**RAG-Heptamer Interaction in the Synaptic Complex Is a Crucial Biochemical Checkpoint for the 12/23 Recombination Rule**

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In V(D)J recombination, the RAG1 and RAG2 protein complex cleaves the recombination signal sequences (RSSs), generating a hairpin structure at the coding end. The cleavage occurs only between two RSSs with different spacer lengths of 12 and 23 bp. Here we report that in the synaptic complex, recombination-activating gene (RAG) proteins interact with the 7-mer and unstack the adjacent base in the coding region. We generated a RAG1 mutant that exhibits reduced RAG-7-mer interaction, unstacking of the coding base, and hairpin formation. Mutation of the 23-RSS at the first position of the 7-mer, which has been reported to impair the cleavage of the partner 12-RSS, demonstrated phenotypes similar to those of the RAG1 mutant; the RAG interaction and base unstacking in the partner 12-RSS are reduced. We propose that the RAG-7-mer interaction is a critical step for coding DNA distortion and hairpin formation in the context of the 12/23 rule.

V(D)J recombination plays key roles in activating and diversifying the antigen receptor genes in mammals (1). In the initial phase of the recombination, the protein products of recombination-activating genes, RAG1 and RAG2 (2, 3), recognize and cleave the recombination signal sequences (RSSs), each consisting of conserved 7-mer (CACAGTG) and 9-mer (ACAAAAACC) motifs, separated by a spacer of either 12 or 23 bp (4–13). V(D)J recombination takes place only between two RSSs with different spacer lengths, one containing a 12-bp spacer and the other containing a 23-bp spacer (14). This is the 12/23 rule for V(D)J recombination.

It has been proposed that V(D)J joining is a reversal of an ancient accidental insertion of a transposable element into a primordial V gene, later exploited by the vertebrate immune system during evolution (6). Consistent with this hypothesis, the postcleavage complex of the RAG proteins is known to possess transposition activity, both in vitro and in vivo (15–21). Furthermore, computational analyses of the fly and mosquito genomes revealed a transposon named Transib, which codes for a RAG1-like transposase (22). In sea urchin Strongylocentrotus purpuratus, a pair of genes (SpRag1L and SpRag2L) resembling RAG1 and RAG2 were found in tandem in the genome (23, 24). Many RAG1-like pseudogenes are also present in the sea urchin genome (22, 24–26). Although the physiological roles and biochemical properties are yet to be studied for the SpRag1/2L proteins, they may represent the evolutionary intermediates of the vertebrate RAG1 and RAG2.

During V(D)J recombination in mice, the RAG proteins cleave RSS DNA in two successive steps, nicking and hairpin formation (27, 28). A nick is first introduced at the coding/7-mer border on the top strand, and the resulting 3′-hydroxyl group (3′-OH) attacks the bottom strand to generate a hairpin structure at the coding end. Although nicking can occur without the partner RSS, the subsequent hairpin formation requires the synaptic formation between 12- and 23-RSSs (29–40). Previous works suggested that the RAG1-RAG2 complex is formed preferentially on the 12-RSS, with subsequent recruitment of the partner 23-RSS (34, 37, 41). In the present study, we performed DMS and KMnO4 footprinting to analyze the RAG-RSS interaction and DNA distortion during the process of synaptic complex formation. It was found that the 7-mer was protected on the bottom strand and that the adjacent coding base was unstacked in the synaptic complex of the 12/23 pair but not the 12/12 pair. We also analyzed both RAG1 and RSS mutants that have defects in the distortion and cleavage of the 7-mer sequence. Our results indicate that the RAG-7-mer interaction is a crucial checkpoint in V(D)J recombination to ensure the 12/23 rule.

**EXPERIMENTAL PROCEDURES**

Preparation of Proteins—The glutathione S-transferase (GST)-tagged truncated RAG1 protein (amino acids 384–1040) was coexpressed with the GST-tagged truncated RAG2 protein (amino acids 1–383) in HEK-293T cells (42), purified with glutathione-agarose affinity chromatography (9), and dialyzed against 25 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 150 mM KC1, and 10% glycerol. Plasmids for the RAG1 mutants (HA1, HA2, HA3, and DH3u) were generated by *in vitro* mutagenesis.

DNA Substrates—Oligonucleotides were synthesized and purified as described (43–45). Sequences used were as follows...
Checkpoint for the 12/23 Rule

The 32P-labeled, biotinylated, and 3'-dideoxyoligonucleotides were prepared as described (43–45). The nicked RSS was prepared by annealing three oligonucleotides: an RSS bottom strand (69-mer) for RSS cleavage assay, 5'-ACCTCCTAGGGTGTAAGCTCTGGTCTTACACAGTGGTAGTACTCCACTGTCTGGGTTGCCATGGACTC-3' and a 5'-phosphorylated top strand of the signal end DNA, and a coding top strand (with a 3'-OH end). Annealed DNA was purified by electrophoresis in an 8% polyacrylamide gel, as described (43–45).

**RSS Cleavage Reactions**—The 32P-labeled RSS DNA (400 cpm/μl) and the partner RSS DNA (8 nM) were incubated with RAG1 (12 μg/ml), RAG2 (12 μg/ml), and HMG1 (8 μg/ml) proteins at 37 °C for 45 min in cleavage buffer containing either 10 mM MgCl2 or 10 mM CaCl2. Samples were preheated at 25 °C for 5 min and incubated at 37 °C for 45 min in cleavage buffer containing either 10 mM MgCl2 or 10 mM CaCl2. The biotinylated partner RSS (8 nM) was then added and further incubated at 37 °C for 6 min. Methylation was stopped by adding β-mercaptoethanol (0.2 mM) and sodium acetate (0.3 M). DNA was extracted with phenol/chloroform/isoamylalcohol (25:24:1), precipitated twice with ethanol, dissolved in 10 mM sodium phosphate (pH 6.8) and 1 mM EDTA, and heated at 90 °C for 15 min. Sodium hydroxide (0.1 N) was then added, and heated at 90 °C for 30 min. DNA was precipitated with ethanol, washed with 70% ethanol, dissolved in formamide dye mix, and electrophoresed in a 10% denaturing polyacrylamide gel.

For KMnO4 footprinting, 32P-labeled RSS DNA (400 cpm/μl) was incubated with RAG1 (12 μg/ml), RAG2 (12 μg/ml), and HMG1 (8 μg/ml) proteins at 37 °C for 45 min in cleavage buffer containing either 10 mM MgCl2 or 10 mM CaCl2. Samples were preheated at 25 °C for 5 min. KMnO4 (10 mM) was then added and further incubated at 25 °C for 6 min. Oxidation was stopped by adding β-mercaptoethanol (1.2 M). SDS (0.556%) and proteinase K (0.556 mg/ml) were added to the sample and incubated at 50 °C for 60 min. DNA was precipitated twice with ethanol, dissolved in 10% piperidine, heated at 90 °C for 30 min, and freeze-dried three times. Samples were dissolved in formamide dye mix and electrophoresed in a 10% denaturing polyacrylamide gel.

**RESULTS**

**RAG1 Mutants for Synapsis-dependent RSS Cleavage**—To analyze the synopsis-dependent RSS cleavage, we tried to isolate the RAG1 mutants defective in partner-dependent hairpin formation. Mutants were generated by swapping short stretches of the murine RAG1 sequence with the corresponding regions from sea urchin proteins deduced from the RAG1-like genes (22–26), expecting that such swappings may alter the regulatory functions without disrupting the overall protein structure. Among the mutants analyzed, three showed up-regulated RSS cleavage in vitro. These hyperactive (HA) mutants, HA1, HA2, and HA3 (Fig. 1A), underwent hairpin formation on the 12-RSS substrate even in the absence of the partner 23-RSS under the Mg2+ condition. It should be noted that hairpin formation usually requires both 12- and 23-RSSs in the synaptic complex (Fig. 1B). With the HA3 mutant, where residues 978–984 were replaced, more than 75% of total 12-RSS was converted to the hairpin form, and the addition of the partner 23-RSS did not enhance the reaction (Fig. 1B). The HA3 phenotype is similar to that for the previously reported RAG1
mutant (E649A) that cleaves RSS DNA independent of the synapsis formation (46). Two other mutants, HA1 and HA2, demonstrated elevated levels of hairpin formation by severalfold in the absence of the partner RSS, which could be further enhanced with the addition of partner RSS (Fig. 1B).

Another group of RAG1 mutants identified in the present study demonstrated defective hairpin formation (DH) even in the absence of partner RSS. With one of the mutants, DH3u, hairpin formation was not enhanced by the addition of the partner RSS, although the partner-independent basal hairpin formation activity was comparable with wild type (Fig. 1B). Under the relaxed condition using Mn²⁺/H₁₁₀₀₁ (27, 31, 47), instead of Mg²⁺/H₁₁₀₀₁, the DH3u cleaved RSS DNA as efficiently as the wild-type RAG1 (Fig. S1).

RAG-7-Mer Interaction Is Enhanced by the Synapsis Formation—We then studied the RAG-RSS interaction at different stages of synaptic complex formation. Using the nicked RSS as an intermediary substrate, both the single RSS complex and the synaptic complex were analyzed in parallel by DMS footprinting (Figs. 2 and S2). Hairpin formation, which usually hampers the footprinting, was blocked by two different methods. First we utilized a catalytic mutant of RAG1 (D600A) that can form the RAG-RSS complex but not the hairpin structure (Fig. 2) (48, 49). In the other method, the 3'-OH at the nick was reduced to the 3'-dideoxy form, and the reaction was performed under the Ca²⁺ condition (Fig. S2) (43). In each experiment, either the 12- or 23-RSS was labeled with ³²P at the 5'-end of the bottom strand and incubated with HMG1 and GST-fused RAG1/2 proteins with or without the partner RSS. The resulting complex was isolated with glutathione-Sepharose (Figs. 2A and S2). The addition of the partner RSS satisfying the 12/23 rule increased the yield of the complex, indicating that synapsis formation stabilizes the binding of RSS to the RAG proteins (Figs. 2A and S2).

For footprinting, the isolated complex was treated with DMS, which methylates G residues in the major grooves of...
double-stranded DNA and A residues in the minor grooves (Figs. 2B and S2). Methylated residues were then chemically cleaved. Fig. 2B shows the DMS footprints of 12- and 23-RSSs, where the hairpin formation is blocked by the D600A mutation. Protection was seen at the second G residue of the 9-mer on the bottom strand, in both the single RSS complex and 12/23 synaptic complex (Fig. 2B). In contrast, protection of the first and third G residues in the 7-mer was found only when the partner RSS was present, satisfying the 12/23 rule (Fig. 2B). The same results were obtained when the hairpin formation was blocked with the dideoxy end using the Ca\(^{2+}\) condition (Fig. S2). These results indicate that synaptic complex formation facilitates the interaction of RAG with the 7-mer end, which flanks the scissile phosphate on the bottom strand.

We next examined whether the RAG-7-mer interaction is altered with the HA mutants of RAG1. To block the hairpin formation, double mutants were generated, carrying both D600A and one of the HA mutations. The RAG1–7-mer interaction was analyzed by footprinting under the Mg\(^{2+}\) condition (Figs. 2C and S3). We used the Mg\(^{2+}\) condition, because the disregulated hairpin formation by HA was observed with Mg\(^{2+}\) but not with Ca\(^{2+}\) (Fig. S4); although Ca\(^{2+}\) cannot support the nicking reaction, it supports hairpin formation on nicked RSS in a 12/23 synaptic complex (50). Among the HA RAG1 mutants, only HA2 showed significant changes in the DMS footprint patterns. With the HA2 mutant, the first G residue in the 7-mer became hypersensitive to DMS, whereas the third G residue on the bottom strand was protected in the single 12-RSS complex (Fig. 2C). These changes may account, at least in part, for the increased RSS cleavage by the HA2 mutant in the absence of partner RSS (Fig. 1B).

\(^4\) T. Nishihara, F. Nagawa, T. Imai, and H. Sakano, unpublished results.
Synaptic Complex Formation Facilitates Base Unstacking in the Coding Region—RAG proteins have been suggested to induce DNA distortion near the cleavage site in both coding and 7-mer sequences (4, 5, 51–56). Because the hairpin tip is unpaired and unstacked, a similar distortion is expected to occur in the coding region before the hairpin forms. In order to determine when such distortion starts to take place, we employed another footprinting method using KMnO₄, which modifies unstacked thymine bases. For footprinting, nicked 12-RSS was incubated with the RAG1, RAG2, and HMG proteins with or without the 23-RSS (Figs. 3 and S6). After the addition of KMnO₄, modified T residues were chemically cleaved. In the presence of 23-RSS as a partner, RAG proteins greatly enhanced the KMnO₄ sensitivity of the 12-RSS in the coding base adjacent to the 7-mer on the bottom strand, where the 3′-OH end of the top strand attacks to form a hairpin structure. This hypersensitivity was less prominent in the absence of an appropriate partner RSS. The sensitivity of another residue, the second base in the 7-mer, was not enhanced by the partner RSS. In contrast to the nicked RSS, intact 12-RSS did not show KMnO₄ sensitivity, suggesting that the nick enhances the distortion of the 7-mer by RAG proteins (Fig. S7). These results may suggest that the nicking in a single RSS complex relaxes the 7-mer region and that the subsequent 12/23 synapsis formation facilitates the base unstacking in the coding region, leading to hairpin formation (52).

It should be noted that two HA mutants (HA3m and HA2) exhibited enhanced DNA distortion in the single RSS complex (Figs. S6 and S8). The HA3m mutant increased the unstacking of the coding base without affecting the unstacking of the 7-mer base in the absence of the partner RSS (Fig. S6). Unlike the D600A mutant, the D600A/HA3 double mutant rendered the coding base more sensitive to KMnO₄ than the 7-mer base (Fig. S6). We assume that the enhanced unstacking of the coding base may explain, at least in part, the efficient hairpin formation in the single RSS complex with the HA3 and HA3m mutants (Figs. 1B and S5).

RAG1 Mutants of Hairpin Formation Impair the 7-Mer Interaction and Base Unstacking in the Coding Region—We analyzed the base unstacking in the coding region with the DH3u mutant of RAG1. This mutant, defective in the hairpin formation, retains the ability to unstack the 7-mer base but loses the synapsis-dependent unstacking of the coding base (Fig. 3). We found that the DH3u mutant is capable of forming the synaptic complex following the 12/23 rule (Fig. 4A). In the pull-down assays using the ³²P-labeled RSS and biotinylated RSS, the 12/23 pair was detected much more efficiently than the 23/23 pair and slightly more efficiently than the 12/12 pair. In the DMS footprinting with the isolated 12/23 complex, protection of the first and third G residues on the bottom strand 7-mer was much reduced when the DH3u mutant was used, although the protection patterns in the 9-mer and spacer regions were similar to those with the wild-type RAG1 (Fig. 4B). Thus, the RAG-RSS interaction with the DH3u mutant in the synaptic complex appears to be...
FIGURE 4. The 7-mer interaction and coding base unstacking in the synaptic complex. A, isolation of the synaptic complex. RSS DNA was reduced to convert the 3'-OH at the nick to the 3'-dideoxy form (ddA) and then labeled with $^{32}$P at the 5'-end of the bottom strand (asterisk). The wild type (WT) RAG1 or the mutant (DH3u), RAG2, and HMG proteins were mixed and incubated with $^{32}$P-labeled RSS and biotinylated (Bio) partner RSS. The synaptic complex was isolated with avidin beads under the Ca$^{2+}$ condition. $^{32}$P counts incorporated into the complex were measured and divided by the input counts (percentage, pull-down of $^{32}$P). Pull-downs of 12-RSS and 23-RSS through biotinylated 23-RSS with the wild-type proteins were 17.3 ± 0.7% and 1.33 ± 0.38% (mean ± S.D. in three experiments), respectively. B, DMS footprints of isolated synaptic complexes. The complex containing either the WT or DH3u RAG1 was treated with DMS and electrophoresed in the sequencing gel for footprinting. C, effects of the C-to-A change at the first position of the 23-RSS 7-mer. The synaptic complex containing the biotinylated 23-RSS with the C-to-A change was treated with DMS or KMnO$_4$ for footprintings. D, KMnO$_4$ footprints of synaptic complexes. All combinations of two different RSSs, 12/12, 12/23, 23/12, 23/23, were analyzed. The filled triangles in the DMS footprints indicate the first and third G residues in the 7-mer. The filled triangles in the KMnO$_4$ footprints indicate the coding base adjacent to the 7-mer. The open triangles indicate the second G residue in the 9-mer. The 7- and 9-mer regions are indicated on the right. The substrate DNA (free) was subjected to DMS or KMnO$_4$ treatment as a control.
Checkpoint for the 12/23 Rule

base was evident in the 12/23 complex, slightly reduced in the 23/23 complex, and much reduced in the 12/12 complex (Fig. 4D). Thus, the RAG-7-mer interaction and unstacking of the coding base are blocked in the 12/12 synaptic complex. This prohibition may contribute to the maintenance of the 12/23 rule at the initial stage of synaptic complex formation and hairpin formation during V(D)J recombination.

DISCUSSION

In the present study, we performed DMS and KMnO₄ footprinting to analyze the changes in the RAG-RSS interaction and DNA distortions during the 12/23 synaptic complex formation. The 7-mer is distorted first in a single RSS complex, and then its bottom strand is tightly protected by RAG proteins in the synaptic complex, and the flanking coding base is unstacked. This distortion may be an important step for the hairpin formation at the coding end. These synopsis-dependent processes are strictly blocked in the 12/12 pair, violating the 12/23 joining rule.

Nicking and 7-Mer Distortion—In the present study, it was found that the second residue of the 7-mer on the bottom strand becomes sensitive to KMnO₄ in the single RSS complex with RAG proteins. It appears that distortion of the 7-mer is initiated before the synaptic complex formation. KMnO₄ oxidizes the 5–6 double bond of unstacked thymines, and the reactivity is much higher with single-stranded DNA than with double-stranded. Since the central domain of the core RAG1 is known to bind single-stranded 7-mer (54, 57), DNA distortion in the 7-mer may involve the local unwinding of DNA (Fig. 5). Nicking the RSS top strand may increase the flexibility of the DNA structure and allow for DNA distortion.

RAG-7-Mer Interaction and Synaptic Complex Formation—The synaptic complex formation allows the interaction of RAG proteins with the 7-mer in the bottom strand (Fig. 5). This interaction appears to be key in stabilizing the synaptic complex, because both C-to-A change at the first position of the 23-7-mer and the DH3u RAG1 mutation significantly reduced the interaction and synaptic complex formation (Fig. 4, A and B). The 7-mer recognition occurs in a concerted manner with two RSSs in the synaptic complex. The 7-mer mutation in the 23-RSS affected the protection of 12-RSS 7-mer. This mutation in the 23-RSS 7-mer and the DH3u RAG1 mutation significantly blocked in the 12/12 complex, and much reduced in the 12/12 complex (Fig. 4D). Thus, the RAG-7-mer interaction and unstacking of the coding base are blocked in the 12/12 synaptic complex. This prohibition may contribute to the maintenance of the 12/23 rule at the initial stage of synaptic complex formation and hairpin formation during V(D)J recombination.
recognition of the 12- and 23-RSS 7-mers. The DH3u mutation may interfere with this conformational change, although it is also possible that the mutated region is directly involved in the 7-mer interaction in the synaptic complex.

The 7-mer interaction of RAG proteins occurs efficiently with 12/23 and 23/23 pairs but not with the 12/12 pair. Our results indicate that the 7-mer interaction is required not only for the stabilization of the 12/23 synaptic complex but also for the unstacking of the coding base. Thus, the 7-mer interaction may be important in preventing the hairpin formations with an undesirable combination of RSSs, 12/12. In contrast, the 7-mer interaction does not appear to be important in checking for the 23/23 combination. This is consistent with the less tight inhibition of hairpin formation with 23/23 combination (61). Since the RAG complex first binds to the 12-RSS in vivo (41), prohibition of 12/12 pairing of RSSs may take priority in cells.

Distortion of the Coding Region DNA and Hairpin Formation—An abasic nucleotide, when incorporated 3' to the scissile phosphate, is more stimulatory to the hairpin formation than mismatched bases (52, 55). It is possible that the coding base adjacent to the scissile phosphate is not only unpaired but also flipped out of the helix during hairpin formation (52). Such a flipped-out base is observed in the hairpin-like structure of the Tn5 transposon (58). The flipped-out base is assumed to be sensitive to KMnO₄. We observed that the coding base adjacent to the scissile phosphate is indeed oxidized by KMnO₄ in the synaptic complex. Our data indicate that this distortion occurs after the synaptic complex formation that is based on the RAG-7-mer interaction.

It has been proposed that nicking and hairpin formation are mediated by the same active site in RAG1 (50, 57, 59, 60). In this scenario, the 3'-OH end of the top strand should remain in the active site until it acts as a nuclease in the hairpin formation reaction (Fig. 5). We assume that recognition of the 7-mer bottom strand would place the scissile phosphate in the active site of RAG1. To bring the scissile phosphate to the active site, the coding DNA must be unpaired and unstacked. Stabilization of flipped-out bases by the Trp¹⁹³ and/or Trp¹⁹⁶ residue(s) of RAG1 may also be important in this process (52, 61).

Regulation of the Hairpin Formation—Since the hairpin formation is found at low levels, even in the single RSS complex, there may be some interaction between the RAG proteins and 7-mer before synthesis formation. Hence, RAG mutants like HA with the elevated catalytic activity and/or RSS interaction may bypass the 12/23 requirement.

The HA2 mutant enhanced DNA distortion of the coding and 7-mer bases as well as the hairpin formation activity. The mutated region includes Trp¹⁹³, which has been proposed to distort DNA by stabilizing the flipped-out bases through stacking interaction (61). Hairpin formation is reduced by changing the Trp¹⁹³ residue to Ala but not to Phe (61). In the HA2 mutant, the Trp¹⁹³ has been changed to Tyr, further confirming the importance of the aromatic character of this residue. It is likely that the HA2 mutation is more effective than the wild-type RAG1 in distorting the coding/7-mer region. With the HA2 mutant, we observed 7-mer protection at the third G residue on the bottom strand even without the partner 23-RSS. It is possible that the mutated region in the RAG1 may directly interact with the 7-mer and enhance the unwinding of adjacent DNA of the bottom strand.

Conclusion—In the present study, we analyzed the RAG-RSS interaction and DNA distortion during the process of synaptic complex formation. It was found that RAG-7-mer interaction is facilitated by pairing two RSSs in the combination of 12/23 and that it induces distortion of the coding region DNA. This step appears to be a critical checkpoint for the 12/23 joining rule, because these interactions do not occur with the 12/12 pair. It is assumed that the RAG1-RAG2 complex first binds to the single 12-RSS in cells, possibly due to the regulation of chromatin structure. Since the RSS cleavage is prohibited with the 12/12 pair, the RAG-7-mer interaction analyzed in the present study may be a crucial step in preventing the undesirable recombination with inappropriate pairs of RSS substrates.

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