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

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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 phosphorothioated 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),[1] 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 (ACAAAAACC) 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_N2-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 phosphorothioated 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 1ΔN, amino acids 330–1040; GST-RAG2 2ΔC, 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-

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[1] The abbreviations used are: RSS, recombination signal sequence; GST, glutathione *S*-transferase; DTT, dithiothreitol; aa, amino acid(s); MOPS, 4-morpholinepropanesulfonic acid; ds, double-stranded; ss, single-stranded.

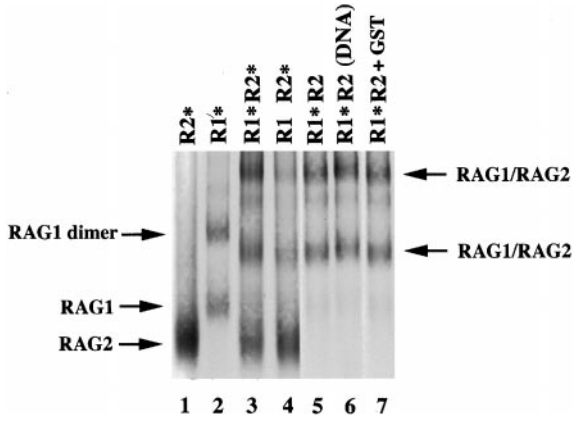

FIG. 1. **RAG1 and RAG2 interactions in solution.** RAG1 and RAG2 were purified as ^{35}S-labeled proteins, mixed together, cross-linked with glutaraldehyde, and resolved on a 5% native polyacrylamide gel. *Lanes 1* and *2* represent purified ^{35}S-labeled RAG2 and RAG1, respectively, after cross-linking. *Lane 3* contains ^{35}S-labeled RAG1 and RAG2, *lane 4* contains ^{35}S-RAG2·RAG1, *lane 5* contains ^{35}S-RAG1·RAG2, and *lane 6* contains ^{35}S-RAG1·RAG2 in the presence of the 12RSS probe. In *lane 7*, RAG1 and RAG2 were mixed with purified GST protein.

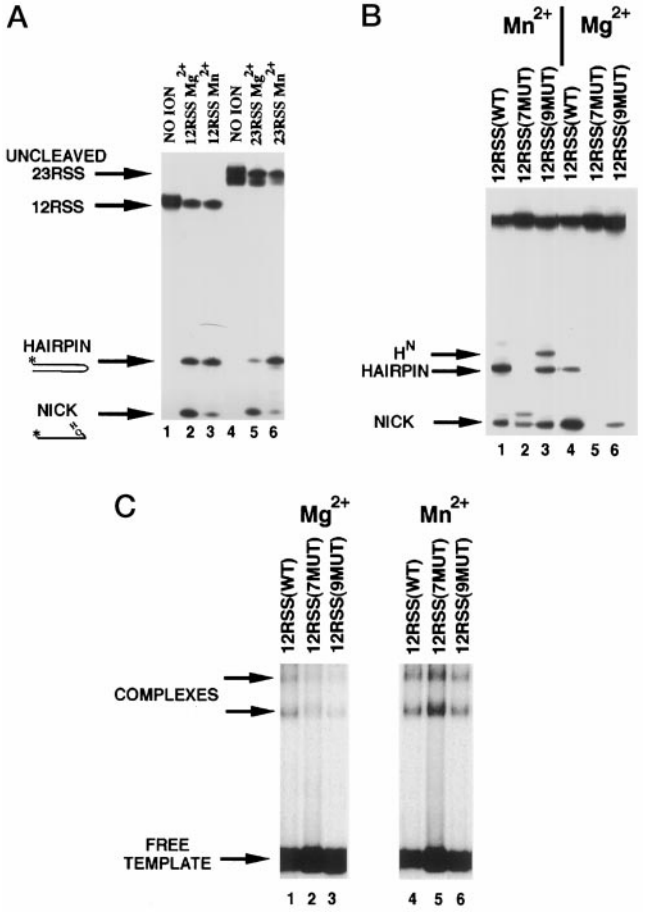

FIG. 2. **RAG1 and RAG2 mediate efficient nicking and transesterification in either Mn^{2+} or Mg^{2+}.** *A*, Mg^{2+}- and Mn^{2+}-based V(D)J cleavage on a single RSS. The 12RSS or 23RSS substrates were ^{32}P 5′ end-labeled to visualize exclusively the nicked products. Purified RAG1 and RAG2 were incubated with the substrates for 60 min. *B*, the effect of heptamer and nonamer mutations on V(D)J cleavage in the presence of Mn^{2+} or Mg^{2+}. Mutant substrates are described under "Experimental Procedures." *C*, mobility shift assays on the heptamer or nonamer mutant substrates. Note that the intensity of the shifted complexes is also dependent on the efficiency of cleavage. A low exposure is purposely shown to reveal the effects of the different mutant templates.

phoresis. ^{35}S-Labeled proteins were transiently expressed in 293T cells. The transfected cells were methionine-deprived for 30 min and subsequently incubated in the presence of [^{35}S]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 ^{32}P end-labeled cleavage substrate in 25 mM MOPS-KOH (pH 7.0), 10 mM Tris-HCl (pH 7.0), 95 mM KCl, 2.2 mM DTT, 4% glycerol (no Me_2SO conditions), 1 mM $MgCl_2$, or 1 mM $MnCl_2$ in a 20-μl final volume. Reactions were incubated at 37 °C for the indicated time points. 10% Me_2SO cleavage reactions were conducted in: 25 mM MOPS-KOH (pH 7.0), 5 mM Tris-HCl (pH 7.0), 120 mM KOAc, 18 mM KCl, 10% Me_2SO, 2.2 mM DTT, 0.5 μM nonspecific single-stranded DNA with 50 ng of each protein and 0.01–0.05 pmol of ^{32}P 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′-ACGCGTCGACGTCTTACACAGTGATACAGCCCTGAACAAAAACCGGATCCGCG-3′
23RSSup:	5′-ACGCGTCGACGTCTTACACAGTGATGCAGGCCAAGTGTGAAGCCATACAAAAACCGGATCCGCG-3′
12RSS(7mut)up:	5′-ACGCGTCGACGTCTTA**ACG**AGTGATACAGCCCTGAACAAAAACCGGATCCGCG-3′
12RSS(9mut)up:	5′-ACGCGTCGACGTCTTACACAGTGATACAGCCCTGAACAA**CCG**CCGGATCCGCG-3′
ICP(1–16):	5′-ACGCGTCGACGTCTTA-3′
ICP(17–53):	5′-CACAGTGATACAGCCCTGAACAAAAACCGGATCCGCG-3′

SEQUENCES 1–6

Standard cleavage reactions employed ^{32}P 5′ 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′-phosphorylated ICP(17–53) and unlabeled 12RSS-lower strand. The spacers for 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 oxygens 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 5′ end-labeled probe in 25 mM MOPS-HCl (pH 8.0), 1 mM $MgCl_2$ or 1 mM $MnCl_2$, 2.2 mM DTT, 1 μg of bovine serum albumin, 0.5 μM nonspecific primer (5′-CCTCGAGCTCATCAGCTTGCCTGTGGGCAGCTCGATCTCTTTGTCG-3′), 20% Me_2SO, 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 ^{35}S-labeled proteins (Fig. 1). After incubation, protein complexes were cross-linked with glutaral-

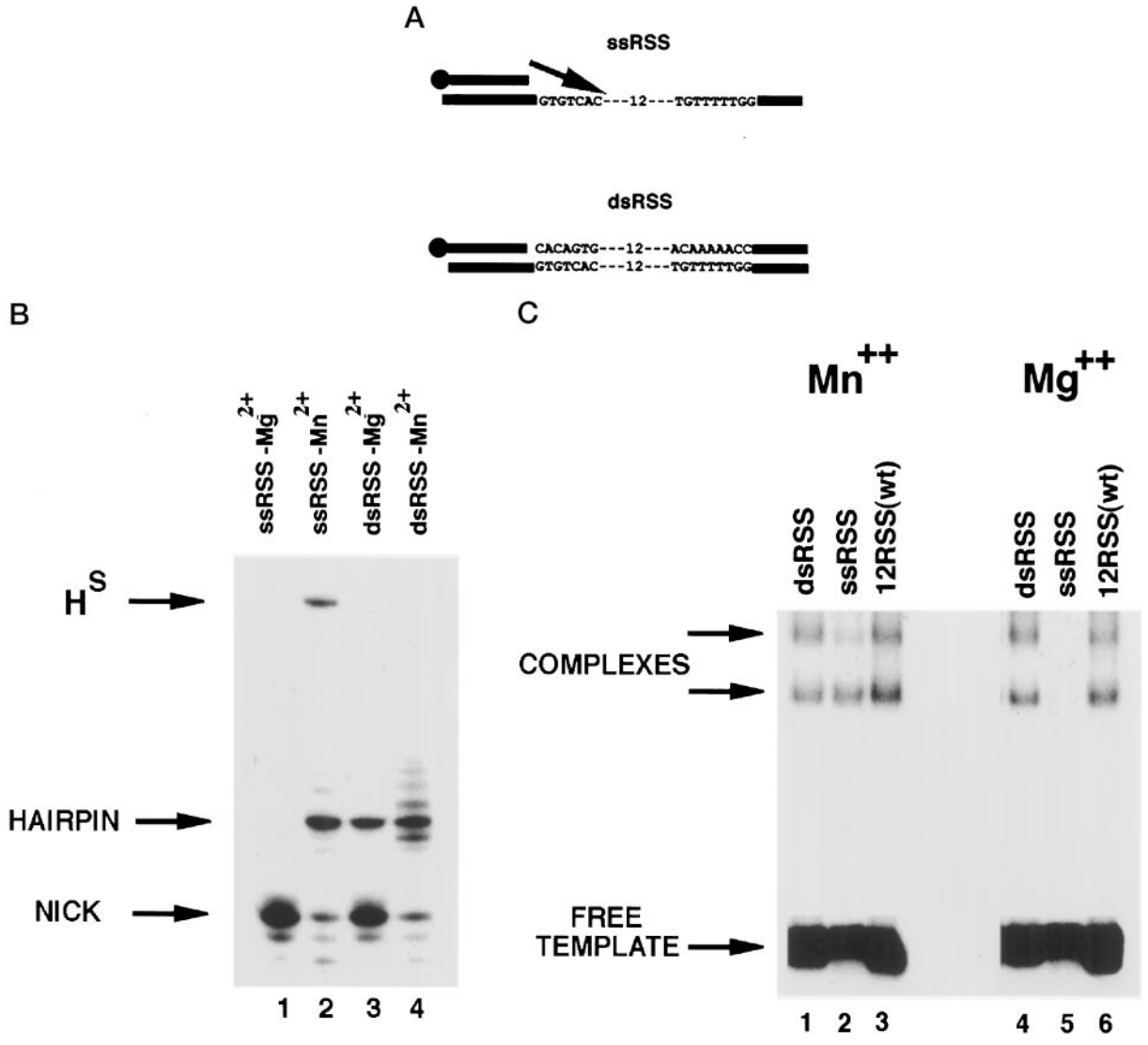

FIG. 3. **Mn^{2+} but not Mg^{2+} allows DNA binding and transesterification on single-stranded RSS.** *A*, single-stranded RSS cleavage substrates (ssRSS) were generated by annealing ^{32}P 5′ end-labeled upper strand coding nucleotides 1–16 with the full-length lower strand (12RSS-lo). The prenicked double-stranded RSS cleavage substrate (dsRSS) was synthesized from ^{32}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^{2+} (*lane 1*) and Mn^{2+} (*lane 2*). H^s 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^{2+} (*lane 3*) and Mn^{2+} (*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^{2+} (*lanes 1–3*) or Mg^{2+} (*lanes 4–6*).

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^{2+} Relaxes DNA Binding and Cleavage Specificity by RAG1·RAG2—To test the effect of Me^{2+} cofactors at the initial stages of V(D)J recombination, cleavage reactions were performed in the presence of either Mn^{2+} or Mg^{2+} (Fig. 2). The active cores of purified RAG1 (aa 330–1040) and RAG2 (aa 1–383) were incubated with ^{32}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^{2+} (Fig. 2*A*, *lanes 2* and *5*), whereas Mn^{2+} accelerated hairpin formation by severalfold (Fig. 2*A*, *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^{2+} prompted us to study the effect of Me^{2+} 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.

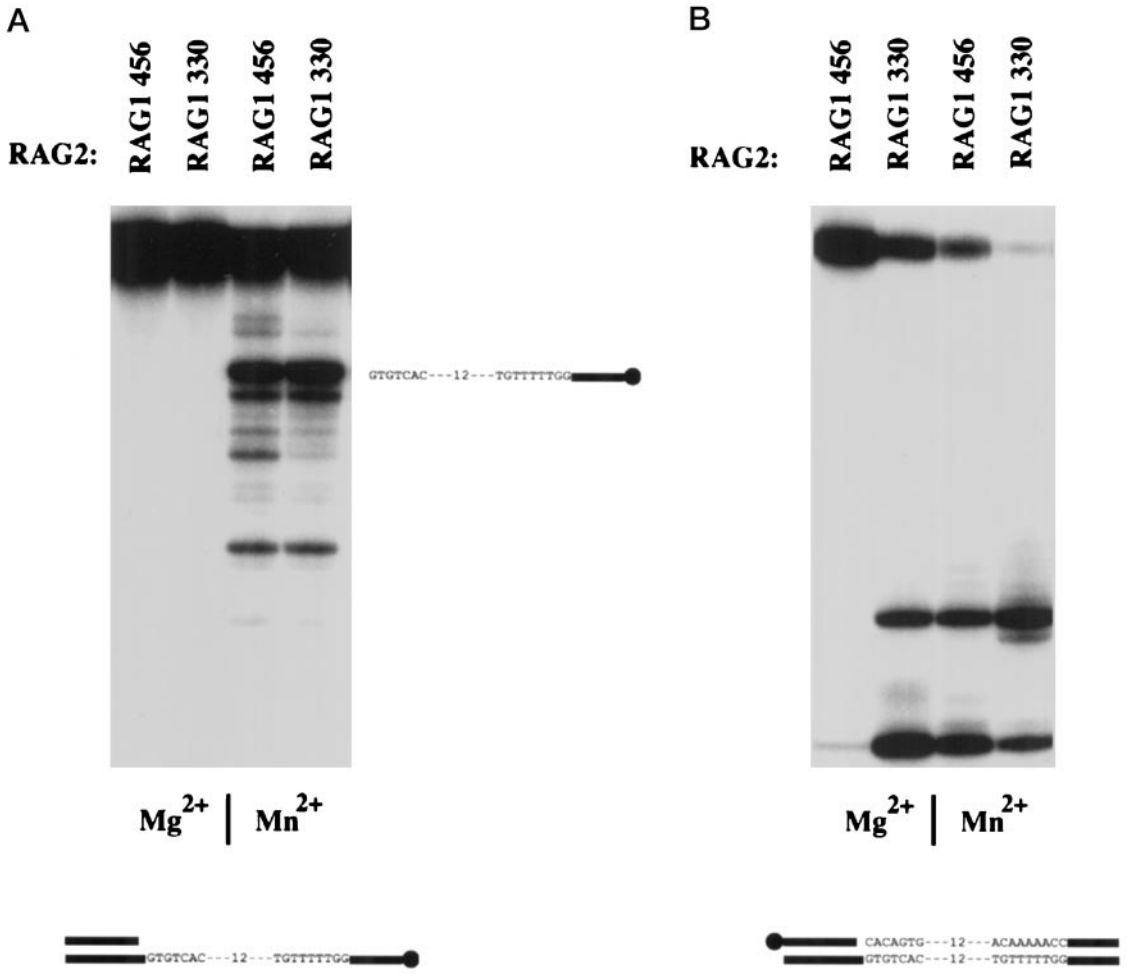

FIG. 4. **RAG1 aa 384–456 are not part of the RAG1·RAG2 active center.** The RAG1 homeodomain is not required for heptamer-mediated cleavage. *A*, RAG1 456–1040 can mediate hairpin formation on ssRSS in the presence of Mn^{2+}. Note that the ssRSS probe was labeled in the lower strand. *B*, RAG1 deletion mutants 456–1040 and RAG1 330–1040 were incubated with RAG2 and a radiolabeled 12RSS substrate for 120 min.

been shown to be critical for the function of the heptamer and nonamer elements of the RSS (40, 41) (Fig. 2*B*). Mutation of the first two residues of the heptamer abolish hairpin formation as reported previously (40, 41). However, RAG1·RAG2 can mediate nicking of the mutant heptamer substrate in the presence of Mn^{2+} but not in Mg^{2+} (Fig. 2*B*, *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. 2*B*, *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·RAG2. Gel retardation assays with purified RAG1·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. 2*C*, *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. 2*C*, *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 transesterification reaction on a single-stranded RSS substrate (Fig. 3*A*) in the presence of either Mg^{2+} or Mn^{2+}. Fig. 3*B* 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. 3*B*, *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. 3*C* 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. 2*B* and 3, *B* 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. 4*A*, in the presence of Mn^{2+} RAG1Δ456/RAG2-mediated efficient

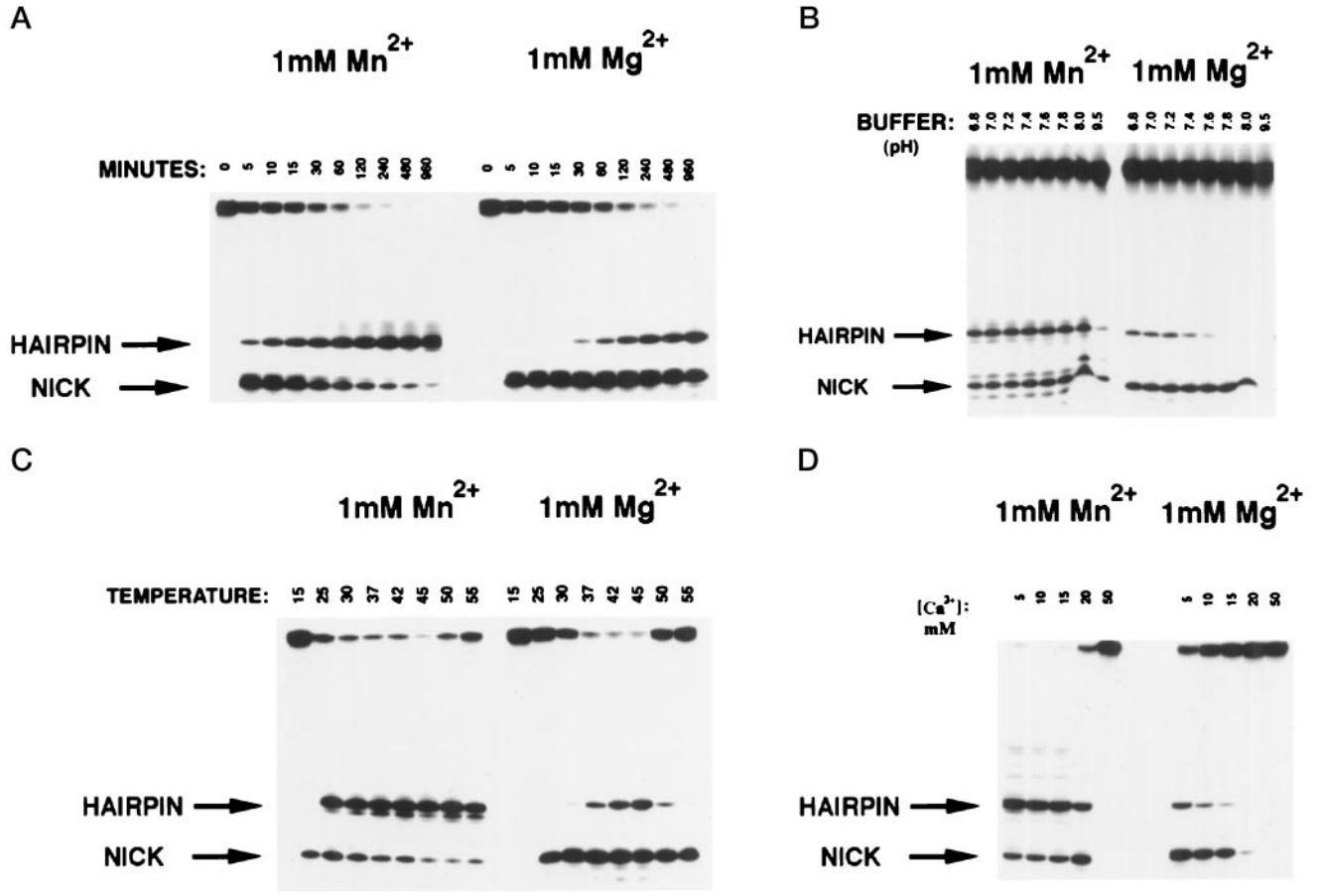

FIG. 5. **Comparison of the kinetics of Mn^{2+}- *versus* Mg^{2+}-based V(D)J cleavage reactions.** *A*, time dependence of the formation of cleavage intermediates. *B*, pH dependence of the transesterification reaction. *C*, temperature (°C) dependence of cleavage intermediate formation. *D*, relative Me^{2+} affinity of RAG1·RAG2 determined by Ca^{2+} titration. Reactions in *B*, *C*, and *D* were allowed to proceed for 60 min.

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. 4*B*). 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. 5*A*) 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. 5*B*). However, Mg^{2+}-driven transesterification exhibited a strong pH dependence. Hairpin generation was eliminated above pH 7.6 (Fig. 5*B*), 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+}. The latter is known to inhibit the cleavage activity of several restriction endonucleases (42) and transposases (43), as well as that of RAG1·RAG2 (10). 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

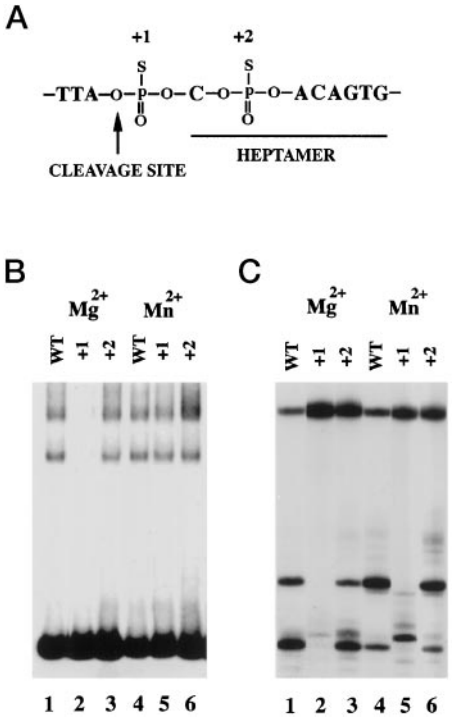

FIG. 6. **Phosphorothioated interference.** *A*, non-bridging oxygens were replaced by sulfurs in the upper strand of the standard cleavage substrate at and around the site of cleavage. The replaced positions are *numbered*. *B*, mobility shift assays were performed with RAG1 and RAG2 in Mn^{2+} or Mg^{2+} using the two phosphorothioated substrates. *C*, cleavage analysis of phosphorothioated substrates. Assays were performed in either Mn^{2+} or Mg^{2+} for 30 min.

with sulfur modifications at the indicated positions (Fig. 6*A*) and analyzed in DNA binding and cleavage assays. Substitution of the non-bridging oxygen at the cleavage site (+1) had a dramatic effect on the DNA binding and cleavage activities of RAG1·RAG2. DNA binding was severely compromised in Mg^{2+} but reconstituted by Mn^{2+} (Fig. 6*B*, *lanes 2* and *5*) or Ca^{2+} (data not shown). However, nicking and transesterification reactions by RAG1·RAG2 were severely reduced on the +1 thiosubstrate (Fig. 6*C*, *lane 2*) and Mn^{2+} only poorly resuscitated the nicking reaction (Fig. 6*C*, *lane 5*). Interestingly, sulfur substitution at the +1 position shifts the nicked product by one nucleotide (Fig. 6*C*, *lane 5*). It should be noted that phosphorothioated substrates were assayed as mixtures of the R_p and S_p forms (48). Thus, both S_p and R_p forms at the +1 position interfere with Mg^{2+}-mediated binding and cleavage by RAG1·RAG2. On the other hand, substitution of the non-bridging oxygen in the heptamer site, +2, reduced overall cleavage activity by 40% but it did not affect the efficiency of hairpin conversion (Fig. 6*C*, *lanes 3* and *6*) or that of DNA binding (Fig. 6*B*, *lanes 3* and *6*).

DISCUSSION

In this study, we analyzed the effect of Me^{2+} cofactors in the function of RAG1·RAG2 during the initial stages of V(D)J recombination. The results show that divalent ions not only modulate the cleavage activity of the complex but they also directly coordinate the binding of the complex to the RSS DNA. In general, Mg^{2+}-based reactions follow more physiological parameters of pH, temperature, and dependence on the heptamer/nonamer RSS motifs. In contrast, Mn^{2+}-mediated assays show relaxed specificity in which RAG1 and RAG2 are able to recognize a ssRSS element and DNA binding and cleavage is independent of nonamer binding. The tolerant phenotype induced by Mn^{2+} has also been observed for other proteins such 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 *Eco*RV 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

transesterification reactions. These data might account for the reduced frequency of V(D)J recombination observed for truncated mutants of RAG1 that lack the homeodomain region (58) and in human patients carrying missense mutations in the Rag-1 homeodomain region.[3]

Mg^{2+} Directly Coordinates DNA Binding of RAG1·RAG2—The analysis of thiosubstrate +1 revealed that Me^{2+} ions directly coordinate the binding of RAG1·RAG2 to the DNA. Sulfur 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 transesterification 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, Me^{2+}-coordinated, domain in proximity with the heptamer might execute both DNA recognition and subsequent cleavage. Such a mechanism has been implicated in Tn*10* transposition, where mutations in the active center of the transposase cancel target DNA capture (55). The role of Me^{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 Me^{2+} binding site of *Eco*RV 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.

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