Joining Mutants of RAG1 and RAG2 Demonstrate Impaired Interactions with the Coding-End DNA

Fumikiyo Nagawa,† Satoshi Hirose,† Hirofumi Nishizumi, Tadashi Nishihara, and Hitoshi Sakano*

Department of Biophysics and Biochemistry,
Graduate School of Science, and CREST Program of JST,
The University of Tokyo, Bunkyo-ku, Tokyo 113-0032, Japan

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*Corresponding author. Mailing address: Department of Biophysics and Biochemistry,
Graduate School of Science, The University of Tokyo, 2-11-16 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Phone: 81 3 5841 7239; Fax: 81 3 5841 7240;
E-mail: sakano@mail.ecc.u-tokyo.ac.jp

†F.N. and S.H. contributed equally to this work.
In V(D)J joining of antigen receptor genes, two recombination signal sequences, 12- and 23-RSSs, form a complex with the protein products of recombination activating genes, RAG1 and RAG2. DNaseI footprinting demonstrates that the interaction of RAG proteins with substrate RSS DNA is not just limited to the signal region but it involves the coding sequence as well. Joining mutants of RAG1 and RAG2 demonstrate impaired interactions with the coding region in both pre- and post-cleavage type complexes. A possible role of this RAG/coding-region interaction is discussed in the context of V(D)J recombination.
V(D)J recombination plays key roles in activating and diversifying the antigen receptor genes in lymphocytes (1). Two sets of recombination signal sequences (RSSs) are required, each consisting of a conserved 7mer (CACAGTG) and a conserved 9mer (ACAAAAACC). V(D)J recombination takes place only between two RSSs with different spacer lengths, one containing a 12bp spacer (12-RSS) and the other, a 23bp spacer (23-RSS) (2).

At the initial step of V(D)J joining, one RSS is bound to the protein products of recombination-activating genes, RAG1 and RAG2 (3-12). This RSS-RAG interaction primarily involves the Hin homeodomain of the RAG1 protein and the 9mer region of RSS DNA (3, 4, 6). The next step is the synaptic complex formation, bringing the two RSSs together (13-15) in the presence of a DNA-bending protein, HMG1 (11, 16-19). Although a nick can be formed on the top strand in the single RSS-RAG complex, the double strand cleavage, which generates a hairpin at the coding end (CE) and a blunt end at the signal end (SE), requires the 12/23 pairing of the two RSSs (20-23). After the 12- and 23-RSSs are cleaved, the hairpin CEs are opened and processed, and then the two CEs and the two SEs recombine to form a coding joint and a signal joint, respectively, by DNA repair mechanisms (24-38). It has been suggested that the RAG proteins also participate in the joining phase of V(D)J recombination (28, 30, 39-47). Although this process is largely unknown, an architectural role has been suggested for the RAG proteins, in holding the two sets of cleaved ends for further processing and ligation in the post cleavage complex (39, 40, 43, 44, 47). In the in vitro reaction, the coding ends are partly retained in the synaptic complex after the double-strand cleavage (39, 40, 43).
Using DNaseI footprinting, we have been studying the interaction between the RAG proteins and recombination signals in both primary and synaptic complexes (6, 48). It was found that RAG-RSS interaction in the 7mer region drastically changes once the synaptic complex is formed for cleavage (48). Here we report the footprint analysis of the coding region in the synaptic complex of V(D)J joining. We have found that the RAG proteins interact with the RSS DNA not only in the signal region, but also in the coding region in the both pre-cleavage and post-cleavage type synaptic complexes. The RAG mutants, K118A/K119A (41) and S723C (43), defective in the post-cleavage process of V(D)J joining demonstrated the reduced interaction with the coding sequence.
EXPERIMENTAL PROCEDURES

Preparation of proteins - GST-tagged, truncated RAG proteins (amino acids 384 to 1040 of RAG1; and 1 to 383 of RAG2) were coexpressed in HEK-293T cells, purified with glutathione-agarose affinity chromatography (4), and dialyzed against 25 mM Tris-HCl (pH 8.0), 2 mM DTT, 150 mM KCl, and 10% glycerol. It has been reported that the cleavage and joining activities of the truncated RAG proteins are comparable with those of the full-length proteins (16-23, 41-47). Purities and concentrations of the RAG proteins used in our studies were determined in the SYPRO Ruby (Invitrogen)-stained gels with a fluorescent imaging analyzer, FLA-2000 (FUJI FILM). The purified RAG proteins were found to be >90% pure. The expression plasmid for the mutant RAG2 (K118A/K119A) was provided by Dr. D. B. Roth. Plasmids for the RAG1 mutants (S723C and E962A) were generated by in vitro mutagenesis.

DNA substrates- Oligonucleotides were synthesized and purified as described (48). The following are the strand sequences with the 7mer and 9mer signals underlined. The 12-RSS top strand (89mer): 5'-CTTCAAACCATCCA
ATAAACCCTGCGCTGAATTCGTCTTTA CACAGTGCTCCAGGGCTGA
ACAAAAACCTCCTAGGGTTGCAGCTGACTC-3'. The 23-RSS top strand (100mer): 5'- CTTCAAACCATCCAATAAACCCTGCGCTGAATTCTCTTTA
CACAGTGCTAGTAGTACTCCACTGTCTGGGTGT ACAAAAACCTCCTAGGGTTGCAGCTGACTC-3'. The 12-SE top strand (59mer): 5'- CACAGTGCTCCAGGGCTGA
GAACAAAAACCTCCTAGGGTTGCAGCTGACTC -3'. The 23-SE top strand (60mer):
The coding top strand (40mer): 5'-CTTCAAACCATCCAATAAACCCCTGCGCTGAATTCGTCTTA-3'. The oligonucleotides containing the 3'-phosphate or the 3'-biotin were synthesized. For the 5'-end labeling, oligonucleotide substrates were incubated with $\gamma^{32}$P]ATP, using the wild-type T4 polynucleotide kinase (New England Biolabs) or the 3'-phosphatase free mutant (Roche Diagnostics). For the 3'-end labeling, the substrates were annealed to appropriate complementary oligonucleotides and filled in one nucleotide with $\alpha^{32}$P)dGTP or dCTP using Klenow Fragment (3' exo-; New England Biolabs). To prepare the 3'-dideoxy oligonucleotide, oligonucleotide was extended with ddATP (Amersham Bioscience) using terminal deoxyribonucleotide transferase (Roche Diagnostics). The labeled oligonucleotides were purified from the denaturing polyacrylamide gel, and reannealed to the indicated complementary strands. The nicked RSS was constructed by annealing three oligonucleotides: a RSS bottom strand, a SE top strand, and a coding top strand with a 3'-dideoxy end. Annealed DNA was purified by electrophoresis in an 8% polyacrylamide gel; eluted from gel slices with an elution buffer containing 0.2M NaCl, 1mM EDTA, and 20mM Tris-HCl (pH7.5); and purified with Elutip-d (Schleicher-Schell).

**Isolation of pre- and post-cleavage complexes**— Biotinylated DNA (8nM) was incubated with RAG1 and RAG2 proteins (4µg/ml) and human HMG1 protein (Sigma) (8µg/ml) at 37°C for 10min in 20µl of binding buffer containing 25mM MOPS- KOH (pH 7.0), 5mM Tris-HCl (pH 8.0), 2.4mM DTT, 90mM KOAc, 30mM KCl, 10mM CaCl2,
0.1mg/ml BSA, and 2% glycerol. As carrier DNA, 2µM double-stranded oligonucleotides, unrelated to the RSS sequence (the top strand is 5’-CTTCAAACCATCCAAATACGGCTGACTGGTGAGCTGACTACCTCCAGGGCTGACACCCCCAATCCTAGGGTTGAGCTGACT-3’), were added. End labeled DNA (~8nM, 0.6 - 1.2 x 10^{18} cpm/mol) was then added and further incubated for 10 min. To isolate the complex, streptavidin-coated magnetic beads (Dynabeads M-280) (10µg/µl) were added, and the reaction mixture was incubated for 15 min at 37°C with occasional mixing. After incubation, magnetic beads and supernatant were separated using a magnet stand. The beads were washed six times at room temperature with 60µl of binding buffer, and then resuspended in 30µl of binding buffer. The washed magnetic beads and the supernatant were subjected to DNaseI footprinting.

**DNaseI footprinting**. DNA samples were pre-heated at 37°C for 4 min, and digested with 5 units of DNaseI (Stratagene) for 4 min. DNA was extracted once with phenol/chloroform/isoamylalcohol (25:24:1), precipitated with ethanol, and washed with 70% ethanol. Samples were dissolved in formamide dye mix and electrophoresed in an 8% denaturing polyacrylamide gel.

**Incorporation of dsDNA into the SE complex**. The SE complex was reconstituted with unlabeled (8nM) or ^32^P-labeled 12-SE DNA (~8nM, 0.6 - 1.2 x 10^{18} cpm/mol), and biotinylated 23-SE DNA (10nM) as described above. The SE complex was purified with magnetic beads, and then incubated with end-labeled dsDNA of various ends (~8nM, 0.6 - 1.2 x 10^{18} cpm/mol) in the presence of Ca^{2+} for 30 min at 37°C. The dsDNA was
constructed by annealing the 40nt top strand of the coding region with the complementary strand. The top strand is labeled at the 5'-end and has either an OH or PO₄ at the 3'-end. After incubation, the bead-bound SE complex was washed several times. DNA was extracted from the bead-bound SE complex and supernatant, separately. Samples were electrophoresed in an 8% denaturing polyacrylamide gel.

**Incorporation of dsDNA into the hybrid complex** - Biotinylated RSS DNA (8nM) was incubated with RAG1 and RAG2 proteins (4µg/ml) and human HMG1 protein (Sigma) (8µg/ml) at 37°C for 10min in 20µl of the binding buffer, as described above. The resulting complex was isolated with streptavidin-coated magnetic beads (Dynabeads M-280) (10µg/µl). End-labeled dsDNA (~8nM, 0.6 - 1.2 x 10¹⁸ cpm/mol) and partner SE or RSS DNA (~8nM, 0.6 - 1.2 x 10¹⁸ cpm/mol) were added and the incubation was continued for another 10 min. After incubation, magnetic beads and supernatant were separated using a magnet stand. The beads were washed with 60µl of binding buffer, six times at room temperature, and then resuspended in 30µl of binding buffer. DNA was extracted from the bead-bound complex, and electrophoresed in an 8% denaturing polyacrylamide gel.
RESULTS

Protection of the coding region in the pre-cleavage synaptic complex—We reconstituted three types of pre-cleavage complexes with unnicked, hemi-nicked, or nicked pair of synthetic 12- and 23-RSSs, both containing identical coding sequences of 40 nucleotides (Fig.1A). Each nick was introduced into the coding/7mer boundary on the top strand of the RSS DNA. Each complex contained $^{32}$P-labeled 12-RSS, biotinylated 23-RSS, and RAG1, RAG2 and HMG1 proteins. To prevent the double strand cleavage of nicked substrates, the complex was formed in the presence of Ca$^{2+}$, and the 3’-OH at the nick was reduced to the 3’-deoxy form. The synaptic complex was isolated with streptavidin-coated magnetic beads as described (48). In Fig.1B, proportions of the $^{32}$P-labeled 12-RSS incorporated into the synaptic complex are compared. Interestingly, the complex formation was facilitated by nicking the top strand, but not the bottom strand. It should be noted that such DNA nicked on the top strand is representative of the RSS cleavage intermediate during V(D)J joining (20). No enhancement was observed by the nick on the bottom strand (data not shown).

We then analyzed the bead-isolated synaptic complex by footprinting (Fig.2). In the 12-RSS, protection was not restricted to the signal region, but was extended into the coding region up to the 12th nucleotide from the 7mer (Fig.2A). When the 12-RSS was nicked at the coding/7mer border on the top strand, the protection was much stronger over all, and it extended 16th nucleotides into the coding region. In the signal region, the footprint pattern was more similar to that of the 12-SE in the SE complex. Basically the same
results were obtained with the 23-RSS (Fig. 2B). It is quite striking that the coding region protection patterns of the 12- and 23-RSSs are virtually indistinguishable from each other. This observation suggests that the two coding regions within the synaptic complex interact with the RAG proteins in basically the same manner. This is in great contrast to the protection patterns of the signal regions, where the interactions with the RAG proteins are asymmetrical between the 12- and 23-RSSs (48). We also analyzed the hemi-nicked complex, in which either the 12- or the 23-RSS is nicked. Fig. 2C shows footprint patterns of two types of hemi-nicked complexes: one where the substrate 12-RSS is nicked, and the other where the partner 23-RSS is nicked. The effect of nicking on protection appears to act in cis, but not in trans. Nicking on the bottom strand did not show any enhancement in protection (data not shown). We also analyzed three other variations of coding sequences for both 12- and 23-RSSs. The protected areas were roughly the same, despite the change in nucleotide sequences (data not shown).

Synaptic complex formation with the RSSs containing the shorter coding regions—We then studied whether the length of the coding region affects the synaptic complex formation. $^{32}$P-labeled 12-RSSs with coding sequences of various lengths (Fig. 3A) were paired with the biotinylated 23-RSS (nicked) containing the 40bp coding region. As shown in Fig. 3B, when the coding regions were longer than 15bp, the 12-RSS was incorporated into the synaptic complex at a level comparable to that of the original coding sequence of 40bp. When the coding region was shortened to 10bp, the complex formation was lowered, and even further when it was 6bp (Fig. 3B). These results indicate that at least 15bp is necessary for the coding region to be incorporated efficiently into the hemi-nicked synaptic
complex. This is consistent with the observation that about 12bp of coding region, proximal to the 7mer, is protected in the DNaseI footprinting (Fig.2). Curiously, however, when the coding region was shorter than 6bp, incorporation of the 12-RSS increased (Fig.3B). We speculate that the 12-RSS with an extremely short coding sequence behaves like an SE DNA in the SE complex where the 7mer end appears to facilitate the complex formation (48).

Protection of dsDNA incorporated into the post-cleavage SE complex—The double strand cleavage in V(D)J recombination generates a hairpin structure at the CE and a blunt end at the SE in the synaptic complex. After the cleavage of the two RSSs, the SEs as well as the CEs appear to remain, at least transiently, in the post-cleavage complex (39, 40, 43, 44) for further processing. To study how the RAG proteins would interact with the coding region after the cleavage, we examined the reconstitution of various post-cleavage complexes (Fig.4A). The bead-isolated SE complex was incubated under the Ca\(^{2+}\) condition with \(^{32}\)P-labeled dsDNA (as artificial CE DNA) with different right-end structures: hairpin, 3'-OH, and 3'-P (Fig.4B). The radioactivity of the SE complex after washing was measured. The dsDNA with a hairpin structure or 3'-OH end was retained in the complex, but at low levels. In contrast, dsDNA with 3'-phosphorylated end is incorporated into the SE complex efficiently (Fig.4B).

Since the 3'-phosphorylated dsDNA was shown to bind to the SE complex, we performed footprinting to examine whether the interaction is specific and how the dsDNA is bound to the complex. As shown in Fig.4C, each strand was analyzed in both directions. About 16 nucleotides proximal to the right-side end (3'-phosphorylated side) of the dsDNA
were protected on the both bottom and top strands. It should be noted that no protection was seen on the other side of the dsDNA, where the 3'-end has an OH group. The 3'-phosphorylated dsDNA may occupy the same site as the coding region of the RSS DNA does in the pre-cleavage synaptic complex. Supporting this idea, the 3'-phosphorylated dsDNA was incorporated into the hybrid complex with the SE DNA, but not into the pre-cleavage complex with the uncleaved RSS DNA (Fig.5A,B). This observation supports the idea that the 3'-phosphorylated dsDNA occupies the same site in the synaptic complex as does the coding region of the uncleaved RSS DNA. This notion was confirmed by DNaseI footprinting. Protection patterns of the 3'-phosphorylated dsDNA in the SE and hybrid complexes resembles that of the coding region in the RSS DNA (Fig.5C). Both the 12-coding and the 23-coding regions were analyzed for interactions with RAG protein using the 12- and 23-SEs, respectively. These results indicate that the 3'-phosphorylated dsDNA retained in the SE or in the hybrid complex, interacts with the RAG proteins in a manner similar to that of the RSS coding region in the pre-cleavage synaptic complex.

RAG mutants that alter the coding interaction in the synaptic complex—It has been proposed that the RAG proteins may be involved in the post-cleavage phase of V(D)J recombination (28, 30, 39, 41-44, 45-47). RAG mutants capable of cleaving the substrate, but defective in the joining process have been isolated for each RAG1 and RAG2 protein (41-43, 45). To examine whether such mutants show any alterations in their ability to interact with RSS DNA, we reconstituted the synaptic complex with the mutant RAGs and examined the protection of the signal and coding regions (Fig.6). Footprint analysis
revealed that the SE DNA is protected normally in the SE complex containing either the mutant RAG1 (S723C) (43) or the mutant RAG2 (K118A/K119A) (41) (Fig. 6A). However, when the pre-cleavage complex was analyzed, protection in the coding region was significantly reduced with the joining mutants (Fig. 6B). In contrast, the cleavage mutants of RAG1, e.g., E962A (49), showed no reduction of coding region protection (Fig. 6B). We then studied the reconstitution of the post-cleavage type complex, using the joining mutant proteins, since their ability to interact with the coding region was shown to be impaired by footprinting (Fig. 6C). The SE complex was formed first, bead-isolated, and then incubated with the 3'-phosphorylated dsDNA as an artificial CE. As shown in Fig. 6C, the SE complex containing the joining mutant S723C or K118A/K119A, but not the cleavage mutant E962A, failed to incorporate the 3'-phosphorylated dsDNA. The wild-type SE complex retained roughly the same amount of dsDNA as SE. SE DNA stably stayed in the complex even with the joining mutant proteins. The loss of the coding-region interaction may explain, at least in part, the defective phenotype of the joining mutants, S723C and K118A/K119A, in the post-cleavage phase of recombination.
DISCUSSION

In the present study, we have demonstrated that RSS DNA is protected not only in the signal region but also in the adjacent coding region in the synaptic complex. In contrast to the signal regions, protection patterns in the coding regions are indistinguishable between the 12- and 23-RSSs (Fig.2A,B and Fig.5C), suggesting that the mode of interaction with RAG proteins in the coding region are basically identical for the 12- and 23-RSSs.

Footprint analysis revealed that the nick on the top strand, but not on the bottom, facilitates the interaction of RSS DNA with the RAG proteins (Fig.2). It appears that nicking of the RSSs leads to a more stable structure of the synaptic complex. Such a structural change may help activate the next cleavage reaction, i.e., double-strand cleavage/hairpin formation. It is possible that nicking at the 7mer end provides more flexibility for the substrate DNA to interact with the RAG proteins in the signal and coding regions on the same molecule. It is also possible that the nicked 7mer on the top strand, which represents a natural cleavage intermediate, may resemble the SE in structure and interaction with RAGs: The SE shows higher affinity to the complex than the 7mer in the intact RSS.

In the single RSS-RAG complex, before the synaptic complex is formed, only a few nucleotides adjacent to the 7mer are protected by the RAG proteins. Our present study demonstrated that much larger coding region, at least 12bp, is protected in the synaptic complex. What causes such a drastic change in the interaction, and what would be a possible role of the coding-RAG interaction? When we analyzed the synaptic complex...
containing the shorter coding sequence for the coupled cleavage, we found that the RSS DNA with a 6bp coding region was efficiently cleaved (data not shown). This is consistent with the previous observation by others (22). In contrast, the longer (>12bp) coding sequence was necessary for the stable formation of the synaptic complex (Fig.3B), and at least 12bp coding region was protected in the pre-cleavage synaptic complex (Fig.2). As mentioned above, when the RSS DNA contained the coding region of 6bp, the complex formation was at the lowest level, but the cleavage took place in the isolated complex at more or less the same level as the control (40bp coding). Curiously, when the coding sequence was further shortened, RSS DNA was not nicked (our unpublished observation), but, the synaptic complex formation was increased (Fig.3B). There seems to be two types of interactions for the RSS DNA in the synaptic complex: one is the SE type and the other is the pre-cleavage type. Pre-cleavage type complex requires the coding region, while the SE type complex requires the 7mer sequence near the end. The SE type complex is inactive for the nicking. We also examined the effect of the coding sequence on the complex formation. Four different coding sequences were analyzed by footprinting for protection in the both 12- and 23-RSSs. In all eight samples, the same extent of the coding region was protected regardless of their sequences. It has been reported that not only the recombination signals but also some coding sequences are involved in the regulation of V(D)J recombination (50). Our present results support such a notion.

It has been proposed that the RAG proteins play an architectural role in holding the substrate DNA or cleaved ends in the synaptic complex (39, 40, 43). This notion was supported in our present study not only by the footprint analysis but also by the binding
experiment with dsDNA. We found that the SE complex efficiently incorporates the dsDNA when its 3’-end is phosphorylated (Fig. 4B). Since the footprint pattern of dsDNA is analogous to that of the coding region of the pre-cleavage complex, it is unlikely that the binding of dsDNA to the post-cleavage complex is due to nonspecific interaction. The transposition or the target capture activity does not explain the incorporation of the 3’-phosphorylated dsDNA into the SE complex, because the incorporation is so efficient: by these activities, the dsDNA with a hairpin structure or 3’-OH end could be incorporated into the complex, but with low efficiency (Fig. 4B). Why is the dsDNA incorporated so efficiently into the SE complex only when the 3’-end is phosphorylated? Is the 3’-end of the processed CE phosphorylated in vivo? Although some nucleases are known to generate the 3’-phosphorylated end (51), involvement of such enzymes in V(D)J joining is not yet clear. Both Artemis and RAG proteins may be involved in the CE processing. However, these proteins produce the 3’-OH end after the 3’-end processing (36, 46, 52). After the double strand cleavage, repair factors such as DNA-PKcs, Artemis, Ku70/80, LigaseIV, and XRCC4 are recruited to the cleaved ends. These repair factors may help to maintain the cleaved OH ends in the post-cleavage complex. In our in vitro system, it is possible that the 3’-phosphate of dsDNA may mimic such in vivo interactions, at least in part, although further studies are needed to clarify this issue.

The RAG1 mutant S723C, defective in the joining process, is known to have a reduced hairpin retention after the coupled cleavage (43). Our present results demonstrated that the SE complex containing the S723C protein failed to incorporate the 3’-phosphorylated dsDNA. In the footprint analysis, protection of the coding region was reduced in the
pre-cleavage complex containing the S723C RAG1. It is possible that the low hairpin retention by the mutant protein is due to the reduced interaction with the coding region DNA. Similar observations were made with the RAG2 joining mutant, K118A/K119A. It has been reported that the amino acid residue K119 in the RAG2 protein may contact with the substrate DNA, using the phosphate backbone rather than specific bases (53). This is consistent with our observation that the protection of the coding region occurs in a sequence independent manner. Although the exact nature of the mutants' defect is still unknown, the failure to retain the coding sequence in the post-cleavage complex may be reason for the impaired joining observed with mutant proteins. Our present results suggest that the coding-region interaction with the RAG proteins is important not only in the pre-cleavage, but also in the post-cleavage process of V(D)J recombination.
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FIGURE LEGENDS

FIG. 1. **Pre-cleavage synaptic complexes in V(D)J joining.**  
*A,* Three types of pre-cleavage complexes are schematically shown; unnicked, hemi-nicked and nicked. Triangles are recombination signal sequences (12- and 23-RSSs).  
*B,* Synaptic complex formation is facilitated by a nick at the coding/7mer border.  
$^{32}$P-labeled 12-RSS DNA was paired with the biotinylated (BIO) 23-RSS, and complexed with (+RAG1/2) or without (-RAG1/2) the RAG1 and RAG2 proteins in the presence of the DNA bending protein, HMG1.  
The complex was isolated with streptavidin-coated magnetic beads.  After washing, $^{32}$P counts incorporated into the complex were measured and divided by the input counts (% bound).  Presence (+) or absence (-) of nicking is indicated.

FIG. 2. **Footprint analysis of RSS DNA in the pre-cleavage synaptic complex.**  
*A,* The 12-RSS was analyzed for the protection of the coding region in the nicked and unnicked synaptic complexes.  
$^{32}$P-labeled 12-RSS was paired with the biotinylated (BIO) 23-RSS, and incubated with RAG1, RAG2, and HMG1 proteins in the presence of Ca$^{2+}$.  
The substrate (12-RSS) and the partner (23-RSS) DNAs were both nicked or both unnicked. The 12-SE in the SE complex was analyzed as a control.  
*B,* The 23-RSS was analyzed in the same manner as in A.  
*C,* The 12-RSS in the hemi-nicked synaptic complex was analyzed by footprinting.  
$^{32}$P-labeled unnicked or nicked 12-RSS was paired with nicked (+) or unnicked (-) biotinylated (BIO) 23-RSS.  Positions for the nick (\(\downarrow\)) and $^{32}$P-label (*) are indicated.
FIG. 3. **Synaptic complex formation with various coding DNAs.**  

**A,** The 12-RSS DNAs containing short coding regions. Top-strand sequences are compared. The coding regions lengths are shown on the left (nt). The 7mer and 9mer signals are underlined.  

**B,** Formation of the hemi-nicked synaptic complex. ^32^P-labeled 12-RSS was paired with a nicked, biotinylated (BIO) 23-RSS containing a 40bp coding sequence. The complex was isolated with streptavidin-coated magnetic beads. After washing, ^32^P counts incorporated into the complex were measured and divided by the input counts (%). Three independent experiments are performed, and both the average and the standard deviation were shown.

FIG. 4. **Footprint analysis of dsDNA incorporated into the post-cleavage SE complex.**  

**A,** Schematic diagrams of three post-cleavage complexes. Triangles are recombination signals. Double strand cleavage of RSS generates a hairpin structure at the coding end (CE) and a blunt end at the signal end (SE). The hairpin CE is opened, processed, and then ligated, generating the coding joint. The SEs stay in the SE complex until the signal joint is formed.  

**B,** Binding of dsDNA to the SE complex. ^32^P-labeled dsDNAs were incubated with the bead-isolated SE complex in the presence of Ca^{2+}. ^32^P counts that persist with the SE complex were measured and divided by the input counts (%). Binding to the beads without RAG complex is a negative control. DsDNA with different structures at the right end were examined: hairpin, 3'-OH and 3'-P.  

**C,** DNaseI protection of the 3'-phosphorylated dsDNA bound to the SE complex. The 3'-end was phosphorylated on the top strand in the dsDNA of 40bp. Input DNA and supernatant (sup) after the wash were footprinted in parallel as controls. Both the top and bottom
FIG. 5. **The 3’-phosphorylated dsDNA incorporated into the synaptic complex.**  

A, The 3’-phosphorylated dsDNA is incorporated into the synaptic complex with the SE, but not with the RSS. Biotinylated (BIO) RSS (12- or 23-alone) was complexed with the RAG1, RAG2, and HMG1 proteins in the presence of Ca\(^{2+}\) to form the single-RSS complex. The bead-isolated complex was then incubated with the \(^{32}\)P-labeled partner RSS or SE in the presence of \(^{32}\)P-labeled 3’-phosphorylated dsDNA. The resulting synaptic complex was re-isolated with magnetic beads. After washing, DNA was extracted from the complex and electrophoresed in an 8% polyacrylamide gel. The 3’-phosphorylated dsDNA was detected in the synaptic complex with the SE (hybrid complex), but not with the RSS (pre-cleavage complex).  

B, Schematic diagrams of the pre-cleavage, hybrid and SE complexes. Triangles represent recombination signals.  

C, DNaseI protection of the 3’-phosphorylated dsDNA incorporated into the synaptic complex. Both the SE and hybrid complexes were analyzed. RSS DNA was also analyzed for the protection of the coding region in the hybrid and pre-cleavage complexes. The dsDNA sequence was attached to the 12- and 23-signals as their coding regions. Biotinylated (BIO) partner RSSs or SEs are shown.

FIG. 6. **Interaction of RSS DNA with the mutant RAG proteins.**  

A, Protection of the signal region. The 12-SE in the SE complex was analyzed by DNaseI footprinting. \(^{32}\)P-labeled 12-SE and the biotinylated 23-SE were complexed with four different
combinations of RAG1 and RAG2 proteins: wild-type RAG1 and wild-type RAG2 (WT); wild type RAG2 and mutant RAG1 (S723C); wild-type RAG1 and mutant RAG2 (K118/9A); and wild-type RAG2 and mutant RAG1 (E962A). The complex was formed in the presence of HMG1 and Ca^{2+}, and isolated with magnetic beads. Footprint patterns of the signal regions are indistinguishable between wild-type and mutant-type complexes.

**B,** Footprint analysis of the pre-cleavage synaptic complexes. 32P-labeled 12- and biotinylated 23-RSSs (both nicked) were complexed with four different combinations of RAG1 and RAG2 proteins as in **A.** Positions for the 32P-label (*) and nick (↓) are indicated. Lossed protection in the coding region is indicated by dotted lines. **C,** Incorporation of the 3'-phosphorylated dsDNA into the SE complex. 32P-labeled 12-SE and biotinylated 23-SE were complexed with RAG1 and RAG2 proteins in four different combinations. The bead-isolated complex was incubated with the 32P-labeled 3'-phosphorylated dsDNA. The complex was re-isolated with magnetic beads and washed. DNA was extracted from the complex and electrophoresed in an 8% polyacrylamide gel.
A
Pre-cleavage complexes

Unnicked → Hemi-nicked → Nicked

▶ 12-RSS, ▼ 23-RSS

B

% bound

+RAG1/2

20
15
10
5
0

12-RSS (32P)
23-RSS (BIO)

- + - + +
- - + + +

nicking

Fig. 1.
A

12-RSS ($^{32}$P)

coding region

| 7mer | 12nt | 9mer |
|------|------|------|
| GCTGACACAGTGTCCTGCCAGGGCTGAACAAAACCCTCTAGGGTTGCAGCTGACT | | |
| ATTCGACAGTGTCCTGCCAGGGCTGAACAAAACCCTCTAGGGTTGCAGCTGACT | | |
| GTCTTACACAGTGTCCTGCCAGGGCTGAACAAAACCCTCTAGGGTTGCAGCTGACT | | |
| TACACAGTGTCCTGCCAGGGCTGAACAAAACCCTCTAGGGTTGCAGCTGACT | | |
| ACACAGTGTCCTGCCAGGGCTGAACAAAACCCTCTAGGGTTGCAGCTGACT | | |
| CACAGTGTCCTGCCAGGGCTGAACAAAACCCTCTAGGGTTGCAGCTGACT | | |
| (nt) | 40 | 15 | 10 | 6 | 3 | 1 | 0 |

B

Fig. 3.
Fig. 4.
Fig. 6.
Joining mutants of RAG1 and RAG2 demonstrate impaired interactions with the coding-end DNA
Fumikiyo Nagawa, Satoshi Hirose, Hirofumi Nishizumi, Tadashi Nishihara and Hitoshi Sakano

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