RAG1 and RAG2 Cooperate in Specific Binding to the Recombination Signal Sequence in Vitro*

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An essential step in the development of the vertebrate immune system is the DNA level rearrangement of the antigen receptor genes. This process, termed “V(D)J recombination,” begins with DNA cleavage at the appropriate sites mediated by the two proteins RAG1 and RAG2. We report here that the two proteins cooperate to bind DNA with significantly higher specificity than either protein alone. Gel purification of the triple complex is performed in the absence of any cross-linking agents. Both proteins remain present in the complex, and UV cross-linking using iodouridine-containing probes shows that RAG1 makes close contacts in both the heptamer and nonamer motifs. The two proteins are also shown to associate with each other in the absence of any DNA. These findings refine our understanding of the protein-DNA interactions that accompany cleavage at the recombination signals.

The antigen receptors expressed on B-cells and T-cells in animals are encoded by genes that are assembled at the DNA level from arrays of inherited segments in a process termed “V(D)J recombination” (reviewed in Refs. 1 and 2). Targets for this recombination reaction are identified by recombination signal sequences (RSSs) immediately adjacent to the recombination site in the DNA. The RSS is composed of conserved heptamer (CACAGTG) and nonamer (ACAAAAACC) motifs, separated by a spacer of either 12 or 23 base pairs in length. Recombination in vitro occurs between pairs of DNA segments bordered by RSSs of the two different spacer lengths (the 12/23 rule; Ref. 3). Similar segments always carry RSSs of the same length, and as a consequence of this organization, a V segment (for example) could be permitted to join to a D segment in the same locus, but not a second V segment.

V(D)J recombination occurs through a cutting and pasting mechanism in which specific double-strand DNA breaks are generated adjacent to each RSS. The first step in this reaction, cleavage of DNA containing an RSS, can be reconstituted in vitro (4, 5) in a reaction that requires the two proteins RAG1 and RAG2 (6, 7). Cleavage at either of the RSSs alone will occur with Mn²⁺ as divalent cation cofactor (8). This coupled reaction is stimulated by the nonspecific DNA bending protein HMG1 (9). The biochemistry of the cleavage reaction has been studied by means of the expression and purification of truncated “core” regions of these proteins, which maintain all the activities currently measurable.

The specificity in the reaction must be derived by direct contacts between the RAG proteins and the RSS. The individual roles of the proteins have not been fully explored, although several studies have implicated RAG1 in binding to the RSS. Previously, we showed an alteration in the specificity of recombination by a RAG1 mutant, which suggested that RAG1 could interact with the heptamer and the immediately adjacent coding DNA (10). The binding of RAG1 and RAG2 to a reiterated RSS target was tested in a one-hybrid assay in mammalian cells (11). RAG1 bound the DNA with specificity for the nonamer, and a mild enhancement of binding was found in the presence of the heptamer. RAG2 would also bind the RSS, but only in the presence of RAG1. DNA binding in vitro by RAG proteins was assayed by surface plasmon resonance, which showed RAG1 binding to the nonamer, a low affinity interaction with the heptamer, and no binding by RAG2 under the high salt conditions studied (12). Protein-DNA interactions were also observed in complexes cross-linked by glutaraldehyde (13); however, no binding by RAG1 alone was observed.

Because our mutant studies had suggested the importance of the heptamer in mediating recombination by RAG1, and physical approaches have demonstrated only slight heptamer effects in RAG1 binding, we were motivated to explore, in more detail, the contacts between RAG1 and target DNA alone, and in the context of a protein complex containing RAG2. We chose to examine the behavior of RAG1 and RAG2 individually and together using gel mobility shift and UV cross-linking. Here we report that RAG1 and RAG2 proteins, purified individually, can associate to form a protein complex in the absence of DNA. We also find that in an electrophoretic mobility shift assay (EMSA), each protein by itself can bind DNA, albeit at low specificity. However, together the two proteins and DNA form a different complex with significantly higher specificity for the RSS. Sequence alteration of the oligonucleotides reveals that the complex is dependent on the presence of the nonamer motif, and somewhat more binding occurs when the heptamer is also present. Both proteins are present in the ternary complex. Most important, using a series of probes substituting iodouracil at individual positions, we find that UV exposure of the mixed protein complex generates cross-links to RAG1 not only in the nonamer but also in the heptamer, while RAG2 is not cross-linked. The interaction of RAG1 at both the nonamer and the heptamer alters our view of the protein-DNA complex that must form during cleavage. The precise configuration and biochemical roles of these proteins in the cleavage step and subsequent processing and joining steps remain to be determined.

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* The abbreviations used are: RSS, recombination signal sequence; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; MBP, maltose-binding protein; MOPS, 4-morpholinepropanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Proteins—Baculovirus stocks for MR1 and MR2 were obtained from Martin Gellert (National Institutes of Health, Bethesda, MD), and proteins produced as described previously (5). MR1 and MR2 are fusion proteins, each containing an N-terminal maltose-binding protein (MBP), followed by the functional core region of mouse RAG1 (residues 384–1008) or RAG2 (residues 1–587) respectively. The C termini carry a poly-histidine tag, created by three tandem copies of the c-Myc epitope tag as used previously (14). The related baculovirus-produced protein, here designated R1, is identical to MR1 but for the absence of the MBP fusion partner. The virus was constructed by M. S. in collaboration with Joanne Hesle while in the laboratory of Martin Gellert. The protein produced was purified by metal chelate chromatography and, where appropriate, by amylase affinity, from Sf9 cells, and all proteins were stored in a buffer containing 25 mM Tris-HCl (pH 8.0), 150 mM KCl, 2 mM dithiothreitol, and 50% glycerol. Proteins purified through one chromatography step were 90% pure while double-affinity-purified proteins were homogeneous on Coomassie Brilliant Blue-stained gels.

Binding and Cleavage Assays—Binding mixture (typically 10 μl) containing 0.02–0.1 pmol of 32P-labeled oligonucleotide substrate DNA in 25 mM MOPS (pH 7.0), 10 mM MgCl2, 2 mM dithiothreitol, 50 mg/ml bovine serum albumin, 16% dimethyl sulfoxide, and 50 mM KCl. Non-specific DNA pdf-dCpdf-dC (Amersham Pharmacia Biotech) was added to 50 μM final concentration in most reactions, but to only 5 μg/ml in reactions containing RAG2 alone, unless otherwise indicated. Each RAG protein was usually added in the range of 10–200 ng per reaction. Typically, binding reactions were assembled on ice, with protein always the last added component and incubated on ice for 10 min. To each reaction containing binding buffer (without dithiothreitol), 50% glycerol, 0.07% bromphenol blue, and 0.01% xylene cyanol) was added, and samples were analyzed by electrophoresis through a polyacrylamide gel using a Tris borate buffer system. Probes were detected by autoradiography and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software (version 2.1).

Cleavage was assayed under binding conditions except for the substitution of 1 M NaCl in place of the MgCl2, and reactions were incubated for 1 h at 37 °C. Products were analyzed by electrophoresis through 8% polyacrylamide gel using a Tris borate-EDTA system.

MBP “Pull-down” Assay and Western Blotting—The proteins were incubated in DNA binding buffer for 10 min on ice and centrifuged at 15,000 rpm for 5 min at 4 °C. The supernatants were bound to amylose resin cubated in DNA binding buffer for 10 min on ice and centrifuged at 15,000 rpm for 5 min at 4 °C. The supernatants were bound to amylose resin cubated in DNA binding buffer (without dithiothreitol) for 5 min on ice in a 12-μl volume. UV exposure was performed on ice for 20 min at 3 cm from a 6-watt UV lamp equipped with 302 nm filter. Samples for gel analysis were adjusted to 1% SDS, 0.1 M dithiothreitol, 10% glycerol and heated to 70 °C. Sample were analyzed on 6% PAGE, Tris borate-EDTA gels.

RESULTS

It has been shown previously that the truncated forms of RAG1 and RAG2 used here are active in V(D)J recombination and capable of supporting the complete reaction in cells (14–16). Furthermore, these truncated forms are more easily expressed in the baculovirus system, purified, and assayed for cleavage activity in vitro. Most commonly these proteins are expressed as fusions to the bacterial MBP, which increases the protein solubility and provides an affinity purification method. It should be noted that the truncated RAG1 is entirely lacking in a N-terminal zinc-binding ring-finger motif and the RAG2 lacks a C-terminal acidic region. A function for these regions in the reaction mechanism has yet to be described. Previous studies have coexpressed the two truncated proteins in a single mixed infection allowing the copurification of RAG1 and RAG2 (5). We felt it would be useful to express the proteins individually so as to better characterize their behaviors and perhaps to demonstrate that a mixed complex could form from purified components. The activity of these proteins was demonstrated by testing them individually and together in the oligonucleotide cleavage assay (5). Only the combination of the two proteins cleaved the end-labeled oligonucleotide to liberate the shorter hairpinned coding end (Fig. 1A).

RAG1 or RAG2 Alone Binds DNA Nonspecifically—DNA binding was demonstrated for each of these individual proteins by EMSA. In the absence of competitor DNA, both proteins bound probes in a manner suggestive of nonspecific binding. We observed that, as either protein alone was titrated into the binding reaction, complexes were formed with slower mobility, ultimately creating large aggregates that were trapped in the sample well (data not shown). This behavior is typical of DNA binding that is not specific for sequence, and indeed this proved to be the case when tested with probes of differing sequence. Discrete bands could be obtained by adding the nonspecific competitor dI-dC, at 50 or 5 μM, respectively, for MR1 and MR2. Fig. 1D shows the DNA binding behavior of each protein with a variety of RSS-related probes under these conditions. For MR1, binding is detected with all probes, though is some what reduced (about 2-fold in these and other data not shown) and MR2 probes in which the hexamer was notomer, or both are fully replaced (lanes 2–4) compared with the intact 12-RSS (lane 1). Similarly MR2 does not exhibit a significant difference in binding to these different probes. No change in specificity was observed with additional probes containing either 12-RSS or 23-RSS related sequences (data not shown). It is worth noting that, at higher concentrations of dI-dC than shown in

Substrates with dU (listed as U) are as follows (antisense strand shown, sense strand is all normal DNA): 3: 5’-GGAGGACCTCGAG GTTTTTTCT TCCAGTCGTTAG CACTG TGTAATGGCAGCGC-3’; 4: 5’-GGAGGACCTCGAG GTTTTTTCT TCCAGTCGTTAG CACTG TGTAATGGCAGCGC-3’; 5: 5’-GGAGGACCTCGAG GTTTTTTCT TCCAGTCGTTAG CACTG TGTAATGGCAGCGC-3’; 6: 5’-GGAGGACCTCGAG GTTTTTTCT TCCAGTCGTTAG CACTG TGTAATGGCAGCGC-3’; 7: 5’-GGAGGACCTCGAG GTTTTTTCT TCCAGTCGTTAG CACTG TGTAATGGCAGCGC-3’; 8: 5’-GGAGGACCTCGAG GTTTTTTCT TCCAGTCGTTAG CACTG TGTAATGGCAGCGC-3’; 9: 5’-GGAGGACCTCGAG GTTTTTTCT TCCAGTCGTTAG CACTG TGTAATGGCAGCGC-3’.
Fig. 1. A, individually purified MR1 and MR2 do not cleave the oligonucleotide substrate duplex oligo-245, which contains a 12-RSS (lanes 1 and 2). Combining these purified proteins restores cleavage activity (lane 3). DNA from reactions are displayed here by PAGE. Lane 4 is a mock reaction without protein. B, MR1 (lanes 1–5) or MR2 (lanes 6–8) individually bind DNA with low specificity. The proteins bind to each of the probes in this EMSA analysis. Competitor dI-dC was present in these reactions at 50 μg/ml for MR1 and 5 μg/ml for MR2. This figure is a composite of lanes from a single gel for each panel.

Fig. 2. MR1 plus MR2 together form a complex with higher specificity for the RSS. A, in the absence of dI-dC, MR1 forms a nonspecific smear on both 12-RSS (duplex oligo-245, lane 2) or the 23-RSS (duplex oligo-36, lane 5). MR1 plus MR2, at the same concentration, forms a new discrete band of different mobility, whether mixed as isolated proteins or purified from a mixed infection (lanes 3, 4, and 6). B, titration of increasing amounts of MR1 + MR2 in the presence of dI-dC competitor (50 μg/ml) shows preferred binding to 23-RSS probe (duplex oligo-36, lanes 2–4) over the probe in which the nonamer is replaced (duplex oligo-40, lanes 8–10). The probe in which the heptamer is replaced (duplex oligo-38, lanes 5–7) shows binding more like the first probe. Each protein is at the level indicated in μg/ml. C, graph of the binding data from panel B.

Both Proteins Together Bind DNA Specifically—The behavior changed markedly when MR1 and MR2 together were tested by EMSA. Even in the absence of dI-dC (Fig. 2A), the mixed proteins formed a DNA complex that migrated more discretely and at different mobility than those produced by the individual proteins. Complexes with similar mobility assembled on probes containing either a 12- or 23-RSS. We note here that MR1 plus MR2 generated the same binding pattern whether the proteins were purified individually from separate infections and then mixed (lane 3) or copurified from coinfecected insect cells (lane 4). In the presence of dI-dC competitor (50 μg/ml), the faster migrating species predominated. Furthermore, a stronger sequence dependence was revealed when the mixed proteins were bound to the probes of altered sequence (Fig. 2B). In this titration experiment, increasing amounts of the two proteins were bound to each of three related probes. Binding to a probe in which the nonamer had been extensively mutated was much weaker than to the other two probes. The results are quantified in Fig. 2C. A set of similar 12-RSS probes, when tested with RAG1 plus RAG2 protein together, also showed binding with specific dependence on the intact nonamer (data not shown).

We performed competitive binding experiments to determine the relative affinities of the MR1 plus MR2 complex for the canonical signals over those in which the heptamer or nonamer was altered. Binding to a 12-RSS probe is shown (Fig. 3A) with competition by the same unlabeled DNA, and with related DNA in which the heptamer or nonamer was entirely replaced. Similarly, binding to a 23-RSS probe was performed with competition by the same DNA or related heptamer and nonamer replacements (Fig. 3B). Both experiments yielded comparable results. In both cases, the heptamer-mutated DNA competed almost as well as the self-competition by unlabeled probe DNA. Again, in both cases, the nonamer-mutated DNA competed significantly less well. Comparing the sets of data shows that the MR1 plus MR2 complex bound the intact RSS between 5-
and 10-fold better than the equivalent DNA lacking the nonamer. A weak but consistent effect was also apparent favoring binding to the intact RSS over the heptamer mutants.

Since a fairly strong dependence on the nonamer was evident in this assay, we explored the effect of more modest alterations in the sequence. These EMSAs were performed at a fixed protein concentration with a series of 12-RSS probes that differed by only two base pairs each and with dI-dC as competitor. Fig. 3 shows a comparison of the intact 12-RSS (lane 4) with three probes in which the nonamer was modified at its first two base pairs, the second two, or positions 6 and 7. The results show that each of these nonamer mutations decreased the binding of the protein complex, with the most profound effect found at the sixth and seventh positions of the nonamer.

**RAG1 Associates with RAG2 in Vitro**—We wished to describe this new complex that assembled in the presence of both proteins. We have shown that MR2 binds DNA only weakly, and it seemed possible that the complex obtained in the presence of both proteins could contain only MR1. The role of RAG2 could be, for example, to catalyze a change in conformation in RAG1 while not remaining integral to the complex. We therefore tested whether MR1 and MR2 could associate stably with each other in the absence or presence of DNA. Fig. 4A is the result of a two-dimensional electrophoretic separation of MR1 and MR2 that had been coincubated in the standard reaction buffer, but in the absence of DNA. Complexes were collected by affinity chromatography to amylose resin, which will retain R1 only if it associates with the other proteins. Retained proteins were eluted and analyzed by Western blot. C, protein analysis of EMSA bands. The specific complex assembled on 12-RSS probe (duplex oligo-245) and 23-RSS probe (duplex oligo-36) were excised, denatured, and analyzed for protein content by Western blotting. Both proteins contain the same epitope tag so the signal is a direct measure of relative protein abundance. Both proteins were present in apparent equimolar representation by densitometric analysis of this image. Lanes marked M are markers.
were applied to one side of the gel during the second run to aid in the identification of the final proteins. Finally, the gel was transferred (through its third dimension) to nitrocellulose and probed with the anti-epitope tag antibody, which binds to both proteins. Displayed on the blot, from right to left, are two prominent bands of MR2 that migrate by themselves (positions marked D and E), followed by a band of MR1, which also migrated alone (position C). These are followed by two sets of spots where MR1 and MR2 appear to migrate coincidentally in the first dimension (positions A and B). The last spots (other than the markers) represent MR1 and MR2 that failed to enter the gel in the first dimension, dissociated upon treatment with SDS, and therefore appear at the position of the origin. The coincident bands (representing approximately 10% of the input protein) are evidence that slower migrating components in the native gel contained both MR1 and MR2.

To demonstrate complex formation by a second approach, we designed the MBP pull-down assay (Fig. 4B). For this experiment, we purified a protein (R1) that was identical to MR1 with its epitope tag, but did not contain the maltose-binding fusion partner. A 3-fold molar excess of R1 over MR2, purified separately, was mixed in the reaction buffer used for the cleavage assay. One sample remained free of DNA, while to the second the 12-RSS DNA (duplex oligo-245) was added. Parallel samples were assembled replacing MR2 with purified MBP (New England Biolabs) to test for any interaction that could occur through the fusion partner. This protein does not contain the c-Myc epitope tag. After incubation on ice, the samples were then exposed to the amylose resin, washed extensively in reaction buffer, and finally eluted in the same buffer supplemented with 10 mM maltose. The eluted fractions were analyzed by Western blot visualized with the anti-Myc antibody. Since the MBP protein itself does not contain the c-Myc epitope tag, it does not appear on the blot. The key observation, however, is that the tagged R1 does appear. Since it does not bind directly to the amylose beads, and interacts minimally with the MBP protein alone (Fig. 4B, lanes 1 and 2), the strong positive R1 band in those lanes that contain MR2 (lanes 3 and 4) indicates that the R1 protein is associating with the MR2 specifically. The association of MR2 with R1 is only slightly increased by the presence of the DNA (lane 4).

We next analyzed the protein content of the gel-shift band that is assembled in the presence of MR1, MR2, the RSS DNA probe, and competitor dI-dC. Preparative amounts of the complexes on both 12- and 23-RSS probes (duplex oligonucleotides 245 and 36) were assembled using copurified MR1 and MR2. The major protein-DNA complex of each was excised from a native gel. Each gel slice was denatured in SDS and the eluted proteins analyzed by Western blotting. Both MR1 and MR2 were found in apparent equal molar ratio in each case (Fig. 4C). The adjacent gel slice from a lane in which both proteins were loaded without DNA yielded only a trace amount of protein when treated the same way (data not shown). Taken together, these data show that each of these proteins derived from RAG1 and RAG2 are capable of binding DNA alone, but that their specificity for each RSS is increased significantly when the two proteins are present simultaneously. Both proteins are present in the specific complex formed on the 12- and 23-RSS. Furthermore, this complex appears to contain an equal molar ratio of the two proteins.

**RAG1 Contacts Both the Heptamer and the Nonamer—**Since specific base changes in the nonamer significantly reduced the binding of MR1 and MR2 in the EMSAs, we wished to use additional methods to detect contacts between the protein and DNA. One such method is UV cross-linking. Ordinary DNA will cross-link to proteins at low efficiency upon UV exposure. We first attempted to cross-link ordinary DNA to MR1 or the mixture of MR1 plus MR2. While cross-linked products dependent on the presence of protein was obtained (at a level of less than 0.1% of the DNA), we could not demonstrate sequence specificity in this interaction and dl-dC quenched the cross-linking to unacceptable low levels (data not shown). We therefore utilized the ability of modified DNA bases to increase the cross-linking efficiency at the site of modification. In these experiments, we used 5-iodouracil in place of thymine at individual positions along one strand of the probe DNA. The substitution of the iodine for the 5-methyl of thymine does not interfere with the normal structure of DNA but increases the reactivity of that base more than 10-fold upon UV exposure. A series of five probes were synthesized of the identical sequence (containing a 12-RSS) save for the positioning of a single 5-IdU residue in place of the normal T on the bottom strand of the DNA as depicted in Fig. 5A. This placed the IdU residues at three positions in the nonamer, at one position in the heptamer, and at one position within the spacer. The substituted strands were end-labeled, assembled into duplex DNA, and used as probes for both EMSA and UV cross-linking assays. The sample was exposed to UV and then split into two fractions. One was analyzed directly under native conditions while the other was disassembled with SDS and heat, then analyzed by SDS-PAGE. Experiments were performed with MR1 alone or with both MR1 and MR2 under the same conditions (including dl-dC competition) that showed specific binding previously (Fig. 2B). In contrast to the UV cross-linking of unnmodified DNA, here we were able to obtain cross-linking efficiencies as high as 1–2%. The EMSA results for both sets of proteins show that equal binding occurred with each probe, as expected (top panels in Fig. 5B). The UV cross-linking with MR1 alone (Fig. 5B, bottom left) confirms the previous interpretation of non-specific binding. The cross-links formed with this protein using each of the probes were essentially equal in intensity across all five positions. This is the expected result if RAG1 is binding non-specifically, and therefore forming a population of com-
plexes with protein distributed equally over the entire DNA probe. Any single position in the DNA would have an equal probability of cross-linking to protein. Contrast this with the behavior of the mixed MR1 plus MR2 gel (Fig. 5B, bottom right). Here, distinct inhomogeneities in cross-linking efficiency exist. Within the nonamer, there is a considerably less cross-linking at the third base pair (probe 3) compared with the adjacent fourth and fifth positions of the nonamer (probes 4 and 5). There is essentially no cross-linking to probe 6, which places the iodine in the spacer. The inhomogeneities in cross-linking are the anticipated behavior of specific binding since, in this case, certain positions should be in close contact with protein and other positions relatively unbound.

Most interesting is the strong signal obtained with probe 7. This probe is substituted at the second position of the heptamer. It is apparent, therefore, that in the presence of MR2, a complex forms in which MR1 is in close contact to both the nonamer and heptamer. All of the contacts visualized in these experiments represent cross-links to MR1. The complexes seen in the SDS gels all possess mobilities expected for MR1 (120 kDa) and not for MR2 (92 kDa). This interpretation has been confirmed by using the R1 version of RAG1 and obtaining a more rapidly moving species (data not shown). We fail to see MR2 cross-linking to any of these probes under these conditions, although we are convinced that it is in contact with DNA. These points will be discussed below.

**DISCUSSION**

The V(D)J recombination mechanism has been divided into two phases. The initial step requires recognition of the RSS and cleavage of the DNA backbone immediately adjacent to the heptamer. There are two different RSS sequences that differ in the length of the spacer region, but the proteins RAG1 and RAG2 together are sufficient to act at either one alone and also participate in creating a synaptic structure that couples one of each type RSS into a larger complex (8, 17, 18). The two proteins also seem to play a necessary role in stabilizing the post-cleavage DNA ends for subsequent processing and rejoining in later steps in the reaction (19–21). It is not known whether these proteins have additional enzymatic activities. Thus, these two proteins must play several roles in the reaction, and teasing apart the DNA-protein and protein-protein interactions is likely to be quite a challenge. We are focusing on the very first step in this report. It is already appreciated that cleavage of individual RSS-containing DNA molecules can be achieved in vitro in a reaction that is dependent on the presence of both proteins. We show here that preceding cleavage we can measure RAG protein binding to each RSS in an EMSA in the absence of any chemical cross-linking agents. We have purified each protein component in isolation as MBP fusion derivatives of the functional core regions (called MR1 and MR2 respectively). Each protein seems to have intrinsic DNA binding ability when measured by EMSA in the absence of competitor. The affinity of MR2 for DNA is relatively weak, however, as any more than 50 ng of dI-dC competitor in our standard 10-μl binding reaction abolishes the signal. MR1 also shows DNA binding alone, and this binding is more stable, persisting at dI-dC competitor levels 10 times higher. Neither protein alone shows strong sequence preferences in these assays, although subtle reduction (2-fold effects) in binding is observed in some cases.

The story becomes more interesting as the two proteins are tested together. The mixed proteins form a complex of increased specificity for RSS DNA and shows an altered mobility in EMSA compared with the behavior of the individual proteins. Similar behavior is detected on both 12- and 23-RSS-containing probes. In both cases the complex is primarily dependent on the presence of the nonamer, with small additional effects noted when the heptamer was replaced by unrelated sequence. Where tested, the assay demonstrated a severalfold reduction in binding to probes in which the nonamer was altered 2 base pairs at a time. The most severe reduction in binding occurred with probe 16, which replaced base pairs 6 and 7 of the nonamer. This corresponds well with the sensitivity of recombination to single base changes in the RSS, as measured in a cellular assay (22). In that study, a single G replacement at position 6 reduced recombination to 3%. Similarly replacement with a G at position 7 reduced recombination to 14% of that observed with the consensus nonamer. The new complex that forms in the presence of both proteins was directly excised from an EMSA gel and analyzed for its component proteins. Both MR1 and MR2 were found, in what appears to be an equal molar ratio. Since we already knew that MR2 alone did not have sufficient affinity to stably associate with the probe in the presence of the high amount of competitor dI-dC used in this isolation, we suspected that protein-protein interactions between MR1 and MR2 were acting to stabilize MR2 in the triple complex. We obtained evidence for direct interaction in the absence of DNA by two means. A two-dimensional gel system was used to show that a protein mixture containing both MR1 and MR2 partitioned in the native dimension into several fractions. Some of these contain only the individual proteins, but in two of them, both proteins were present simultaneously. Direct contacts were also implicated by the MBP pull-down assay. The MBP-RAG2 fusion was able to retain a RAG1 core region on amylose beads, while MBP in the absence of the fusion partner was not.

Only a small fraction of the RAG1 protein was stably associated with MR2 in these assays. In the two-dimensional gel analysis, about 10% of the MR2 was in the slower mobility positions labeled A and B (Fig. 4A). In the MBP pull-down assay, about 20% of the R1 was recovered through association with MR2 (Fig. 4B). This could be owing to the majority of the RAG proteins being in conformational states that makes them unavailable to interact with the other. For example, formation of homodimers of the individual proteins may compete with the assembly of a heterodimer between them. We note that the two-dimensional gel (Fig. 4A) shows two different migration patterns of MR2 in the absence of MR1 (positions labeled D and E), which likely represents two structural forms of RAG2. We are currently studying the multimerization state of these proteins individually and together. RAG1 and RAG2 have been reported to interact in vivo as determined by coimmunoprecipitation (23). The only caveat to these experiments is the concern that complexes isolated from cells may represent separate DNA-binding proteins tethered together by bridging DNA or joined in larger multiprotein complexes without direct contact with each other. Our studies confirm that these interactions can occur between proteins purified separately under high salt conditions that would eliminate DNA.

We used the base analog 5-iodouridine as a cross-linking probe to map specific interactions within the ternary complex of MR1, MR2, and DNA. Halogenated bases have properties that are both attractive and limiting as cross-linkers. The anolog is structurally similar to thymine, and can be incorporated in place of T without distortion of the helix. The 5-position is situated in the major groove, so this cross-linker is only sensitive to contacts there. Furthermore, only close contacts can be linked since this agent has a van der Waals radius of only 2.15 Å (comparable to the 2.0 Å of the methyl group it replaces) (24). Finally, iodine prefers to cross-link to aromatic targets on protein (25). As a consequence, positive results are meaningful, but there can be several explanations for a negative result. We assembled the protein DNA complexes in the presence of Mg2+
while on ice to prevent nucleolytic cleavage of the substrate. Sufficient unlabeled competitor di-dc was present to assure that the majority of labeled complexes were specific. We used five probes in which T bases were replaced with IdU at the complementary position to A bases in the consensus sense sequence. Three positions in the nonamer, one in the spacer, and one in the heptamer were tested. First, the presence of MR2 made a big difference in the distribution of cross-links. When the five probes were tested with MR1 alone, each position cross-linked equally well, which is the expected behavior for a complex in which the protein is binding nonspecifically. In the presence of both proteins, however, there were significant differences in cross-linking efficiency. Of the three positions substituted in the nonamer, position three (probe 3 in Fig. 5) gave very low levels of cross-linking, while positions four and five cross-linked well. No cross-linking was observed with the spacer probe (probe 6). This is good, since one does not expect close contacts to bases that are not conserved. Most significantly, a strong contact was detected using probe 7, with the IdU base positioned at the second base pair of the heptamer. Subtle effects of the heptamer have been detected in RAG1 binding before (11). In this report, we have also seen weak but consistent preference of MR1 for oligonucleotides that contain both the heptamer and nonamer over one that contains only the nonamer. The direct evidence of a strong contact between RAG1 and the heptamer is consistent with an earlier result that measured recombination in a cellular assay using a mutant RAG1 (10). The mutant ("D32") was specifically more sensitive to DNA sequence changes in the two base pairs of coding DNA adjacent to the heptamer and to changes in the heptamer itself, compared with the parent RAG1. A homology to the Hin invertase DNA binding domain has been described at the N terminus of the essential RAG1 core region (12). The bacterial domain has a known mode of binding in the minor groove of DNA. Hence, binding mediated by this motif should not be detected by a cross-linker specific for the major groove. The contacts in the nonamer and the heptamer detected by the IdU cross-linking approach may represent additional interactions with RAG1 aside from those attributable to the previous domain, and help to explain the additional specificity that the recombination reaction requires.

Finally, the UV cross-linking assay did not cross-link to MR2. We are convinced from the experiments in Fig. 4C that MR2 is present in the complexes at the time of UV exposure. We have already described the limitations of the cross-linking strategy. It is possible that RAG2 does not make close contacts in the major groove with an appropriate amino acid in a favorable configuration for cross-linking with IdU. Alternatively, we may not have selected the appropriate positions for the cross-linking agent. Toward that end, additional studies are under way in our laboratory.

The fact that RAG1 makes close contact at both the heptamer and nonamer does not prove that an individual RAG1 molecule makes both contacts concurrently. Indeed, if it were so, then a single RAG1 monomer would be required to contact the 12-RSS over at least a 28-base pair interval and to be capable of also binding to the 23-RSS over 39 base pairs. This does not consider any further interaction with the coding DNA upstream of the heptamer, for which indirect evidence exists (10). This seems to ask much of the protein. An alternative model would have two monomers of RAG1 acting together. One could contact the nonamer and the second, the heptamer and coding flank. This division of labor is fully consistent with the UV cross-linking data, and would allow increased flexibility. The issue of whether monomer or dimer is the active species is under current investigation. One major question yet to be resolved is how the structure that assembles on the 12-RSS is distinguished from that on the 23-RSS. There must be a difference that is recognized by the proteins in higher order interactions to favor the assembly of one each over the homodimerization of two 12-RSS complexes or two 23-RSS complexes. Solving this puzzle will provide a structural answer to the 12/23 rule.

Some of the same conclusions regarding a cooperative interaction between RAG1 and RAG2 have been published recently (26). Our reports are generally consistent but have used different experimental approaches. We have not used any chemical cross-linking in the EMSAs, and we have used UV cross-linking rather than footprinting assays to map sites of protein contact. The most important new observation presented in our report is the evidence that RAG1 makes close protein contacts at both the heptamer and nonamer. This complements the footprinting data (26), which indicate that protection is achieved at the nonamer by a mixture of RAG1 and RAG2, while enhanced cleavage was obtained at the heptamer. Our work also demonstrates that RAG1 and RAG2 are physically present in the excised gel shift complex (Fig. 4C). While this fact is presumed true in the previous work, the altered mobility of a complex formed in the presence of RAG2 could formally owe to a change in conformation or stoichiometry without demanding persistent association of both protein species. Since we show that RAG1 and RAG2 can associate with each other in the absence of DNA (Fig. 4B), we find it more plausible that the protein preassociates prior to binding DNA. However, we do find weak nonspecific binding of RAG2 to DNA (Fig. 1B), so sequential or simultaneous but independent binding by the individual proteins cannot be excluded.

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