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The Homeodomain Region of Rag-1 Reveals the Parallel Mechanisms of Bacterial and V(D)J Recombination

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Summary

The V(D)J recombinase subunits Rag-1 and Rag-2 mediate assembly of antigen receptor gene segments. We studied the mechanisms of DNA recognition by Rag-1/Rag-2 using surface plasmon resonance. The critical step for signal recognition is binding of Rag-1 to the nonamer. This is achieved by a region of Rag-1 homologous to the DNA-binding domain of the Hin family of bacterial invertases and to homeodomain proteins. Strikingly, the Hin homeodomain can functionally substitute for the Rag-1 homologous region. Rag-1 also interacts with the heptamer but with low affinity. Rag-2 shows no direct binding to DNA. Once the Rag-1/Rag-2 complex is engaged on the DNA, subsequent cleavage is directed by the heptamer sequence. This order of events remarkably parallels mechanisms that mediate transposition in bacteria and nematodes.

Introduction

The immune system encounters foreign antigens through antigen receptors (immunoglobulin, Ig; T cell receptor, TcR) present on the surfaces of B and T cells. The diverse structure of each receptor is generated by the somatic assembly of their composite gene segments in a process termed V(D)J recombination (Tonegawa, 1983). This is the only site-specific recombination process identified thus far in vertebrates (Lieber, 1991). Rearrangement is directed by recombination signal sequences (RSSs) that flank each antigen receptor gene segment (Lewis, 1994). The consensus RSS consists of a heptamer sequence (CACAGTG) directly adjacent to the coding element and an A/T-rich nonamer site (ACAAAAACC) separated from the heptamer by a spacer of either 12 bp or 23 bp (Hesse et al., 1989; Ramsden et al., 1994). It has been noted that the V(D)J RSS motifs are reminiscent of DNA sequences that direct excision by the Tc1 family of invertebrate transposases (Dreyfus, 1992) and inversion by the Hin family of bacterial site-specific recombinases (Simon et al., 1980; Rathbun and Tucker, 1987).

The sequential stages of V(D)J recombination are mediated by both lymphoid-specific and ubiquitous proteins. The two key lymphoid activities are the recombination activating proteins Rag-1 (Schatz et al., 1989) and Rag-2 (Oettinger et al., 1990). The two proteins coexist in a complex that localizes in the periphery of the nucleus (Spanopoulou et al., 1995). This implies that Rag-1 and Rag-2 act coordinately during V(D)J recombination. Indeed, disruption of either the *Rag-1* or *Rag-2* gene by homologous recombination produces an identical phenotype in which lymphoid development is arrested prior to the rearrangement of the antigen receptor loci (Shinkai et al., 1992; Mombaerts et al., 1992; Spanopoulou et al., 1994; Spanopoulou, 1996). No recombination intermediates are found in the Rag−/− lymphoid cells, suggesting that Rag-1 and Rag-2 act at the initial stages of recombination (Schlissel et al., 1993).

Purified Rag-1 and Rag-2 are sufficient to mediate the initial stages of V(D)J recombination. The two proteins establish the formation of a synaptic complex between the two recombining RSSs, which allows specific cleavage of the DNA (Eastman et al., 1996; van Gent et al., 1996a). The cleavage reaction is initiated by nicking at the coding/heptamer border of the upper strand followed by nucleophilic attack of the bottom strand and formation of covalently sealed coding ends (hairpins) and blunt signal ends (McBlane et al., 1995). It has been suggested that this mechanism of cleavage by Rag-1/Rag-2 is similar to DNA cleavage by transposases and retroviral integrases (van Gent et al., 1996b).

Subsequent to cleavage by Rag-1 and Rag-2, several ubiquitous DNA repair proteins participate in completion of the V(D)J recombination process. These include the DNA-dependent protein kinase (*scid*), its DNA binding subunits Ku70 and Ku80, and XR-1 (reviewed by Jackson and Jeggo, 1995; Bogue and Roth, 1996). Mutations in DNA-dependent protein kinase lead to the accummulation of hairpin coding ends (Roth et al., 1992), while mutations in Ku70/80 affect processing of both coding and signal ends (Taccioli et al., 1993; Pergola et al., 1993). In the organism, the indispensable function of DNA-dependent protein kinase and Ku70/80 in V(D)J recombination is manifested by the lack of antigen receptors in mice deficient for the DNA-dependent protein kinase (*scid*) or Ku80 genes (Bosma and Carroll, 1991; Nussenzweig et al., 1996; Zhu et al., 1996).

Here we describe the mechanisms that underlie DNA recognition by the Rag-1–Rag-2 complex at the initial stages of V(D)J recombination. We show that the event that initiates V(D)J recombination is the recognition of the nonamer RSS by Rag-1. Specific recognition of the A/T-rich nonamer site is mediated by a domain of Rag-1 that shows distinct homology to the helix-turn-helix homeodomain structure (Gehring et al., 1994a, 1994b). Homeodomains recognize A/T-rich DNA sequences and constitute the DNA-binding domain of proteins that act as gene regulators specifying cell differentiation and development of the organism (Scott et al., 1989; Gehring et al., 1994a, 1994b). This domain of Rag-1 has been highly conserved during evolution and is most closely related to the homeodomain of the Salmonella typhimurium Hin invertase, which also recognizes a nonamer-like sequence (Simon et al., 1980; Feng et al., 1994). Substitution of the Rag-1 homeodomain with that of the

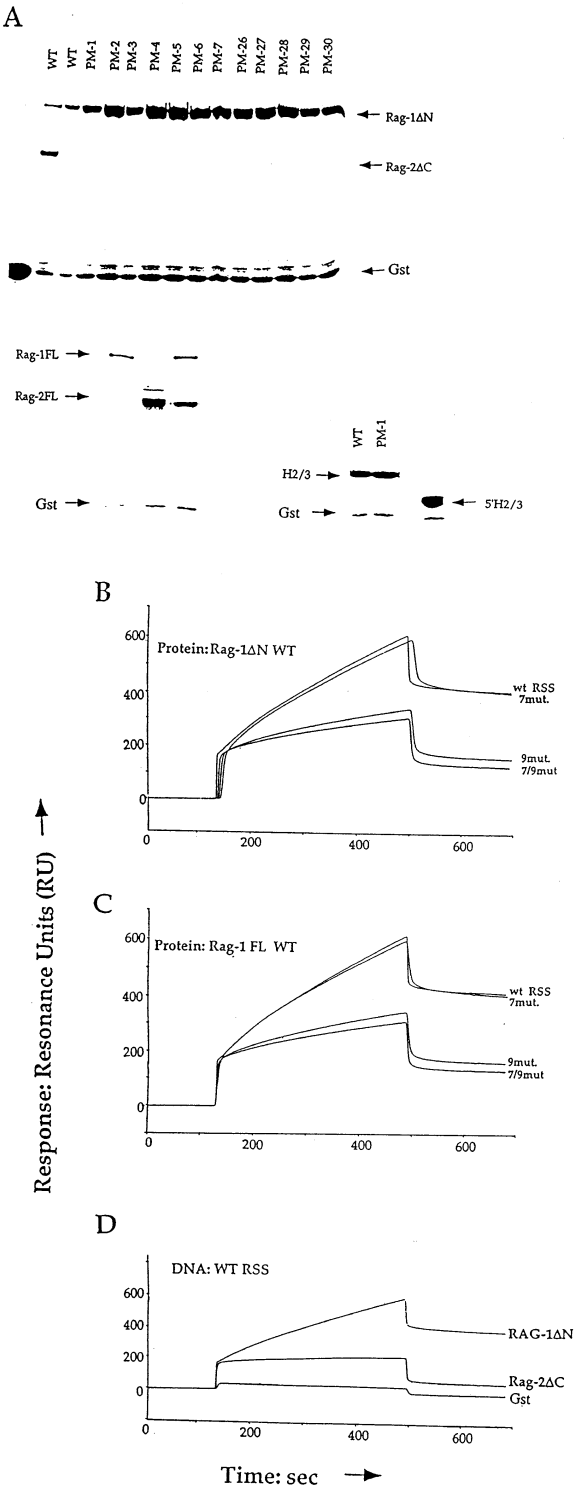

Figure 1. DNA-Binding Properties of Rag-1 and Rag-2 Analyzed by SPR

(A) Detection of GST–Rag fusion proteins by Coomassie blue staining. The structure of each recombinant protein is described in the text. "Rag-1FL" and "Rag-2FL" indicate full-length proteins, while "Rag-1ΔN" (amino acids 330–1040) and "Rag-2ΔC" (amino acids 1–388) represent the active cores of the two proteins (Silver et al., 1993; Sadofsky et al., 1993, 1994; Cuomo et al., 1994). Polypeptides H2/3 and 5′-H2/3 represent amino acids 358–477 and 376–477 of the Rag-1 protein, respectively, expressed as GST–fusion proteins. 5′-H2/3 is expressed in bacterial cells.

(B) Sensorgram of interacting wild-type Rag-1 protein with the V(D)J RSS sequences immobilized on the BIAcore matrix. Wild-type Rag-1 protein (amino acids 330–1040; Rag-1ΔN), at a concentration of 10 pM, was introduced by constant flow on a four-surface matrix that carried 2,500 resonance units of each of the four 12 RSS oligonucleotides: WT.7mer/9mer (WT RSS), 7mut., 9mut., and 7/9mut. Binding was allowed to proceed for 6 min (association phase), after which the unbound protein was removed by a constant flow of the same binding buffer (dissociation phase). Thus, the beginning of the dissociation phase represents the amount of protein bound during the association phase.

(C) Binding of full-length wild-type Rag-1 protein (amino acids 1–1040) on the four surfaces.

(D) DNA-binding profiles of wild-type, Rag-1ΔN, Rag-2ΔC, and GST proteins on the wild-type V(D)J RSS (WT.7mer/9mer).

Hin invertase produces a hybrid protein that is functional in V(D)J recombination. Rag-1 appears also to interact with the heptamer motif, albeit with lower affinity, while Rag-2 alone is unable to interact with the RSS. The initial "anchoring" of the Rag-1–Rag-2 complex on the nonamer allows subsequent cleavage of the DNA that requires Rag-2 and is dependent on the presence of an intact heptamer motif. This sequence of events remarkably parallels the initial stages of transposition mediated by the Caenorhabditis elegans transposase Tc1A (Vos et al., 1993; Vos and Plasterk, 1994). These findings uncover a homology between the mechanisms of genomic recombination in lower organisms and V(D)J recombination in vertebrates that may imply an evolutionary relationship.

Results

Preparation of Purified Recombinant Rag Proteins

Rag-1 and Rag-2 proteins were produced as glutathione S-transferase (GST) N-terminal fusion products. Proteins were expressed as full-length products or as the minimal sequences required for recombination (active cores; Silver et al., 1993; Sadofsky et al., 1993, 1994; Cuomo et al., 1994). The recombinant GST–Rag proteins were transiently overexpressed in the human kidney cell line 293T and purified on glutathione–agarose beads. Purified Rag proteins were 95% homogeneous with respect to other contaminating proteins and 80% homogeneous with respect to the copurifying endogenous GST protein (Figure 1A). The GST–Rag fusion proteins are soluble and comparable to the wild-type Rag proteins in their ability to activate V(D)J recombination in vivo when coexpressed with recombination substrates in fibroblasts (Figure 2) and in their ability to mediate cleavage and hairpin formation in vitro (see Figure 5).

DNA Binding Properties of Rag-1 and Rag-2

The Rag-1–Rag-2 complex mediates efficient DNA cleavage only in the presence of an intact heptamer and nonamer motif (McBlane et al., 1995; Ramsden et al., 1996). This implies that the complex has specific DNA-binding activities. Previous studies on the DNA-binding properties of the two proteins were hampered by the high nonspecific affinity of Rag-1 for DNA. To circumvent

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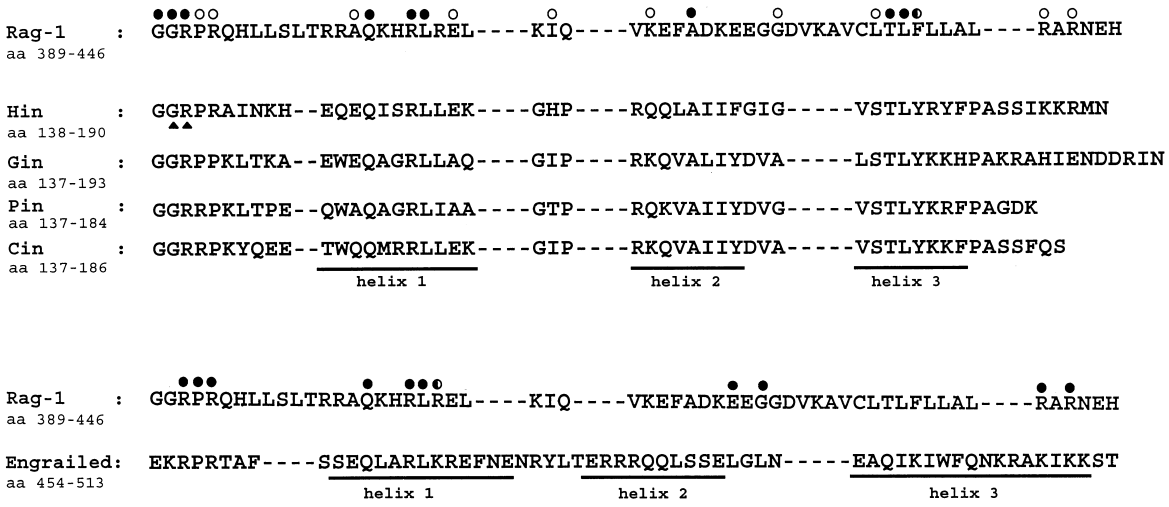

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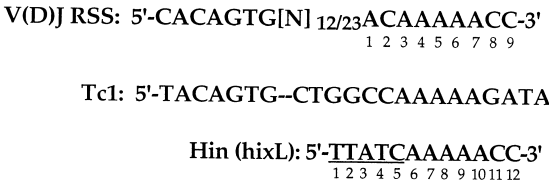


Figure 2. Homology of Rag-1 to Salmonella Hin Recombinase and to Homeodomain Proteins

(A) Homology of Rag-1 (amino acids 389–442) to the DNA-binding domains of the Hin family of invertases and to the engrailed homeodomain. Closed circles indicate conserved amino acid residues, and half-open circles indicate conservative substitutions. Open circles show homology of Rag-1 to other enteric invertases.

(B) The cognate DNA-binding half-site of Hin (*hix*; Hughes et al., 1992), the 26 bp terminal nucleotides of transposon Tc1 (Vos and Plasterk, 1994), and the most conserved form of the nonamer RSS motif of V(D)J recombination (Hesse et al., 1989; Ramsden et al., 1994). Underlined are nucleotides contacted by Hin helix III in the major groove (Feng et al., 1994).

these problems, we have studied the specific affinity of Rag-1/Rag-2 for DNA using surface plasmon resonance (SPR), which effectively monitors interactions between macromolecules in real time and allows a broad spectrum of experimental conditions to be tested (Jönsson et al., 1991; Panayotou et al., 1993). Several protein–DNA interactions have been studied using this system (for example, Bondeson et al., 1993; Fisher et al., 1994). One of the interacting components is immobilized on a dextran layer bound to a gold surface (sensor chip), while the other is provided by constant flow. The SPR detector records changes in the refractive index of the medium close to the dextran layer, which is in turn directly proportional to the mass of macromolecules bound to the surface. The response is converted to arbitrary resonance units and plotted against time.

To study DNA recognition by Rag-1/Rag-2, we coupled biotinylated oligonucleotides containing the RSS site or specific mutations within the heptamer (7mer)–nonamer (9mer) sequence (see Experimental Procedures) to streptavidin-coated chips. Identical amounts of wild-type or mutant oligonucleotides were loaded on the chip, and these DNA surfaces were tested for binding of the wild-type Rag-1 and Rag-2 proteins. Purified Rag proteins in binding buffer were passed over the matrix by constant flow, giving an initial rapid increase in the signal (upgoing slope). This increase is mainly due to the detection of unbound protein ("bulk" effect dependent on the concentration of the loaded protein). Binding was terminated by replacing the protein solution with running buffer, producing a drop in the signal (due to the removal of the noninteracting protein), followed by a slow dissociation phase. The relative binding capacity of the different proteins was obtained by comparing the baseline signal before injection with the signal attained at the beginning of the dissociation phase. While in principle it is possible to measure directly the association and dissociation rate constants of interactions using the BIAcore instrument, the results obtained in our experiments did not fit simple kinetic models when analyzed with suitable software, and therefore it was not possible to assign the rate constants. This could be due to the

GST–moiety of the Rag proteins that mediates strong homodimerization of the fusion products. Therefore, the data were interpreted in a semiquantitative way.

Specific interactions became evident when binding reactions were performed in 0.25 M NaCl (see Figure 1B). In a typical experiment, Rag-1 protein (amino acids 330–1040, active core) was tested for binding to four DNA surfaces containing the wild-type RSS motif (7mer/9mer) or mutations of the 7mer, the 9mer, or both. Rag-1 specifically bound to the wild-type RSS signal. This binding was due to interactions with the nonamer, because mutations in that site abolished specific DNA binding of Rag-1 while mutations in the heptamer had only a minor effect (Figure 1B). The full-length Rag-1 protein (amino acids 1–1040) showed a similar DNA-binding profile (Figure 1C). Rag-2 (amino acids 1–388, active core) showed no specific DNA-binding activity and only a very low nonspecific affinity for DNA (Figure 1D). No DNA-binding contributions were made by the GST part of Rags, because GST alone showed no binding activity (Figure 1D). To investigate the potential effect of Rag-2 on the specific binding of Rag-1, the two proteins were mixed and loaded on the four DNA surfaces. However, the presence of Rag-2 did not alter the DNA binding profile of Rag-1 (data not shown).

Homology of Rag-1 to the Hin Homeodomain

In attempting to define the regions of Rag-1 that mediate specific binding to the nonamer motif, we noticed that the very N-terminus of the active core of Rag-1 contained distinct homology to the DNA-binding domain of the bacterial site-specific invertases (van de Putte and Goosen, 1992) and in particular to the Salmonella Hin recombinase (Figure 2A), which mediates flagellar variation (Simon et al., 1980). The cognate DNA-binding site of Hin (*hix*) is a bipartite structure of two motifs (**TTATC**A AAAACC; Simon et al., 1980; Hughes et al., 1993), one of which is strikingly homologous to the nonamer sequence (ACAAAAACC) found in the V(D)J RSSs (Hesse et al., 1989; Ramsden et al., 1994; Figure 2B). The Hin family of recombinases consists of Hin, Cin, Gin, and Pin, all of which share extensive homology in their DNA-binding domains (Figure 2A) and are functionally interchangeable (van de Putte and Goosen, 1992). These domains fold into a helix-turn-helix structure having extensive homology to the DNA-binding domain of homeodomain proteins (Affolter et al., 1991; Feng et al., 1994).

The crystallographic analysis of the Hin homeodomain has shown that residues G139/R140 interact with base pairs 8 and 9 of the nonamer-homologous part in the minor groove, while residues within helix III interact with bases 2–5 in the major groove (Figure 2B; Feng et al., 1994). Given these structural characteristics of Hin, the homology of Rag-1 to Hin becomes remarkable. Rag-1 shares absolute homology with the GGRPR sequence of Hin that binds to the nonamer-like sequence of the *hix* site, and it maintains a helical configuration of the corresponding helix II. However, Rag-1 shares less homology to helix III of Hin (Figure 2A), suggesting that the two regions might determine different DNA-binding specificities. Based on these data, we hypothesized that the Hin-homologous region of Rag-1 might contribute to the specific binding of the protein to the V(D)J nonamer motif. To dissect the functional role of the Rag-1 Hin-homologous sequences, a number of point mutations within this region were analyzed for their V(D)J recombination activity and DNA-binding potential.

Functional Analysis of the Rag-1 Hin-Homologous Region

All mutations of Rag-1 residues corresponding to amino acids (GG389/390, R391, Q404/RL407/408) that are essential for the function of Hin and homeodomain proteins (Feng et al. 1994; Gehring et al., 1994a) abolished the V(D)J recombination activity of Rag-1. On the other hand, mutation of residues that are not conserved in the Hin protein (S398, EF417/418) had no effect on the recombination activity of Rag-1 (Figures 3B and 3C). Interestingly, mutant PM-27, which contains LTLF432–435GILY, gave a recombination efficiency approximately 1.5-fold that of the wild-type protein. In contrast, PM-26, which introduces a set of different mutations in the same region, gave no detectable recombination activity. PM-26 was designed to introduce a KIWFG motif instead of LTLFL (amino acids 432–436), because this motif is evident in Rag-2 at amino acid positions 315–319. KIWF is one of the most highly conserved motifs within helix III of homeodomain proteins (Scott et al., 1989; Gehring et al., 1994b). The levels of expression of each individual mutant were monitored by Western blot analysis, which showed that all recombinant proteins were expressed at similar levels (Figure 3D).

DNA Binding Analysis of Rag-1 Mutants

The contribution of the Hin-homologous region of Rag-1 to the specific binding of the protein to DNA was examined by SPR. Mutations that abolished V(D)J recombination activity also abolished the specific DNA-binding affinity of Rag-1 (Figure 4A). In particular, mutant PM-1, which contains a single change of R391L (Figure 3B), destroyed the specific binding of Rag-1 to the nonamer motif but left intact the nonspecific DNA affinity of the protein (Figure 4B). The corresponding Arg at this position of Hin and homeodomain proteins is absolutely essential for specific contacts with the minor groove (Feng et al., 1994; Gehring et al., 1994a). Mutant PM-2, in which the second Arg of the GGRPR motif is altered (R393L), retained partial affinity for the wild-type RSS (Figure 4A), which was consistent with the intermediate recombination activity of this protein (see Figure 3B). Only Hin and not the other members of the invertase family has an Arg at that position (see Figure 2A), which would explain the moderate effect of the PM-2 mutation. Mutants PM-3, PM-4, PM-5, PM-6, and PM-26 completely failed to bind to DNA, indicating that the mutant proteins were unstructured (Figures 4A and 4B). Mutant PM-7 responded like PM-1, by abolishing the specific DNA-binding activity of Rag-1 to the nonamer but retaining its nonspecific DNA affinity (as indicated by the slight increase in the slope on all four tested surfaces; Figure 4B). Interestingly, mutant PM-27, which showed increased recombination efficiency, also showed increased DNA-binding activity.

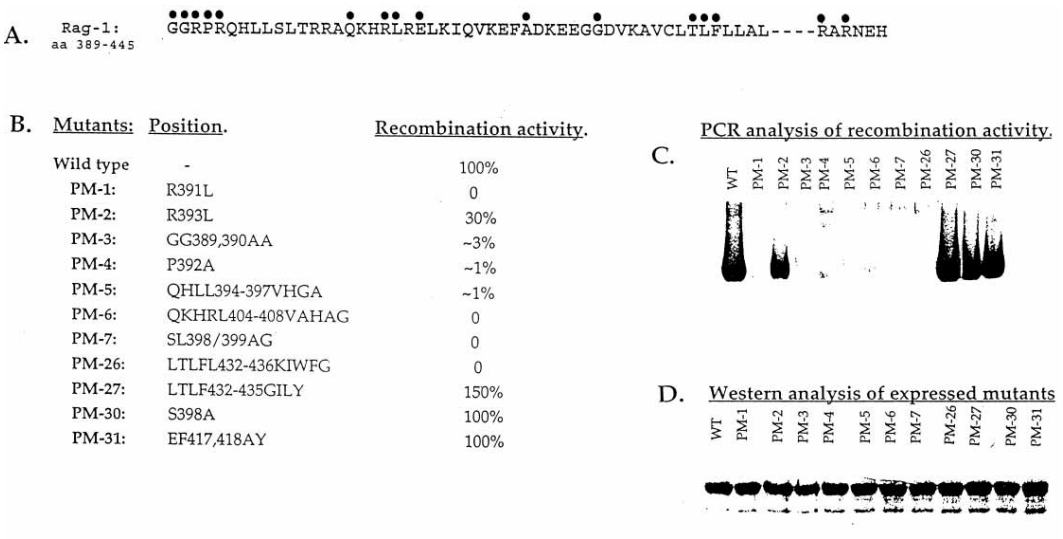


Figure 3. Mutational Analysis of the Hin-Homologous Region of Rag-1

(A) The Hin-homologous region of Rag-1 (amino acids 389–445). Circles indicate homology to Hin.

(B) Functional analysis of mutations within the Hin-homologous domain of Rag-1. Indicated mutants were coexpressed with wild-type Rag-2 protein and an extrachromosomal substrate. Mutant proteins were assayed for deletional and inversional recombination in HeLa cells using the pJH200 and pJH288 substrates, respectively, and for inversional recombination in 293T cells using the pJH288 substrate (Hesse et al., 1987). Recombination products were analyzed both by bacterial transformation (Hesse et al., 1989) and by PCR analysis of the recombined signal sequences. The presented data are the mean values of six independent experiments.

(C) Detection of pJH288 recombination products by PCR analysis (see Experimental Procedures).

(D) Western blot analysis of the Rag-1 mutants expressed in 293T cells.

The functional importance of the Hin-homologous region of Rag-1 for specific binding to the nonamer was corroborated by the fact that an N-terminal deletion (Rag-1ΔHom) that removed amino acids 384–456 of Rag-1 completely abrogated the ability of the protein to bind to DNA with specificity (Figure 4B). The observed binding of Rag-1 to the AT-rich V(D)J nonamer is specific to this sequence because the wild-type Rag-1 protein showed no specific binding to the AT-rich cognate DNA-binding site of the engrailed and sine oculis homeodomains (data not shown; Gehring, 1994b). Subsequently, we examined whether this region of Rag-1 was sufficient for specific binding to the V(D)J nonamer or whether additional sequences were also required.

Residues within Amino Acids 376–477 of Rag-1 Mediate Specific Binding to the Nonamer Motif

A number of C-terminal deletions were generated as GST–fusion proteins that had a fixed N-terminus at amino acid position 330 and C-terminal borders at amino acid positions 997, 877, 773, 698, and 637. Most of these truncated Rag-1 proteins appeared to be unstructured, since they failed to bind to DNA even nonspecifically, while others showed elevated nonspecific binding (data not shown). Hence, we generated truncated Rag-1 proteins encompassing the Hin-homologous region of the protein with the hope that some of them would fold properly. A set of two of these polypeptides (H2/3 amino acids 358–477 and 5′-H2/3 amino acids 376–477) bound to the wild-type V(D)J RSS with specificity for the nonamer site (Figure 4C). Moreover, the single point mutation of R391L (as in mutant PM-1) abolished this specific binding of H2/3, although the protein maintained its nonspecific DNA affinity (Figure 4C).

Previous studies have defined the N-terminal border of the minimal sequences of Rag-1 required for recombination at amino acid position 384 (Silver et al., 1993; Sadofsky et al., 1993), suggesting that the DNA-binding domain of Rag-1 does not extend to the N-terminus of amino acid position 384. Our BIAcore assays mapped the C-terminal border of this domain at amino acid position 477. These data establish that the Hin-homeodomain/homologous region of Rag-1 contained within amino acids 384–477 is sufficient for the specific binding of the protein to the V(D)J nonamer RSS.

The Hin Homeodomain Functionally Replaces the Rag-1 Homologous Region

Given the homology between the DNA-binding domains of Rag-1 and Hin and the similarity of their cognate DNA-binding sites, we examined whether the 52 amino acid Hin homeodomain (Feng et al., 1994) could functionally replace the corresponding Rag-1 region (Figure 5A). The Hin/Rag-1 hybrid protein was analyzed for its ability to mediate nicking and hairpin formation of a 12 RSS oligonucleotide substrate in vitro (Figure 5B) and to complete recombination of the pJH288 inversion substrate in vivo (Figure 5C). Strikingly, the hybrid protein displayed efficient nicking and hairpin conversion activity at 20% of the efficiency of the wild-type Rag-1 protein (Figure 5B). Moreover, the hybrid protein catalyzed bona fide recombination of the pJH288 substrate in vivo at 30% of the efficiency of wild-type Rag-1 (Figure 5C). The demonstration that the sequence homologies between the Rag-1 and Hin homeodomains are such as to allow

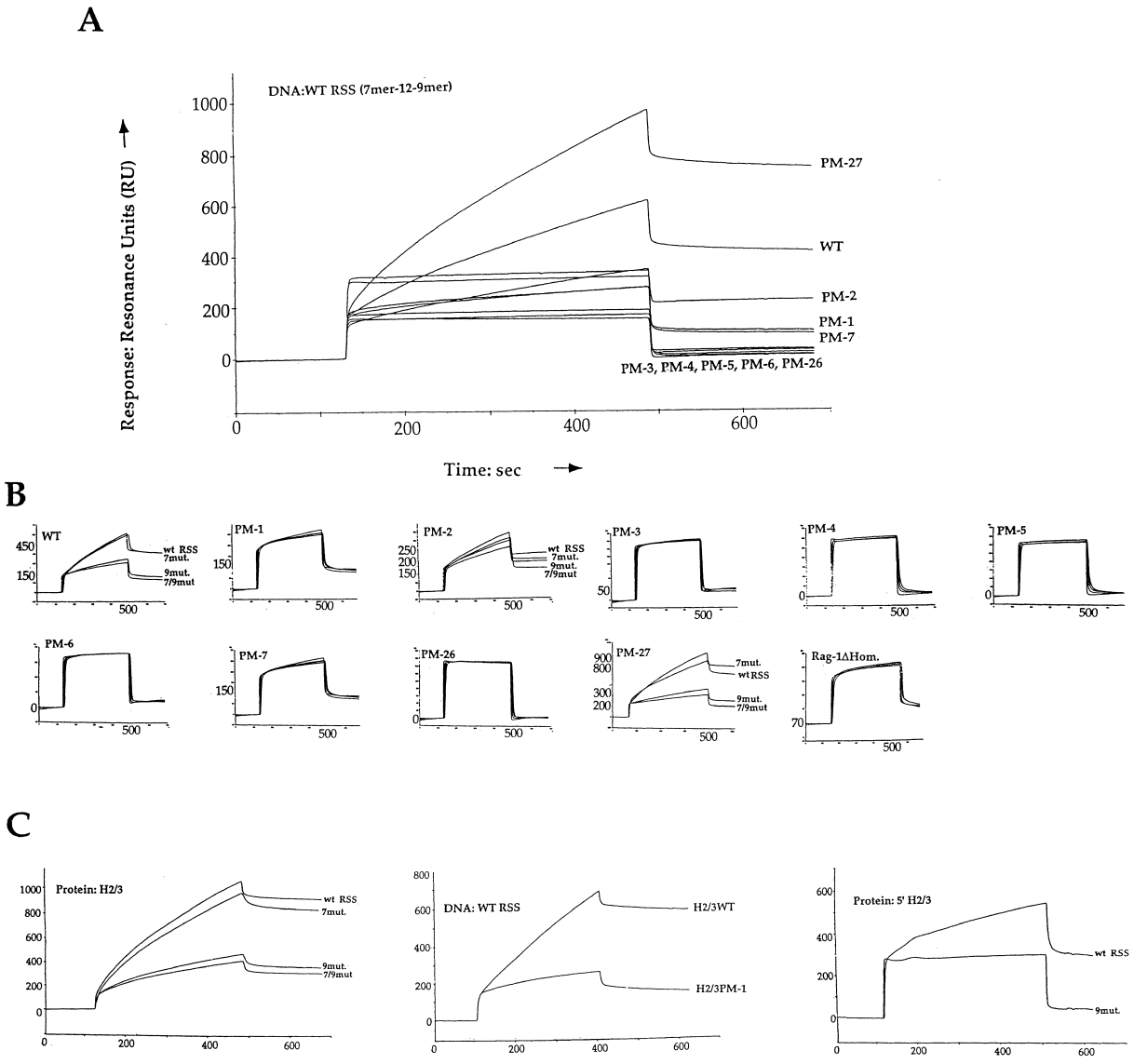


Figure 4. DNA-Binding Profiles of Mutant Rag-1 Proteins

(A) Sensorgrams depicting the DNA binding of mutations within the Hin-homologous region of Rag-1. All mutant proteins were injected at similar concentrations and analyzed under identical experimental conditions on the same WT.7mer/9mer surface (wild-type RSS). The identity of each mutant is described in Figure 3B.

(B) DNA binding of each individual mutant on four equal density surfaces: WT.7mer/9mer (WT RSS), 7mut., 9mut., and 7mut./9mut. DNA surfaces are indicated when necessary. "Rag-1ΔHom" represents an internal deletion of the Hin-homologous region (amino acids 384–456) of Rag-1.

(C) Specific binding of polypeptides H2/3 (amino acids 358–477) and 5′-H2/3 (amino acids 376–477 of Rag-1) to the nonamer RSS. H2/3PM-1 is identical to H2/3 except for a mutation of R391L (see Figure 3B).

functional interconversion strongly argues that the two domains fold in equivalent conformations and that the role of the region in Rag-1 is solely to allow recognition of the nonamer.

Several Mutations within Rag-1 Interfere with Heptamer Binding

Previous studies have implicated the region of Rag-1 around amino acid 604 as a potential domain for the binding of the protein to the heptamer motif (Sadofsky et al., 1995; Roman and Baltimore, 1996). To test the binding of Rag-1 to the heptamer, two GST–fusion Rag-1 mutant proteins (ΔPM-28 and PM-29) were produced that contained mutations in the region previously described for its sensitivity to changes in nucleotides in the coding/heptamer border (see Figure 1A; Figure 6A; Sadofsky et al., 1995; Roman and Baltimore, 1996). Binding of both mutants was sensitive to changes in the heptamer signal (Figure 6B). To test whether this effect is specific, we analyzed several mutations within the

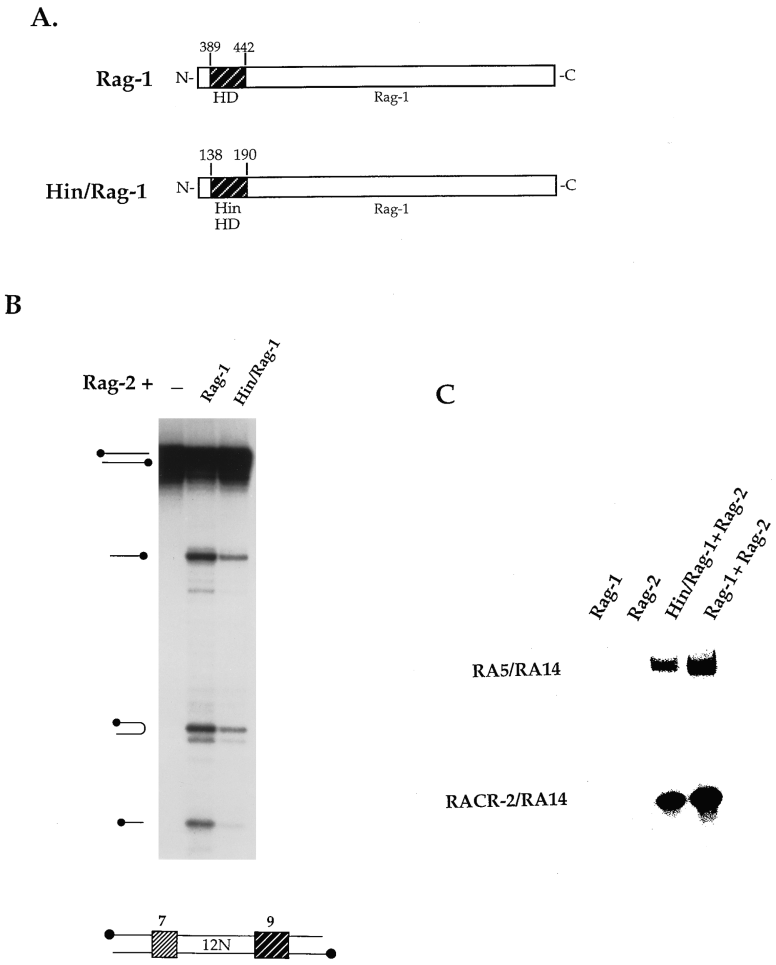


Figure 5. The Hin Homeodomain Can Functionally Replace the Rag-1 Homologous Region

(A) Position of the homeodomain-homologous region within Rag-1 and depiction of the Rag-1/Hin fusion protein. Amino acids 389–444 of Rag-1 were replaced by amino acids 138–190 of the Hin protein.

(B) In vitro cleavage reaction using purified Rag-2 and either Rag-1 or Hin/Rag-1 proteins. The DNA substrate is indicated at the bottom of the panel.

(C) In vivo recombination assay of Rag-1 and Rag-1/Hin proteins. Either of the two proteins were coexpressed with Rag-2 and the inversion substrate pJH288 in 293T cells. Recombinant products were detected by PCR analysis using two different sets of primers, RA5/RA14 and RACR2/RA14.

middle and C-terminal part of the protein. Some mutations were insensitive to changes in the heptamer motif (e.g., see PM-22, Figure 6B), but others had significantly decreased specific DNA binding on the heptamer mutant surface (see mutant Δ51C, Figure 6B). Given that mutant Δ51C carries a deletion that is 214 amino acids away from amino acid position 604, it appears that changes within a large region of Rag-1 render its DNA binding sensitive to changes in the heptamer sequence.

Rag-1 and Rag-2 Together Catalyze Heptamer-Dependent Cleavage on the Chip

To examine the possibility that specific cleavage of the RSS DNA can be monitored by SPR, wild-type Rag-1ΔN and Rag-2ΔC proteins were premixed just prior to their introduction on the chip. Cleavage was indicated by a drop in the signal below the starting point when binding buffer was applied, indicating that DNA was lost from the chip (Figure 7A). Furthermore, the signal remained below baseline after the regeneration phase, when protein bound no longer confounds the mass measurement. Given previous reports that Rag-1/Rag-2 cleavage of an oligonucleotide RSS substrate requires the presence of Mn^{2+} rather than Mg^{2+} (McBlane et al., 1995; Eastman et al., 1996; van Gent et al., 1996a), we tested the activity of the GST–Rag proteins to mediate cleavage of a 12 RSS oligonucleotide substrate in the presence of 1 mM $MgCl_2$ and 100 mM KCl/150 mM NaCl, immitating the BIAcore reaction conditions. The results of the in vitro cleavage assays confirmed the BIAcore data. In the presence of Mg^{2+}, the GST–fusion Rag-1/Rag-2 proteins could mediate efficient nicking but low efficiency hairpin formation compared with identical reactions in the presence of Mn^{2+} (Figure 7B, compare lanes 1 and 6). Moreover, the proteins remained fully active at 100 mM KCl/150 mM NaCl.

The contribution of the heptamer and nonamer motifs to the cleavage reaction was tested by SPR. Cleavage of the 12 RSS was dependent on the presence of an intact heptamer signal, because mutation of the heptamer prevented substantial cleavage (Figure 7A). In contrast, the nonamer sequence was only required for the specific anchoring of the complex to the RSS but not for the subsequent cleavage reaction. This is indicated by the fact that target DNA with a mutant nonamer was also cleaved, presumably owing to the binding of Rag-1/Rag-2 to DNA via the nonspecific DNA-binding activity of Rag-1 (Figure 7A). These data indicate that under given experimental conditions (e.g., excess of

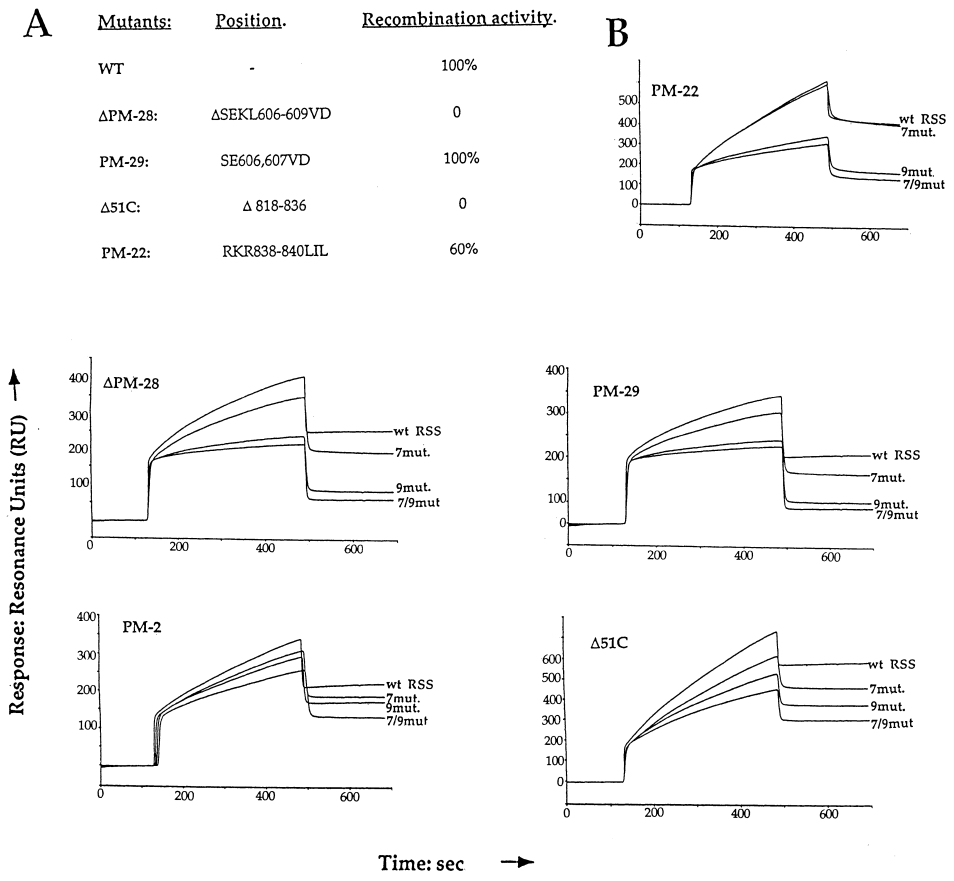


Figure 6. Binding Profiles of Rag-1 Mutants That Show a Dependence on the Heptamer

(A) Description of mutants at the C-terminus of the Rag-1 protein and their respective recombination activity (as defined in Figure 4B).

(B) DNA binding of mutant proteins that respond to changes in the heptamer motif. Mutant PM-22 has a profile identical to the wild-type Rag-1 protein. The structure of PM-2 is described in Figure 3B.

protein), cleavage of target DNA by Rag-1/Rag-2 can occur despite the absence of a functional nonamer. Cleavage requires recognition of the heptamer motif, since very little cleavage was observed on target DNA with mutant sequences.

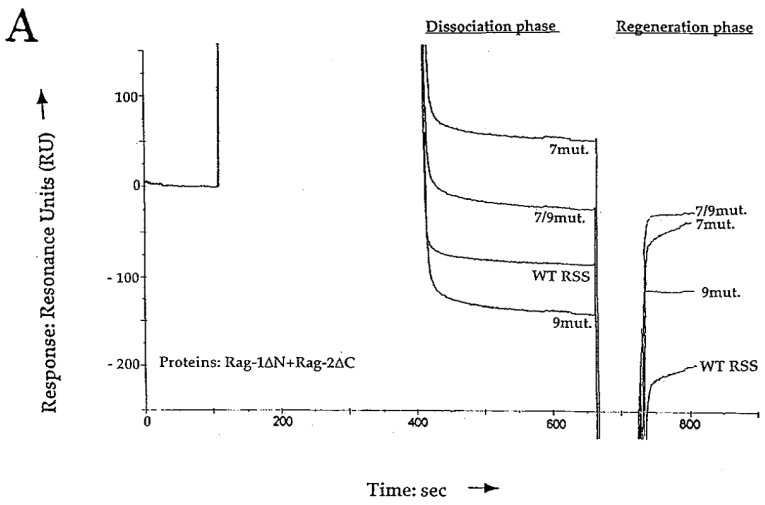
Discussion

The experiments presented in this article define the early stages of V(D)J recombination and provide insights into the parallel or common mechanisms underlying prokaryotic recombination and the rearrangement of antigen receptor. The data establish the following points. First, V(D)J recombination is initiated by the specific binding of Rag-1 to the nonamer V(D)J RSS motif. Second, without a functional nonamer the heptamer is recognized poorly, and heptamer binding may involve a cryptic element in Rag-1. Third, specific binding to the nonamer is mediated by a domain contained within amino acids 384–477 of Rag-1 that shows distinct sequence homology to the homeodomain structure and in particular to the homeodomain of the Salmonella Hin enteric invertase. A polypeptide encompassing the Hin-homologous region of Rag-1 reproduces the specific binding of the Rag-1 protein to the nonamer. The homology between Rag-1 and Hin homeodomains extends at the functional level, since the Hin homeodomain can replace the Rag-1 homologous region in V(D)J recombination inversion reaction in vivo. Fourth, Rag-2 by itself is unable to bind to DNA with specificity. This implies that Rag-2 is recruited into the synaptic complex through its interaction with Rag-1 to form a cleavage-competent complex. Fifth, the V(D)J cleavage reaction requires the presence of a functional heptamer motif.

It should be noted that although in vitro, Rag-1 and Rag-2 bind and cleave efficiently on oligonucleotide targets, in vivo the chromatin configuration of the antigen receptor loci imposes an additional level of complexity. It is conceivable that in vivo, certain regulatory proteins could facilitate the binding of Rag-1/Rag-2 to DNA.

Dominant and Specific Interaction of Rag-1 with the Nonamer RSS

Despite the high nonspecific affinity of Rag-1 for DNA, no cleavage occurs by the Rag-1–Rag-2 complex in the absence of the heptamer motif (Figure 7A; Hesse et al., 1989; Ramsden et al., 1996), while lack of the nonamer sequence drastically lowers the efficiency of cleavage (Figure 7A; Ramsden et al., 1996). This indicates that



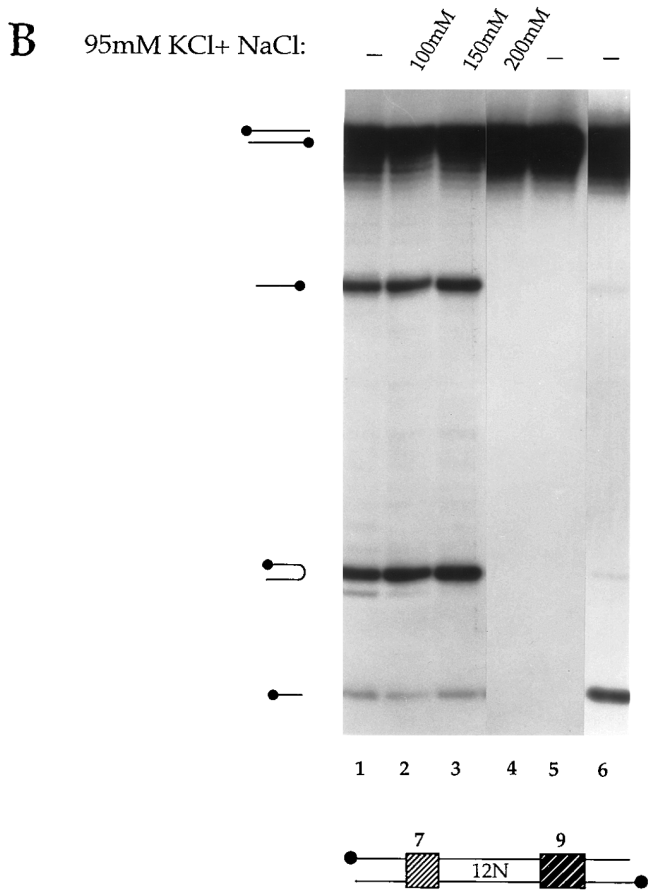


Figure 7. Nonamer-Independent Cleavage by Rag-1/Rag-2

(A) V(D)J cleavage monitored by SPR. Rag-1ΔN was mixed with Rag-2ΔC, and the two proteins were immediately loaded on four surfaces (WT.7mer/9mer [WT RSS], 7mut., 9mut., and 7mut./9mut.) of the BIAcore matrix. Association was allowed to proceed for 7 min, after which the unbound proteins were removed by injection of running buffer. Subsequently, 0.05% SDS was injected for regeneration of the surfaces. The individual values in the dissociation phase reflect the contribution of two factors: binding of the protein and loss of DNA because of cleavage. However, values in the regeneration phase reflect the amount of DNA that was lost from the surface because of cleavage.

(B) In vitro cleavage reactions in the presence of different concentrations of NaCl and cations. Reactions were performed as described in Experimental Procedures (1 mM $MnCl_2$, 95 mM KCl), with the addition of increasing concentrations of NaCl (lanes 1–4). Lane 5 represents reaction conditions as in lane 1 but in the absence of divalent ions. In lane 6, reaction conditions were as in lane 1, but $MnCl_2$ was replaced by 1 mM $MgCl_2$.

Rag-1/Rag-2 must contain specific DNA-binding domains that recognize the heptamer–nonamer DNA sequences and activate the cleavage reaction. Using SPR assays and mutagenic analysis, we identified a dominant region within Rag-1 that mediates the initial specific interaction of the protein with the V(D)J RSS sequence. This domain of Rag-1 interacts with the nonamer motif, and it is restricted to the homeodomain-homologous region of the protein. This was demonstrated by the specific binding to the nonamer RSS of two polypeptides encompassing this domain of Rag-1 (H2/3: amino acids 358–477; 5′-H2/3: amino acids 376–477). Mutation of R391 (PM-1) within this region eliminates the specific binding to the nonamer but not the nonspecific DNA

affinity of Rag-1 (Figure 4A), implying that R391 establishes specific interactions with the nonamer sequence. The equivalent position of R391 in the Hin homeodomain is R140, which along with G139 is one of the two key residues that determine specific interactions of Hin with the nonamer-like sequence in the minor groove (Feng et al., 1994; Figure 2B). It is conceivable that the G390/R391 of Rag-1 could mediate specific interactions with nucleotides 5 and 6 of the nonamer motif situated in the minor groove. This notion is reinforced by the fact that the Hin homeodomain can functionally substitute for the Rag-1 homeodomain, albeit at lower efficiency.

Specificity of DNA binding by Rag-1 is an important issue both for the biochemistry of V(D)J recombination and the potential involvement of V(D)J recombinase in the translocation of oncogenes (Leder et al., 1983). The extensive mapping of antigen receptor RSSs has indicated that while the heptamer motif is very highly conserved, the nonamer RSS sequences are not conserved to the same degree. This had led to suggestions that the nonamer sequence might have a secondary role in the V(D)J reaction. Our data and those of Difilippantonio et al. (1996 [this issue of *Cell*]) indicate that the nonamer site is the dominant element at the initial stages of V(D)J recombination. The answer to how Rags recognize the RSS with a "poor" nonamer sequence might lie with the homeodomain of Rag-1. Homeodomains generally recognize AT-rich motifs, and they can exhibit promiscuity in their interaction with target sites (Gehring et al., 1994a). In several cases, homeodomain proteins achieve specificity of DNA binding by homodimerization or by forming heterodimers with other proteins (White, 1994). It is possible that specificity of Rag-1 DNA binding can be modulated through its interaction with other proteins or by direct homodimerization.

Homology of Rag-1 to Hin and Homeodomain Proteins

The Hin homeodomain represents an intermediate structure between the prototypical helix-turn-helix DNA-binding domains of bacterial regulators and the eukaryotic homeodomain proteins (Feng et al., 1994). The primary sequence of the Rag-1 homeodomain indicates that its sequence is more homologous to Hin than to other homeodomain structures (Figure 2A). In Rag-1, residues RPR391/393 at the N-terminus of putative helix I are found in the same position of most homeodomain proteins and mediate interactions with the minor groove (Gehring et al., 1994a, 1994b). The homology of Rag-1 to Hin and to homeodomain proteins extends to residues Q404, RL407/408, and E410 of helix I (Figure 2A). However, Rag-1 shows restricted homology to helix II of homeodomains and Hin and contains a longer turn between putative helices II and III. Of particular interest is the homology of Rag-1 to helix III of Hin and homeodomain structures. Helix III of homeodomains constitutes the recognition helix that provides critical specific interactions with nucleotides in the major groove (Gehring et al., 1994a, 1994b). Almost invariably, a Trp and a Phe residue are found at positions 7 and 8 of the third helix (Gehring et al., 1994a, 1994b). In contrast, the Hin family of invertases, as well as Rag-1, both lack the Trp and residue at that position (Figure 2A). Hin uses several residues (G172, T175, Y177, and Y179) within helix III to establish nonspecific interactions (Feng et al., 1994). Rag-1 contains T433 and F435 at the equivalent positions that could mediate nonspecific interactions with the RSS spacer region. Specific interactions of Hin with the TTAT motif in the major groove are established by S174 and R178, which are not present in the Rag-1 homologous region. Despite these differences, the Hin domain is able to substitute the Rag-1–homologous region. These observations suggest that Rag-1 might share structural or functional similarities with the N-terminus and helix I of the homeodomain structure but project different characteristics within putative helix III. In this respect, Rag-1 might represent an intermediate homeodomain structure in which specificity is determined by the N-terminal arm of the structure.

Interaction of Rag-1 with the Heptamer Sequence

The undiminished binding of wild-type Rag-1 to the RSS even when the heptamer is altered in key residues indicates that the protein recognizes only the nonamer (Figure 2). However, several Rag-1 mutants appeared to respond to changes in the heptamer sequence. The binding of mutant PM-27 was enhanced even further when the heptamer was mutagenized (Figure 4B). In contrast, the specific binding of mutants PM-2, ΔPM-28, PM-29, and Δ51C was clearly reduced when the heptamer sequence was mutagenized (Figure 6). The fact that several mutations within Rag-1 lead to the same phenotype suggests that they may cause an alteration in the structure of the protein that reveals a covert affinity for the heptamer. These data suggest that Rag-1 can recognize the heptamer as well as the nonamer, but in the wild-type protein this affinity is dominated by the binding of the protein to the nonamer and the nonspecific DNA affinity of Rag-1. It should be noted that in their complementary studies, Schatz and colleagues have reached similar conclusions despite the different experimental methodologies (Difilippantonio et al., 1996). Neither of the two approaches defines the precise domain of Rag-1 that interacts with the heptamer motif. However, if interactions with the heptamer are dependent on the anchoring of Rag-1 on the DNA through the nonamer site, mapping of the heptamer-binding domain of Rag-1 might only be uncovered indirectly. In fact, this seems to be a recurring theme in studying the DNA-binding properties of several transposases (Derbyshire and Grindley, 1992; references therein).

Binding of Rag-1 to both RSS motifs can have important implications for the topology of the V(D)J synaptic complex and can conceivably impose the 12/23 rule. If within one molecule of Rag-1 its homeodomain interacts with the nonamer site in the minor groove and a second domain with the heptamer site, the separation of the two motifs by one or two turns of the helix on the 12 RSS and 23 RSS, respectively, would produce unequal interactions of the protein with the DNA and differential recruitment of Rag-2 on the two sites. Given the dominant affinity of Rag-1 for the nonamer sequence, the presence of a 12 bp or 23 bp spacer would be expected to differentiate the interactions of the Rag-1–Rag-2 complex with the heptamer site.

V(D)J Cleavage Reaction Requires a Functional Heptamer But Not a Nonamer Signal

By following the initial stages of V(D)J recombination on the biosensor chip, we see that the separate functions of the heptamer and nonamer sites become evident. Once Rag-1 is attached to the RSS through its interaction with the nonamer, the cleavage reaction then requires Rag-1/Rag-2 and a functional heptamer signal. The indispensable role of the heptamer in the cleavage reaction has also been observed under different experimental conditions (Ramsden et al., 1996). It appears that the nonamer signal may not directly participate in the cleavage reaction except for anchoring the Rag-1–Rag-2 complex appropriately spaced from the heptamer sequence. Thus, it could be the nonspecific DNA-binding activity of Rag-1 that recruits the Rag-1–Rag-2 complex to the DNA and mediates heptamer-dependent cleavage even when the nonamer signal is mutated (Figure 7A).

These observations dissociate the timing by which the two RSS signals function into nonamer-directed binding and then heptamer-directed cleavage. This order of events constitutes an emerging picture for the mechanisms that govern DNA recognition and cleavage of a large number of transposases. Bacterial transposases IS903, IS10, Tn3, and MuA, the nematode transposase Tc1A, and the resolvases $\gamma\delta$ all recognize two separate sites within their recombination sequences. One site functions as a high affinity DNA-binding region that anchors the protein on the DNA, while the other is the site of cleavage. However, no direct interactions have been detected between the different transposases and their site of cleavage (Derbyshire and Grindley, 1992; Vos et al., 1993; references therein). It has been suggested that the inability of the recombinase to recognize its cleavage site directly can function to prevent cleavage at single ends of a transposon (Derbyshire and Grindley, 1992). This could also be an operating rule for the Rag-1–Rag-2 complex, which could cleave DNA only after the two RSSs to be recombined are coupled in the synaptic complex (Eastman et al., 1996; van Gent et al., 1996). The in vitro cleavage of DNA with only one RSS (McBlane et al., 1995) would then have to be artifactual, as suggested by the preference of this reaction for a Mn^{2+} cofactor (van Gent et al., 1996).

The Parallels of Tc1 Transposition and V(D)J Recombination

Perhaps the most striking resemblance between V(D)J recombination and transposition stems from the similar functional properties of Rag-1/Rag-2 and that of the nematode transposase Tc1A. It was previously recognized that the end sequences of the Tc1 transposon share striking similarity to the V(D)J RSS motifs (Figure 2B; Dreyfus, 1992). The mode of DNA recognition by Rag-1/Rag-2 and that of the Tc1A transposase reveals extensive parallels between the two systems. The C. elegans transposase Tc1A binds to an inverted repeat consisting of a heptamer- and a nonamer-like sequence (Figure 2B). Tc1A establishes high affinity interactions with the nonamer-like motif using the N-terminal domain. Cleavage occurs at the heptamer-like site, yet no interactions could be detected between Tc1A and the heptamer-like motif (Vos et al., 1993; Vos and Plasterk, 1994). The nonamer-binding N-terminal domain of Tc1A shares distinct homology to the paired class of DNA-binding domains (Franz et al., 1994). Interestingly, crystallographic analysis of the Drosophila-paired DNA-binding domain has shown that this domain is structurally related to the homeodomain and in particular to the Hin homeodomain (Xu et al., 1995).

Evolution of V(D)J Recombination

The homology of Rag-1 to the DNA-binding domain of bacterial site-specific recombinases, the presence of prototypic V(D)J RSS motifs in the flagellin promoter (Simon et al., 1980), and the striking similarities between V(D)J recombination and Tc1 transposition invite the question about the evolutionary relationship of these recombination systems to the process of antigen receptor loci rearrangement. Although site-specific recombination and transposition are thought to be mechanistically distinct systems (Craig, 1988), it is possible that they have evolved from interrelated processes. This notion is underlined by the function of recombinases such as TnPR, which is encoded by the Tn3 transposon and mediates site-specific recombination of the inverted repeat sequence elements of Tn3 (Simon et al., 1980).

It has been proposed that V(D)J recombination might have evolved based on mechanisms that mediate transposition in prokaryotes (Bartl et al., 1994; Thompson, 1995). In support of this hypothesis, it has recently been shown that the Rag-1–Rag-2 complex mediates DNA cleavage by a transesterification mechanism in parallel with the cleavage reaction by transposases and retroviral integrases (van Gent et al., 1996b).

The juxtaposition of the *Rag-1* and *Rag-2* genes and the organization of their coding information within one exon has been the basis for the suggestion that Rag-1 and Rag-2 might reflect the evolution of a single transposon (Thompson, 1995). However, while the *Rag-1* gene from amphibians, reptiles, birds, and mammals contains a single coding exon (Schatz et al., 1989; Thompson, 1995), the zebrafish and rainbow trout *Rag-1* genes contain an intron that splits the active core coding information into two separate exons (Hansen and Kaattari, 1995). Remarkably, the border of the two exons is at corresponding amino acid position 458 of the mouse Rag-1 protein (Schatz et al., 1989), which separates the Rag-1 Hin-homologous DNA-binding domain from the rest of the protein. It is therefore possible that during evolution, V(D)J recombination adopted DNA motifs and protein structures from several prokaryotic recombination systems.

Experimental Procedures

Recombinant Plasmids

For the construction of GST–fusion proteins, Rag-1, Rag-2 cDNA fragments, or shorter parts of Rag-1 were cloned as 5′-BamHI–NotI-3′ fragments in the pEBG vector. This vector is based on the eukaryotic expression vector pEF-BOS (Mizushima and Nagata, 1990) and provides the GST at the N-terminus of the fusion proteins. Expression of bacterially expressed GST–fusion proteins (5′-H2/3 and 5′-H2/3–3′) was obtained by subcloning of the corresponding

BamHI–NotI fragments into the expression vector pGEX-4T (Pharmacia). For generation of mutations or deletions within the Rag-1 protein, single-stranded Rag-1 cDNA (a gift of Dr. Christopher Roman) was used as a template for the annealing of an oligonucleotide carrying the desirable mutation or deletion. Second-strand synthesis was achieved by T4 DNA polymerase and T4 DNA ligase, using the Biorad Phagemid kit. All recombinant products were sequenced and transferred into the pEBG vector. Mutant proteins were expressed in the context of the Rag-1 active core (amino acids 330–1040).

Preparation of Biotinylated DNAs

Oligonucleotides were biotinylated at their 5′ end with biotin-dUTP (Applied Biosystems, Inc.) during their synthesis. At the end of synthesis, columns were extensively washed to remove any residual free biotin-dUTP, and oligonucleotides were recovered using standard procedures. The biotinylated strand was then annealed to a 6-fold excess of the complementary nonbiotinylated strand to ensure that no single-stranded biotinylated DNA would be immobilized on the BIAcore chip. The sequence of the four oligonucleotide substrates used in BIAcore assays is as follows: WT 7mer/9mer: 5′-TAGCTCGAGAAGACCTACACAGTGATACAGACCTTAACAAAAA CCCTGCTCCAG-3′; 7mut: 5′-TAGCTCGAGAAGACCTA**AGTCTTG**A TACAGACCTTA **ACAAAAACC**CTGCTCCAG-3′; 9mut: 5′-TAGCTCG AGAAGACCTA**C ACAGTG**ATA CAGACCTTA**ACACCTAAC**CTGCTC CAG-3′; 7mut/9mut: 5′-TAGCTCGAGAAGAC **CTAAGTCTTG**ATACA GACCTTA**ACACCTAAC**CTGCTCCAG-3′.

Expression and Purification of GST–Fusion Proteins

With the exception of 5′-H2/3 and 5′-H2/3–3′, all other recombinant proteins described in this paper were produced in the human kidney cell line 293T, using the pEBG vector that allows high levels of expression in transient transfections (Spanopoulou et al., 1995). Recombinant plasmids were transiently expressed. Cells were harvested in phosphate-buffered saline/1 mM phenylmethylsulfonyl fluoride, pelleted, and resuspended in RSB buffer (RSB: 10 mM Tris [pH 7.4], 10 mM NaCl, 5 mM $MgCl_2$, and 0.5% NP-40, plus protease inhibitors). After lysis, 1.5 vol of LSB was added to the cell suspension (LSB: 20 mM Tris [pH 7.4], 1 M NaCl, 0.2% NP-40, 0.2 mM $MgCl_2$, plus protease inhibitors) and rocked gently for at least 2 hr at 4°C. Cellular debris was pelleted at 30,000 rpm for 2 hr. The supernatant was incubated with glutathione–agarose beads. Subsequently, beads were washed four times. Bound GST–fusion proteins were eluted at 4°C for 30 min by GST–elution buffer (50 mM Tris [pH 8.4], 20 mM glutathione, 1 M NaCl, 10% glycerol, plus protease inhibitors). Elution was repeated four times. The collected proteins were dialyzed against BIAcore running buffer (20 mM Tris–HCl [pH 7.6], 250 mM NaCl, 5 mM $MgCl_2$, 4% glycerol, 1 mM dithiothreitol). Proteins were concentrated on Centricon 50 columns (Amicon), which also removed part of the copurifying GST. The quantity and quality of each protein preparation was determined by Coomassie staining (Figure 1A), in comparison with standard concentrations of bovine serum albumin.

BIAcore Binding Assays

The basic methodology of the BIAcore biosensor has been described previously (Jönsson et al., 1991; Panayotou et al., 1993). Experiments were conducted on BIAcore and BIAcore 2000 instruments (Pharmacia). The running buffer was 20 mM Tris–HCl (pH 7.6), 250 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, and a constant flow of 5 μl/min was maintained at 25°C. Biotinylated DNA was injected over immobilized streptavidin (SA5 sensorchip, Pharmacia) until a level of approximately 2,500 resonance units was obtained. The same amount was immobilized for the different DNAs employed. Proteins were dialyzed in running buffer and stored at 4°C. Stock solutions were diluted in running buffer and injected over the immobilized DNA. For each assay, Rag-1ΔN (or mutant forms of it) were loaded on the chip at a concentration of 10pM over the course of 6 min, followed by injection of running buffer. The resulting resonance units versus time plots (sensorgrams) were analyzed using the evaluation software supplied with the instruments. To regenerate the surface for repeat injections of protein, a pulse of 5 μl of 0.05% SDS was injected. This resulted in complete dissociation of bound proteins without affecting the level of immobilized DNA, which could be used for at least 50 rounds of protein binding and regeneration. All experiments presented in this paper were performed at least three times.

Recombination Assays

Mutant proteins were tested for their recombination activity in 293 and Hela cells by cotransfection of Rag-1 (mutant or wild-type) with wild-type Rag-2 (amino acids 1–388) and the recombination substrates, either pJH288 or pJH200 (Hesse et al., 1987). Cells were transfected by calcium phosphate precipitation and harvested 48 hr later. Recombined products were isolated as described previously (Oettinger et al., 1990) and analyzed for recombination frequency by two different methodologies: first, as a ratio of chloramphenicol/ampicillin-resistant versus ampicillin-resistant colonies (Hesse et al., 1989); and second, by polymerase chain reaction (PCR) analysis using oligonucleotides that detect the recombined products by annealing to the joined heptamer signal sequences (oligo-RA5 and RACR2) and to the CAT gene (oligo-RA14) (RA5: 5′-CCAGTCTGTAG CACTGTGCAC-3′; RACR2: 5′-TTTGTTCCAGTCTGTAGCACTGCG CAC-3′; RA14: 5′-TCCAGCTGAACGGTCTGGT-3′). PCR conditions were as follows: 94°C for 40 s, 65°C for 60 s, and 75°C for 60 s (35 cycles). The reactions incorporated ^{32}P-dCTP. Reaction products were analyzed on a 5% polyacrylamide gel and visualized by autoradiography.

Cleavage Assays

Reaction conditions were based on previously described protocols (McBlane et al., 1995). Purified Rag-1 and Rag-2 were incubated with a ^{32}P-radiolabeled 12 RSS oligonucleotide substrate (upper strand: 5′-ACGCGTCGACGTCTTA**CACAGTG**ATA CAGCCCTGA**A CAAAAACC**GGATCCGCG-3′). Standard reactions were performed in 20 μl in the presence of 25 mM MOPS–KOH (pH 7.0), 5 mM Tris–HCl, 95 mM KCl, 2.2 mM dithiothreitol, 4% glycerol, 1 mM $MnCl_2$ (unless stated otherwise), and 50 ng of each protein. Reactions were incubated at 37°C for 1 hr, and cleavage products were resolved on 12.5% denaturing polyacrylamide gels and visualized by autoradiography.

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