McBlane, D. A. Ramsden, M. J. Sadofsky, J. E. Hesse, and M. Gellert.** 1995. Initiation of V(D)J recombination in a cell-free system. Cell **81:**925–934.
29. **van Gent, D. C., K. Mizuuchi, and M. Gellert.** 1996. Similarities between initiation of V(D)J recombination and retroviral integration. Science **271:** 1592–1594.
30. **van Gent, D. C., D. A. Ramsden, and M. Gellert.** 1996. The RAG1 and RAG2 proteins establish the 12/23 rule in V(D)J recombination. Cell **85:** 107–113.
31. **Zdzienicka, M. Z.** 1995. Mammalian mutants defective in the response to ionizing radiation-induced DNA damage. Mutat. Res. **336:**203–213.