The Effect of Me$_{2}^{+}$ Cofactors at the Initial Stages of V(D)J Recombination

(Received for publication, March 5, 1998, and in revised form, April 21, 1998)

Sandro Santagata, Vassilis Aidinis, and Eugenia Spanopoulou†

From the Mount Sinai School of Medicine, Howard Hughes Medical Institute, Ruttenberg Cancer Center, New York, New York 10029

V(D)J site-specific recombination mediates the somatic assembly of the antigen receptor gene segments. This process is initiated by the recombination activating proteins RAG1 and RAG2, which recognize the recombination signal sequences (RSS) and cleave the DNA at the coding/RSS junction. In this study, we show that RAG1 and RAG2 have the ability to directly interact in solution before binding to the DNA. RAG1 forms a homodimer, which leads to the appearance of two distinct RAG1-RAG2 complexes bound to DNA. To investigate the properties of the two RAG1-RAG2 complexes in the presence of different Me$_{2}^{+}$ cofactors, we established an in vitro Mg$_{2}^{+}$-based cleavage reaction on a single RSS. Using this system, we found that Mg$_{2}^{+}$ confers a specific pattern of DNA binding and cleavage. In contrast, Mn$_{2}^{+}$ allows aberrant binding of RAG1-RAG2 to single-stranded RSS and permits cleavage independent of binding to the nonamer. To determine the contribution of Me$_{2}^{+}$ ions at the early stages of V(D)J recombination, we analyzed specific DNA recognition and cleavage by RAG1-RAG2 on phosphorothioate substrates. These experiments revealed that Me$_{2}^{+}$ ions directly coordinate the binding of RAG1-RAG2 to the RSS DNA.

Diversity of the immunoglobulin and T cell receptor repertoire is generated by site-specific rearrangement of V, D, and J gene segments in a process termed V(D)J recombination (1). Each antigen receptor coding segment is flanked by highly conserved recombination signal sequences (RSS), which direct the site of reciprocal recombination (2). The consensus RSS consists of a heptamer sequence (CACAGTG) directly adjacent to the coding element and an A/T-rich nonamer site (ACAAAACCC) separated from the heptamer by a spacer of either 12 or 23 base pairs (3–5). Recombination typically occurs between a 12-base pair and a 23-base pair RSS, a phenomenon referred to as the 12/23 rule (1, 4). V(D)J recombination is initiated by two lymphoid-specific proteins RAG1 and RAG2 (6, 7) that bind to the RSS with specificity (8–10). Recognition of the nonamer motif is mediated by a region of RAG1 that exhibits distinct homology to homeodomain proteins (8, 9, 11) whereas the heptamer DNA binding domain of RAG1-RAG2 remains to be identified. Upon binding, RAG1, RAG2, and other as yet unidentified cellular activities mediate synaptic complex formation that brings together a pair of 12RSS and 23RSS signals (12–14). Subsequently, RAG1 and RAG2 cleave the DNA at the junction between the coding/heptamer sequences (15–17). After the generation of double-stranded broken ends by RAG1-RAG2, several ubiquitously expressed DNA repair proteins are engaged in the reaction including Ku70, Ku80, and DNA-PK (18, 19). Null mutations in RAG1, RAG2, or any of the three DNA repair genes lead to immunodeficiency, demonstrating that all of the above activities are indispensable for V(D)J recombination to occur (20–28).

The initial stages of V(D)J recombination have been successfully reproduced in in vitro assays. Using purified RAG proteins and an oligonucleotide substrate with a single RSS, it was demonstrated that RAG1 and RAG2 mediate site-specific cleavage in a two-step process (15, 16). First, a nick is introduced between the coding flank and the heptamer sequence. Second, a double strand break is generated by the liberated 3' hydroxyl group, which serves as a nucleophile in a direct S$_{N}$2-type transesterification reaction of the lower strand (15–17). The cleavage intermediates are a covalently sealed hairpin coding end and a 5' phosphorylated blunt signal end, as also observed in vivo (29–32). Upon cleavage of the 12/23 pair of signals, RAG1 and RAG2 remain stably bound to the signal ends (33).

A notable aspect of the in vitro cleavage system is the differential activity of RAG1-RAG2 in the presence of different Me$_{2}^{+}$ cofactors. It was shown previously that only Mn$_{2}^{+}$ mediates efficient cleavage on a single RSS, whereas coupled 12/23 cleavage happens only in the presence of Mg$_{2}^{+}$ (12–16). In this study, we report that Mg$_{2}^{+}$ does allow efficient cleavage on a single RSS whereas Mn$_{2}^{+}$ accelerates the second phase of the reaction, hairpin formation. The two metals enforce different kinetics of the V(D)J cleavage reaction and differential DNA binding properties of the RAG1-RAG2 complexes. Using phosphorothioate substrates, we find that the binding of RAG1-RAG2 to the RSS is directly coordinated by the Me$_{2}^{+}$ cofactor.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Glutathione S-transferase (GST) fusion recombinant forms of the RAG1 and RAG2 active cores (GST-RAG1 1AN, amino acids 330–1040; GST-RAG2 2AC, amino acids 1–383) (34–37) were overexpressed in 293T cells and purified as described previously (8). Proteins were dialyzed in storage buffer (25 mM Tris (pH 8.0), 150 mM KCl, 2 mM DTT, and 20% glycerol) and quantified by Coomassie Blue staining following SDS-polyacrylamide gel electro-

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: RSS, recombination signal sequence; GST, glutathione S-transferase; DTT, dithiothreitol; aa, amino acid(s); MOPS, 4-morpholinopropanesulfonic acid; ds, double-stranded; ss, single-stranded.
phosphorothioated oligonucleotides occurring nucleotides as determined by data base analysis of multiple loci from numerous species (5). Phosphorothioated oligonucleotides maintained the 12RSS nucleotide sequence but had nonamer mutations. The substrate ssRSS was generated by hybridizing end-labeled ICP(1–16), unlabeled 12RSS-lower strand. dsRSS was created by annealing end-labeled ICP(1–16), unlabeled 12RSS-lower strand. The spacers for substrates are as follows.

**DNA Cleavage Substrates**—The upper strand sequences of cleavage substrates are as follows.

**12RSS-up:**
5'-ACCCGTCGACGTCAGACACATAGCACGAGCTCGTCATCTCTTGTGGGCAGCTCGATCTCTTT-

**23RSS-up:**
5'-ACCCGTCGACGTCAGACACATAGCACGAGCTCGTCATCTCTTGTGGGCAGCTCGATCTCTTT-

**12RSS(7mut)up:**
5'-ACCCGTCGACGTCAGACACATAGCACGAGCTCGTCATCTCTTGTGGGCAGCTCGATCTCTTT-

**12RSS(9mut)up:**
5'-ACCCGTCGACGTCAGACACATAGCACGAGCTCGTCATCTCTTGTGGGCAGCTCGATCTCTTT-

**ICP(1–16):**
5'-ACCCGTCGACGTCAGACACATAGCACGAGCTCGTCATCTCTTGTGGGCAGCTCGATCTCTTT-

**ICP(17–53):**
5'-ACCCGTCGACGTCAGACACATAGCACGAGCTCGTCATCTCTTGTGGGCAGCTCGATCTCTTT-

Standard cleavage reactions employed 32P end-labeled upper strands annealed to unlabeled, complementary lower strand oligonucleotides. The substrate ssRSS was generated by hybridizing end-labeled ICP(1–16) to the unlabeled 12RSS-lower strand. dsRSS was created by annealing end-labeled ICP(1–16), unlabeled 5' phosphorothioated ICP(17–53) and unlabeled 12RSS-lower strand. The spacers for substrate 12RSS and 23RSS were optimized to include the most frequently occurring nucleotides. The substrate 12RSS and 23RSS were optimized to include the most frequently occurring nucleotides as determined by data base analysis of multiple loci from numerous species (5). Phosphorothioated oligonucleotides (Genelink) maintained the 12RSS nucleotide sequence but had nonbridging oxygen replaced by sulfurs at the indicated positions.

**Electrophoretic Mobility Shift Assays**—Conditions were based on the previously published protocol (10). 50 ng of each protein were incubated for 10 min at 30 °C with 0.01–0.05 pmol of 32P end-labeled probe in 25 mM MOPS-KOH (pH 7.0), 10 mM Tris-HCl (pH 7.0), 120 mM potassium acetate, 18 mM KCl, 10% Me2SO, 2.2 mM DTT, 0.5 μM nonspecific primer (5'-ACCCGTCGACGTCAGACACATAGCACGAGCTCGTCATCTCTTGTGGGCAGCTCGATCTCTTT-GTCG-3'), 20% Me2SO, and 120 mM potassium acetate. Cross-linking was achieved by glutaraldehyde (final concentration 0.1%, v/v) treatment for 10 min at 30 °C. Complexes were resolved on a 4.0% native polyacrylamide gel.

**RESULTS**

Detection of RAG1–RAG2 Complexes in Solution—The ability of RAG1 and RAG2 to bind and cleave the DNA is dependent on the simultaneous presence of both proteins (15, 16, 31). This implies that RAG1 and RAG2 might interact either in the presence or absence of the RSS DNA. Although in vivo the two proteins have been found in the same complex (38, 39), in vitro RAG1 and RAG2 have only been observed together in the stable complex formed upon cleavage of the DNA (33). The ability of RAG1 and RAG2 to interact in solution was probed in mixing experiments using 35S-labeled proteins (Fig. 1). After incubation, protein complexes were cross-linked with glutaraldehyde and detected by denaturing gel electrophoresis. 35S-Labeled proteins were transiently expressed in 293T cells. The transfected cells were methionine-deprived for 30 min and subsequently incubated in the presence of [35S]methionine for 2 h prior to harvest.

**In Vitro Cleavage Reactions**—Standard reaction conditions were modifications of the reactions developed by McBlane et al. (16). 50 ng of each protein, RAG1 and RAG2, were incubated with 0.01–0.05 pmol of 32P end-labeled cleavage substrate in 25 mM MOPS-KOH (pH 7.0), 10 mM Tris-HCl (pH 7.0), 95 mM potassium chloride, 2.2 mM DTT, 4% glycerol (no Me2SO conditions), 1 mM MgCl2, or 1 mM MnCl2 in a 20-μl final volume. Reactions were incubated at 37 °C for the indicated time points. 10% Me2SO cleavage reactions were conducted in: 25 mM MOPS-KOH (pH 7.0), 5 mM Tris-HCl (pH 7.0), 120 mM potassium acetate, 18 mM KCl, 10% Me2SO, 2.2 mM DTT, 0.5 μM nonspecific single-stranded DNA with 50 ng of each protein and 0.01–0.05 pmol of 32P end-labeled probe in a final volume of 20 μl. Reactions were stopped by the addition of 0.1% SDS and loading buffer. Samples were resolved on 16% polyacrylamide denaturing gels.

**DNA Cleavage Substrates**—The upper strand sequences of cleavage substrates are as follows.

**12RSS-up:**
5'-ACCGCTCGAGCTCATCAGCTTGCCTGTGGGCAGCTCGATCTCTTT-

**23RSS-up:**
5'-ACCGCTCGAGCTCATCAGCTTGCCTGTGGGCAGCTCGATCTCTTT-

**12RSS(7mut)up:**
5'-ACCGCTCGAGCTCATCAGCTTGCCTGTGGGCAGCTCGATCTCTTT-

**12RSS(9mut)up:**
5'-ACCGCTCGAGCTCATCAGCTTGCCTGTGGGCAGCTCGATCTCTTT-

**ICP(1–16):**
5'-ACCGCTCGAGCTCATCAGCTTGCCTGTGGGCAGCTCGATCTCTTT-

**ICP(17–53):**
5'-ACCGCTCGAGCTCATCAGCTTGCCTGTGGGCAGCTCGATCTCTTT-

**SEQUENCES 1–6**

**A**

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

**J**

**K**

**L**

**M**

**N**

**O**

**P**

**Q**

**R**

**S**

**T**

**U**

**V**

**W**

**X**

**Y**

**Z**

FIG. 1. RAG1 and RAG2 interactions in solution. RAG1 and RAG2 were purified as 35S-labeled proteins, mixed together, cross-linked with glutaraldehyde, and resolved on a 5% native polyacrylamide gel. Lanes 1 and 2 represent purified 35S-labeled RAG2 and RAG1, respectively, after cross-linking. Lane 3 contains 35S-labeled RAG1 and RAG2, lane 4 contains 35S-RAG2/RAG1, lane 5 contains 35S-RAG1-RAG2, and lane 6 contains 35S-RAG1-RAG2 in the presence of the 12RSS probe. In lane 7, RAG1 and RAG2 were mixed with purified 12RSS probe.
dehyde and resolved on a native polyacrylamide gel. RAG2 alone produces one complex that migrates at the apparent molecular weight for the protein (Fig. 1, lane 1). However, RAG1 produced two complexes that corresponded to a monomeric and dimeric form of the protein (Fig. 1, lane 2). RAG1 homodimerization is mediated by the homeodomain part of the protein.2 Incubation of RAG1 with RAG2 produces two new complexes that contain both proteins (Fig. 1, lanes 3–5). The observed RAG1-RAG2 interaction is independent of the presence of RSS DNA (Fig. 1, compare lanes 5 and 6). In addition, formation of the two complexes is specific to the RAG1-RAG2 interaction because addition of GST protein does not change the stoichiometry of the complexes (Fig. 1, lane 7).

**Mn**<sup>2+</sup> Dependence of RAG1-RAG2 Function

Me<sup>2+</sup> Dependence of RAG1-RAG2 Function

![Fig. 3. Mn<sup>2+</sup> but not Mg<sup>2+</sup> allows DNA binding and transesterification on single-stranded RSS.](image)

**A** single-stranded RSS cleavage substrates (ssRSS) were generated by annealing <sup>32</sup>P 5′ end-labeled upper strand coding nucleotides 1–16 with the full-length lower strand (12RSS-lo). The prenicked double-stranded RSS cleavage substrate (dsRSS) was synthesized from <sup>32</sup>P 5′ end-labeled ICP(1–16), unlabeled 5′ phosphorylated ICP(17–53), and 12RSS-lo. B, cleavage assays. ssRSS was incubated with RAG1 and RAG2 in Mg<sup>2+</sup> (lane 2) and Mn<sup>2+</sup> (lane 3). H<sup>S</sup> represents aberrant hairpin production stemming from inappropriate nucleophilic attack at the 5′ end of the lower strand heptamer (40). dsRSS was incubated with RAG1 and RAG2 in Mg<sup>2+</sup> (lane 3) and Mn<sup>2+</sup> (lane 4). C, binding of RAG1-RAG2 to dsRSS (lanes 1 and 4), ssRSS (lanes 2 and 5), and wild type 12RSS (lanes 3 and 6) in either Mn<sup>2+</sup> (lanes 1–3) or Mg<sup>2+</sup> (lanes 4–6).

**Mn**<sup>2+</sup> Relaxes DNA Binding and Cleavage Specificity by RAG1-RAG2—To test the effect of Mn<sup>2+</sup> cofactors at the initial stages of V(D)J recombination, cleavage reactions were performed in the presence of either Mn<sup>2+</sup> or Mg<sup>2+</sup> (Fig. 2). The active cores of purified RAG1 (aa 330–1040) and RAG2 (aa 1–383) were incubated with <sup>32</sup>P-labeled 12RSS or 23RSS substrates. Surprisingly, in contrast to previous reports (12, 16), we found substantial cleavage of a single RSS substrate in the presence of Mg<sup>2+</sup> (Fig. 2A, lanes 2 and 5), whereas Mn<sup>2+</sup> accelerated hairpin formation by severalfold (Fig. 2A, lanes 3 and 6). Identical results were obtained on oligonucleotide templates used in the previous reports (12, 16, 40, 41) (data not shown) indicating that these differences are not due to the DNA composition of the substrates. One major factor that could account for the observed differences is the use of RAG1 and RAG2 proteins expressed in mammalian cells that may carry posttranslational modifications required for their cleavage activity.

The availability of an *in vitro* assay that allows single-site cleavage in the presence of Mg<sup>2+</sup> prompted us to study the effect of Mn<sup>2+</sup> on the specificity of DNA binding and cleavage by RAG1-RAG2. To that extent, specificity of the cleavage reaction was tested by analyzing mutations of nucleotides that have

---

2 V. Aidinis and E. Spanopoulou, manuscript in preparation.
been shown to be critical for the function of the heptamer and nonamer elements of the RSS (40, 41) (Fig. 2B). Mutation of the first two residues of the heptamer abolish hairpin formation as reported previously (40, 41). However, RAG1 z RAG2 can mediate nicking of the mutant heptamer substrate in the presence of Mn$^{2+}$ but not in Mg$^{2+}$ (Fig. 2B, lanes 2 and 5). Similarly, mutations in the nonamer element have a profound effect with Mg$^{2+}$ as a cofactor, whereas Mn$^{2+}$ permits efficient nick/hairpin formation on the mutant nonamer substrate (Fig. 2B, lanes 3 and 6).

The differential effect of the two metals on the cleavage reaction is also reflected on the DNA binding specificity of RAG1 z RAG2. Gel retardation assays with purified RAG1 z RAG2 and a single RSS probe show two complexes that both contain RAG1 and RAG2 interacting in solution (Fig. 2). In Mn$^{2+}$, both complexes bind avidly to the DNA despite mutations in the heptamer or nonamer motifs (Fig. 2C, lanes 4–6). In contrast, Mg$^{2+}$ reduces binding of RAG1-RAG2 to the heptamer mutant by 2-fold and to the nonamer mutant by 5-fold (Fig. 2C, lanes 1–3).

Mn$^{2+}$ but Not Mg$^{2+}$ Allows DNA Recognition and Cleavage of a Single-stranded RSS—It was shown previously that in the presence of Mn$^{2+}$, RAG1 and RAG2 can form hairpins utilizing a substrate with a double-stranded coding flank and a single-stranded lower strand RSS (40, 41). This finding suggested that RAG1 and RAG2 unwind the RSS providing the DNA distortion required for hairpin formation. The question arose however, as to how RAG1 and RAG2, unlike other DNA-binding proteins, can recognize ssDNA by establishing specific contacts only with nucleotides of the lower strand. We thus examined the ability of RAG1-RAG2 to mediate the trans-esterification reaction on a single-stranded RSS substrate (Fig. 3A) in the presence of either Mg$^{2+}$ or Mn$^{2+}$. Fig. 3B shows that RAG1 and RAG2 are unable to mediate hairpin formation in the presence of Mg$^{2+}$ but they could effectively do so when Mn$^{2+}$ was used as the cofactor. When the upper strand of the RSS was replaced to recreate a double-stranded substrate with a nick remaining in the upper strand (Fig. 3B, dsRSS), the capacity of RAG1-RAG2 to mediate hairpin formation was reconstituted irrespective of the divalent cation employed. Mobility shift assays were performed to assess whether the basis of this differential usage of a single-stranded RSS resulted from modified DNA recognition in Mn$^{2+}$ or Mg$^{2+}$. Fig. 3C reveals essentially equivalent levels of Mn$^{2+}$-induced DNA recognition of templates dsRSS, ssRSS, and 12RSS (wild type). However, in Mg$^{2+}$, RAG1 and RAG2 can form a stable ternary complex only with the wild type 12RSS and the nicked dsRSS but are unable to recognize ssRSS.

ssRSS Recognition and Catalytic Activity of RAG1 Is Mediated by aa 456–1040—Given the ability of RAG1-RAG2 to mediate nonamer-independent cleavage and ssRSS recognition when assayed in Mn$^{2+}$ (Figs. 2B and 3B and C), RAG1 456–1040, which lacks the homeodomain region of the protein, was assayed for its ability to recognize the heptamer and mediate hairpin formation on the ssRSS substrate. As shown in Fig. 4A, in the presence of Mn$^{2+}$ RAG1Δ456/RAG2-mediated efficient
transesterification of the ssRSS template. The same protein was also capable of mediating DNA recognition and cleavage of the wild type 12RSS substrate in Mn$^{2+}$ but not in Mg$^{2+}$ (Fig. 4B). Thus, recognition of the heptamer motif and the subsequent transesterification reaction does not require aa 330–456 of RAG1 including the homeodomain region.

**Mg$^{2+}$- but Not Mn$^{2+}$-based Reactions Follow Physiological Parameters**—Given the differential activity of RAG1-RAG2 in the presence of different divalent ions, we studied the effect of Mn$^{2+}$ and Mg$^{2+}$ on the kinetics of the initial stages of V(D)J recombination. Cleavage of a 12RSS was analyzed by altering various physiological parameters. A time course of the reaction (Fig. 5A) revealed that total substrate conversion into cleavage intermediates (either nick or hairpin) is virtually equivalent between reactions conducted in 1 mM Mn$^{2+}$ and in 1 mM Mg$^{2+}$. However, the kinetics of nick conversion into hairpin is accelerated at least 12-fold by Mn$^{2+}$. The biochemical requirements for Mn$^{2+}$- and Mg$^{2+}$-based cleavage were analyzed through a range of pH and temperature. In Mn$^{2+}$, nicking and transesterification were efficient within a wide pH range (Fig. 5B). However, Mg$^{2+}$-driven transesterification exhibited a strong pH dependence. Hairpin generation was eliminated above pH 7.6 (Fig. 5B), whereas nicking was virtually unaffected. Thus, the transesterification reaction can only occur within a narrow window of pH. A differential profile for Mn$^{2+}$- versus Mg$^{2+}$-based reactions was also observed by ranging temperature points (Fig. 5C). Mn$^{2+}$-driven reactions were not inhibited by temperature variations from 25 °C to 55 °C, whereas hairpin formation in Mg$^{2+}$ was most effective at more physiological temperatures, 37 °C to 45 °C, and was repressed below 37 °C and above 50 °C. Interestingly, although total substrate cleavage is equivalent at 37 °C, 42 °C, and 45 °C in Mg$^{2+}$, the amount of hairpin formation is increased 3-fold over this temperature range. Conceivably, this effect could be due to more efficient melting of the DNA required for hairpin conversion.

To estimate the relative affinities of Mn$^{2+}$ and Mg$^{2+}$ for their binding sites, Me$^{2+}$ binding was titrated out by increasing concentrations of Ca$^{2+}$. Although 20 mM Ca$^{2+}$ inhibits the Mn$^{2+}$-based reaction by only 5%, the same Ca$^{2+}$ concentration almost completely arrests the Mg$^{2+}$-based reaction.

**Phosphorothioate Substitutions Reveal a Role for Divalent Cations in DNA Recognition**—The role of divalent cations during site-specific cleavage by RAG1 and RAG2 was explored using phosphorothioated oligonucleotides in which non-bridging oxygens around the site of cleavage were individually replaced by sulfurs. The differential coordination of metal-oxygen and metal-sulfur interactions (44) maintains that coordination of sulfur by Mn$^{2+}$ is stronger than coordination by Mg$^{2+}$. Hence, involvement of a divalent cation at the catalytic site results in reduced cleavage of a phosphorothioated substrate in Mg$^{2+}$ while leaving Mn$^{2+}$-based cleavage predominantly uninhibited. Exploration of the catalytic mechanisms of ribozymes using phosphorothioated methodology has demonstrated a role for metal ions in transition state stabilization during sequence-specific endonuclease cleavage (45–47).

Two phosphorothioated oligonucleotides were synthesized
as restriction endonucleases, retroviral integrases, and transposases. In the presence of Mn$^{2+}$, several of these proteins exhibit relaxed DNA target specificity (49–54). In addition, Mn$^{2+}$ resuscitates the activity of IS10 and Mu transposase and EcoRV endonuclease mutants that are catalytically inactive in Mg$^{2+}$ (48, 50–52).

Direct Interaction of RAG1 and RAG2—Using direct mixing experiments, we found that RAG1 and RAG2 interact in the absence of DNA to form two complexes that are generated by RAG1 homodimerization. The functional significance of RAG1 homodimerization is currently under study. The two RAG1-RAG2 complexes are formed in the absence of DNA, demonstrating that the two proteins interact directly. Although previous experiments had revealed that RAG1 and RAG2 can be co-precipitated from lymphoid extracts (37, 38), a direct interaction between the two proteins had not been shown. This is perhaps because of the transient nature of the interaction, which can, however, be stabilized by glutaraldehyde cross-linking. The latter also stabilizes the RAG1-RAG2 DNA ternary complexes formed during gel-retardation assays (10).

Mg$^{2+}$-mediated Cleavage on a Single RSS—Using RAG1 and RAG2 expressed in mammalian cells, we found that the two proteins are able to mediate efficient cleavage on a single RSS in the presence of Mg$^{2+}$. This is in contrast to the previous notion that single-site cleavage is only permitted by Mn$^{2+}$ (12). It is therefore possible that, under physiological conditions, RAG1 and RAG2 have the ability to cleave on a single RSS. Presumably their 12/23 mode action (1, 13) might be regulated by additional cellular activities that prohibit uncontrolled cleavage on a single RSS. The involvement of such cellular activities has been indicated by previous experiments (13, 14).

The Nonamer Motif Modulates Efficiency of the V(D)J Cleavage Reaction—In vivo V(D)J recombination is critically dependent upon the integrity of both the heptamer and nonamer RSS motifs (3, 56, 57). The role of these two elements has been addressed in in vitro DNA binding and cleavage assays. The nonamer motif mediates recognition of the RSS by RAG1 (8, 9) whereas the heptamer stabilizes binding of the complex to the DNA (9, 10) and guides the subsequent transesterification reaction (8, 40, 41). Using Mn$^{2+}$-based cleavage assays, it was shown previously that mutations in the nonamer motif permit reduced but substantial nicking and hairpin formation (40, 41). These results raised the issue about the importance of the nonamer element during the initial stages of V(D)J recombination. Sequence comparison of RSS motifs from the Ig and T cell receptor loci has shown that in contrast to the heptamer motif, the nonamer element is not as highly conserved (5). Collectively, these observations suggested that the nonamer might affect the efficiency of V(D)J recombination whereas the heptamer is essential for the catalysis of the reaction. The results of our in vitro assays support this hypothesis. Both the DNA binding and cleavage assays demonstrate that, although the heptamer is indispensable for the cleavage reaction, the nonamer modulates the efficiency of RAG1-RAG2 binding to DNA. When tested in Mg$^{2+}$-based assays, mutation of the nonamer impairs the DNA binding potential of RAG1-RAG2 and consequently drastically reduces their cleavage activity but mutation of the heptamer eliminates cleavage by RAG1-RAG2. In further support of the hypothesis that the nonamer modulates the efficiency of V(D)J cleavage, elimination of the nonamer DNA binding domain of RAG1 does not affect the cleavage activity of the protein when assayed in Mn$^{2+}$. Thus, the RAG1 homeodomain seems to affect efficiency and perhaps topology of the initial stages of V(D)J recombination, but it does not participate in the subsequent nicking and
transrepression reactions. These data might account for the reduced frequency of V(D)J recombination observed for trunc
ated mutants of RAG1 that lack the homeodomain region (58) and in human patients carrying nonsense mutations in the Rag-1 homeodomain region.2

**Mg**^2+** Directly Coordinates DNA Binding of RAG1-RAG2—
The analysis of this substrate +1 revealed that **Mg**^2+ ions directly coordinate the binding of RAG1-RAG2 to the DNA. Sulfor substitution at the site of cleavage severely reduced the DNA binding activity of RAG1-RAG2 in **Mg**^2+. However, binding was reconstituted to wild type levels by **Mn**^2+ or **Ca**^2+. The differential behavior of this substrate in **Mg**^2+ or **Mn**^2+ strongly indicates that **Mg**^2+ directly mediates DNA recognition by RAG1-RAG2 through its coordination with the nonbridging oxygens. Thus, the site of cleavage not only coordinates the nicking and transrepression reactions but also guides the binding of RAG1-RAG2 to DNA via coordination of **Mg**^2+. Given that the two proteins specifically recognize the heptamer sequence and cleave in its vicinity, it is possible that a single, **Mg**^2+ -coordinated, domain in proximity with the heptamer might execute both DNA recognition and subsequent cleavage. Such a mechanism has been implicated in Tn10 transposition, where mutations in the active center of the transposase cancel target DNA capture (55). The role of **Mg**^2+ ions in mediating the binding of proteins to the DNA is poorly characterized with the exception of zinc-finger proteins. In one case, it has been shown that **Mg**^2+ maintains the structure of the DNA binding domain of transcription factor HNF3 (59). **Mg**^2+ also binds to a **Mg**^2+ binding site of EcoRV distinct from the catalytic center of the enzyme and determines specificity of DNA binding (60). It can be envisaged that the mode of RAG1-RAG2 binding to the DNA through the coordination of **Mg**^2+ ions is not restricted to this class of proteins but constitutes a global mode by which transcription factors bind to DNA.

Acknowledgment—We thank Dr. Kenji Adzuma for insightful suggestions.

REFERENCES
1. Tonegawa, S. (1983) Nature 302, 575–581
2. Lewis, S., and Gellert, M. (1989) Cell 59, 585–588
3. Hesse, J. E., Lieber, M. R., Mizuuchi, K., and Gellert, M. (1989) Genes Dev. 3, 1035–1061
4. Lewis, S. M. (1994) Adv. Immunol. 56, 27–150
5. Ramsden, D. A., Baetz, K., and Wu, G. E. (1994) Nucleic Acids Res. 22, 1785–1796
6. Schatz, D. G., Oettinger, M. A., and Baltimore, D. (1989) Cell 59, 1035–1048
7. Oettinger, M. A., Schatz, D. G., Gorka, C., and Baltimore, D. (1990) Science 248, 1517–1523
8. Spanopoulou, E., Zaitseva, F., Wang, F.-H., Santagata, S., Baltimore, D., and Panayotou, G. (1996) Cell 87, 263–276
9. Difilippantonio, M. J., McMahan, C. J., Eastman, Q. M., Spanopoulou, E., and Schatz, D. G. (1996) Cell 87, 253–262
10. Homs, K., and Gellert, M. (1997) Cell 86, 65–72
11. Nagawa, F., Ishiguro, K.-I., Ikeda, A., Yoshida, T., Ishikawa, A., Takemori, T., Otuwa, A. J., and Sakano, H. (1998) Mol. Cell. Biol. 18, 655–663
12. van Gent, D. C., Ramsden, D. A., and Gellert, M. (1996) Cell 85, 107–113
13. Eastman, Q. M., Liu, T. M. J., and Schatz, D. G. (1990) Nature 346, 85–88
14. Sawchuck, D. J., Weiss-Garcia, F., Malik, S., Besmer, E., Bustin, M., Nussenzweig, M. C., and Cortes, P. (1997) J. Exp. Med. 185, 2025–2033
15. van Gent, D. C., McBlane, J. F., Ramsden, D. A., Romeo, C., Cuomo, A. C., Gellert, M., and Oettinger, M. A. (1995) Cell 81, 925–934
16. McBlane, J. F., van Gent, D. C., Tsukerblat, B. S., Baltimore, D., and Gellert, M. (1994) Cell 78, 860–869
17. Littauer, J., and Craigie, R. (1995) EMBO J. 16, 2646–2655
18. Akamatsu, Y., Tsurushita, N., Nagawa, F., Matsuoka, M., Okazaki, K., Imai, M., and Sakano, H. (1994) J. Immunol. 153, 4520–4529
19. Stein, S. B., Gomelsky, L., Speidel, S. L., and Roth, D. (1997) EMBO J. 16, 2656–2664
20. Kirch, S. A., Sudarsanam, P., and Oettinger, M. A. (1996) Eur. J. Immunol. 26, 886–891
21. Clark, K. L., Halay, E. D., Lai, E., and Burley, S. K. (1993) Nature 364, 412–420
22. Jeltsch, A., Mashke, H., Selent, U., Wenz, C., Kohler, E., Connolly, B. A., Thornog, H., and Pingoud, A. (1995) Biochemistry 34, 6239–6246

---

3 Villa, A., Santagata, S., Bozzi, F., Gili, S., Frattini, A., Imberti, L., Benerini, L., Ochs, H., Schwarz, K., Notarangelo, L. D., Vezzoni, P., and Spanopoulou, E. (1998) Cell, in press.