Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 139-145

Nonamer Binding Protein Induces a Bend in the Immunoglobulin Gene Recombinational Signal Sequence

Rick Andrews[1], Nadine L. N. Halligan[2], and Brian D. Halligan[1†]

[1] Department of Microbiology, and [2] Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226

Received March 19, 1993

DNA bending has been shown to play a critical role in conservative site-specific DNA recombination reactions such as λ integration. V(D)J recombination, the only mammalian site directed recombination system, is directed by recombinational signal sequences composed of heptamer, nonamer and spacer elements. The nonamer element, GGTTTTTGT, is similar to the consensus sequence for bent DNA. Using the circular permutation electrophoretic mobility assay, we show that the nonamer sequence has a detectable intrinsic bend. The nonamer sequence has been shown to be the binding site for nonamer binding protein (NBP). Binding of NBP to the nonamer site increases the apparent angle of the bend from 32° to 66°. The identification of a protein induced DNA bend near the site of V(D)J recombination may have implications for our understanding of the mechanism of V(D)J recombination.

Immunoglobulin and T-cell antigen receptor genes are unlike most other mammalian genes in that they are encoded in the genome in the form of families of separate gene segments (see 1, 2, 3, 4 for review). The expression of immunoglobulins by mature B-cells or T-cell antigen receptors by mature T-cells requires a series of developmentally regulated recombination events. Immunoglobulin heavy chain genes and T-cell antigen receptor alpha and gamma chain genes are composed of three gene segments: the variable region gene segments (V), the diversity region gene segment (D), and the joining region gene segment (J). The immunoglobulin kappa and lambda light chain genes and the T-cell antigen receptor beta and delta chain genes are composed of two gene segments: the variable region gene segment (V) and the joining region gene segment (J). The recombination process that brings the gene segments of the immunoglobulin or T-cell antigen receptors together has been named V(D)J recombination after the

† To whom correspondence should be addressed.

0006-291X/93 $4.00

segments involved. Analysis of the sequence of a large number of gene segments both before and after recombination has led to the conclusion that V(D)J recombination takes place in a site specific manner and is directed by a motif of DNA sequences, the recombinational signal sequences or RSS, located directly adjacent to the coding regions. The RSS are composed of a conserved heptamer sequence (CACTGTG) separated from a conserved nonamer sequence (GGTTTTTGT) by a defined length spacer region. This motif of DNA sequences has been found to be associated with all functional immunoglobulin and T-cell antigen receptor gene segments and is also well conserved through species expressing immunoglobulins. The high degree of sequence conservation between the noncoding RSS implies that the RSS play a direct role in the recombination reaction. Experiments utilizing defined DNA substrates introduced into pre-B cells in culture have further demonstrated the necessity of these sequence elements for recombination and established these sites as potential protein binding sites (5).

The heptamer DNA sequence has been shown by two groups to be recognized by site specific DNA binding proteins (6, 7). The nonamer DNA sequence, which is separated from the heptamer DNA sequence by one or two turns of the DNA helix, has also been shown to be recognized by a site specific DNA binding protein, the nonamer binding protein, or NBP (8, 9). NBP has been shown to recognize nonamer sequences in RSS from gene segments from different gene families and in RSS with different spacer lengths (8). Studies of the sequence specificity of binding of NBP have shown that the greatest loss in NBP binding affinity occurs in nonamer sequences in which the central stretch of T residues is altered (9). Similarly, a survey of nonamer sequences from different gene segments has revealed that the region of greatest conservation between nonamer sequences is the central T positions (9).

The nonamer sequence, as viewed on the complementary strand, ACAAAAACC, is very similar to DNA sequences that have been demonstrated to be bent or that can be induced to bend by protein binding (10, 11). Oligonucleotides containing five A residues on one strand have been demonstrated by Wu and Crothers (10) to be bent. The nonamer sequence is also similar to DNA sequences that have been shown to form Hoogstein tetrahelical base pairs (12). Similarly, other DNA sequences that have been demonstrated to be bent when bound with protein also contain poly A regions (11).

We have used the system developed by Robertson and Nash (13) to demonstrate that the nonamer sequence is intrinsically bent and can be induced to bend upon the binding of NBP to the nonamer sequence.

Materials and Methods

Cloning

To generate the permuted DNA fragments containing the RSS, the Eco RI-Bam HI fragment from pJκ4 was made blunt by treatment with T4 DNA polymerase.

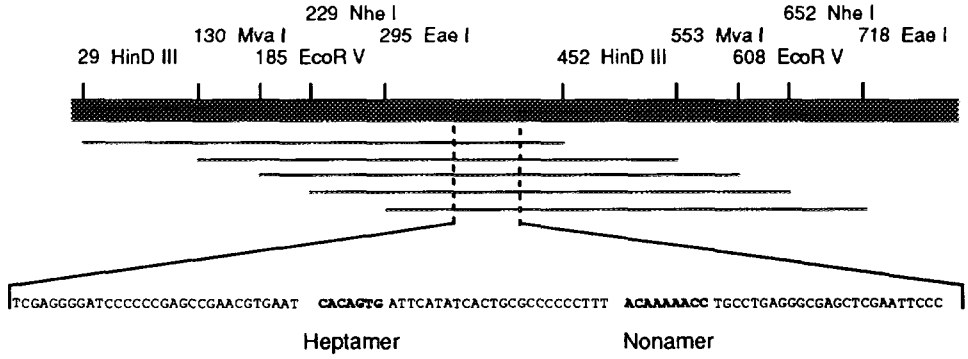

Figure 1. Structure of DNA fragments used for circular permutation and EMSA assays. DNA fragments were generated by digestion of pRA1 with the indicated restriction enzyme at sites in the duplicated region flanking the Jκ4 RSS. Heptamer and nonamer elements are indicated in bold type.

The resulting 84 bp DNA fragment was ligated to Xho I linkers and excess linkers were removed by digestion with Xho I. A plasmid vector designed for the assay of bent DNA, pHN915, was linearized by digestion with Xho I and the RSS containing DNA fragment ligated into the unique Xho I site to produce pRA1. Permuted DNA fragments containing the RSS were produced by digestion of pRA1 with the restriction enzymes Hind III, Nhe I, Mva I, Eco RV and Eae I (Figure 1). For use in protein binding experiments, the fragments were dephosphorylated with calf intestinal alkaline phosphatase, 5′ end labeled with [^{32}P]-γ-ATP using T4 polynucleotide kinase. Radiolabeled DNA fragments were purified by polyacrylamide gel electrophoresis and electroelution.

Extracts

NBP was partially purified from nuclear extracts of calf thymus using ammonium sulfate precipitation and BioRex-70 cation exchange chromatography by the method of Halligan and Desiderio (8). The extracts were adjusted to a protein concentration of 2 mg/ml and 50% (w/v) glycerol.

Bending experiments

To demonstrate intrinsic bending of the nonamer fragment, pRA1 plasmid DNA was digested with the indicated restriction enzyme and electrophoresed through an 8% polyacrylamide 1X TBE gel at 4°C. The gel was stained with 0.5 μg/ml ethidium bromide and photographed.

To demonstrate NBP induced bending of the nonamer fragment, electrophoretic mobility shift assays (EMSA) were performed on the labeled, permuted pRA1 DNA fragments as previously described (8). Briefly, labeled DNA fragments (0.1 ng) were incubated in the presence of NBP (2 μg) and poly(dI-dC)•poly(dI-dC) (10 ng) for 30 minutes at 22°C. The reactions were then loaded on native 6% polyacrylamide gel and electrophoresed at 7.5 V/cm for 2.5 hours. The gels were dried and autoradiographed.

Calculation of bend angles was performed as described by Thompson and Landy (14). by determining the ratio of the mobilities of the fastest and slowest migrating species and using the relationship $\mu_M/\mu_E = \cos(\alpha/2)$.

Results

Cloning

The assay used to detect a DNA bend in the absence or presence of site specific DNA binding proteins was described by Robertson and Nash (13). This assay uses a

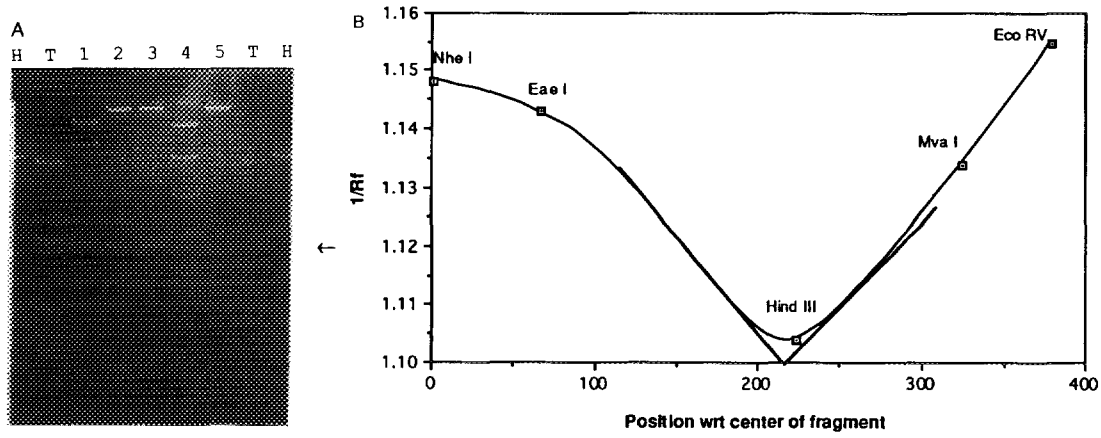

Figure 2. Demonstration of intrinsic bend associated with Jκ4 RSS element.
Panel A. DNA fragments produced by digestion of pRA1 with restriction enzymes at sites in the duplicated region flanking the Jκ4 RSS were electrophoresed through a 8% polyacrylamide gel, stained with ethidium bromide and photographed. Lane 1, Eae I; lane 2, Nhe I; lane 3, Eco RV; lane 4, Mva I; lane 5, Hind III. Marker DNA fragments were run in lanes H (Hpa II digested pBR322) and T (Taq I digested pUC8) for comparison.
Panel B. The inverse of the relative mobilities (R_f) of the DNA fragments containing the RSS region was plotted against the position of the center of the nonamer sequence within the 423 bp fragment.

set of DNA fragments of identical base composition containing the DNA binding site of interest located at different positions with respect to the ends of the fragments. To utilize this assay system, we cloned the full RSS element from the mouse Jκ4 gene segment into the Xho I site of pHN915, to form the plasmid pRA1. Fragments containing the RSS at different relative positions were released by digestion with restriction enzymes located within the duplicated regions. Figure 1 depicts the fragments produced by digestion of pRA1 with Hind III, Nhe I, Eco RV, Mva I and Eae I. Permuted DNA fragments produced by digestion were used directly to assay intrinsic DNA bending of the RSS sequence or were radiolabeled for use in gel retardation assays.

Intrinsic bending

The presence of a slight intrinsic bend in the RSS was detected by gel electrophoresis of permuted DNA fragments containing the RSS, shown in Figure 2, panel A. DNA fragments in which the RSS was located closest to the center of the fragment showed the greatest degree of anomalously slow migration (lanes 2 and 3, Eco RV and Nhe I), while DNA fragments in which the RSS was located most distally showed the least degree of anomalously slow migration (lanes 1 and 5, Eae I and Hind III). Fragments produced by digestion with Mva I (lane 4) have the RSS located at an intermediate position and demonstrate an intermediate migration. From this data, we conclude that there is an intrinsic bend associated with the $J_\kappa 4$ RSS. The magnitude of this bend appears to be approximately 32.5°. This is based on a μ_M/μ_E of 0.96 for the Hind III and Eco RV ended fragments, and the relationship $\mu_M/\mu_E = \cos(\alpha/2)$, where α is the bend angle (14).

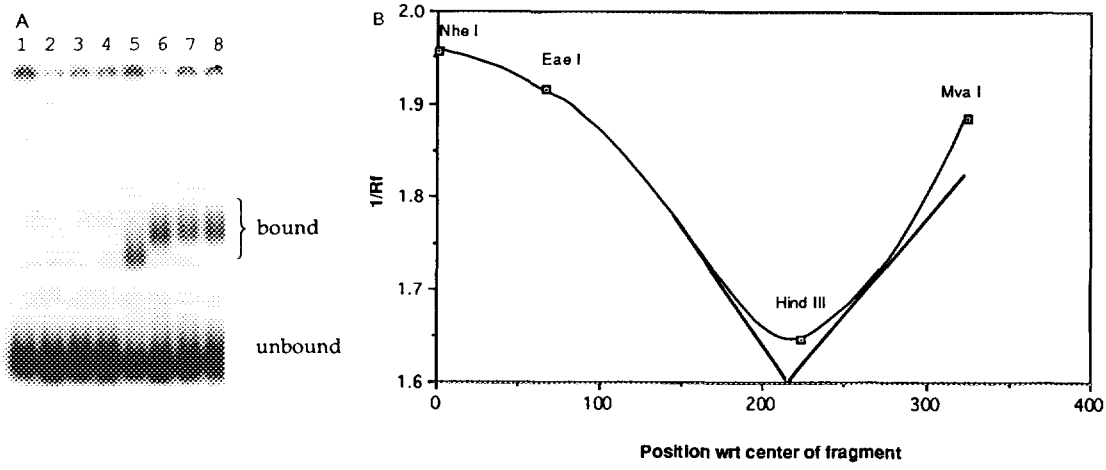

Figure 3. Demonstration of induced bend in Jκ4 RSS by NBP.

Panel A. DNA fragments produced by digestion of pRA1 with restriction enzymes at sites in the duplicated region flanking the Jκ4 RSS were [^{32}P] end labeled and used in EMSA with NBP. Lanes 1 to 4 contain no NBP, lanes 5 to 8 have 2 μg of partially purified NBP added. Lanes 1 and 5, Hind III; lanes 2 and 6, Mva I; lanes 3 and 7, Nhe I; and lanes 4 and 8, Eae I.

Panel B. The inverse of the relative mobilities (R_f) of the NBP bound DNA fragments containing the RSS region was plotted against the position of the center of the nonamer sequence within the 423 bp fragment.

Induced bending

To determine if a bend of larger magnitude could be induced by the binding of NBP to the nonamer sequence, a gel retardation experiment was performed with labeled, permuted DNA fragments as the binding substrate. The results of the experiment are shown in Figure 3. Lanes 1 through 4 depict reactions in which no NBP was added, and lanes 5 through 8 depict reactions in which partially purified NBP was added. The mobilities of the unbound DNA fragments differ only slightly in the 6% polyacrylamide gel system used for the gel retardation assay. The mobilities of the DNA-NBP complexes show a strong dependence on the position of the RSS with respect to the ends of the DNA molecules. The increased mobility of DNA fragments was observed when the RSS was located at the ends of the fragments (lanes 5 and 8), and decreased mobility was observed when the RSS was located at the center of the DNA fragments (lanes 6 and 7). This finding is consistent with the induction of a DNA bend of significantly greater magnitude than the intrinsic bend by the binding of NBP to the nonamer sequence. From these data, we conclude that NBP induces a bend in the RSS sequence of approximately 66° based on a μ_M/μ_E of 0.84. By extrapolation of the R_f vs. position plot (Figure 3, Panel B), the site of the bend is close to the position of the nonamer sequence. This is consistent with the finding that the nonamer is the site of NBP binding within the RSS (8). The magnitude of the bend induced by NBP is less than the >140° angle seen with IHF, but close to the magnitude of bending observed for Int and Xis (14).

Discussion

Studies of DNA recombination systems have shown that the recombination process is catalyzed by a three dimensional array of DNA and proteins (15). Many types of interactions take place in such complexes, including DNA-protein interactions, protein-protein interactions, and the induction of DNA conformational changes by protein. Protein induced DNA bending by Int, IHF, Xis and FIS has been shown to play an important role in the alignment of DNA sequences during the lambda phage integration reaction (16, 17), as well as in the Flp recombination system of yeast (18, 19). Lambda integration is the best studied recombination system involving DNA bending. IHF, integrative host factor, is necessary for recombination and has been demonstrated to bend DNA in a sequence dependent manner. The role of an IHF induced DNA bend in the integration reaction has been directly tested by Goodman and Nash(20). They have replaced the IHF binding site with intrinsically bent DNA sequences or sequences that act as a binding and bending site for other proteins known to bend DNA and re-established recombination in the absence of IHF binding. In this paper, we demonstrate the induction of a similar bend in the immunoglobulin and T-cell antigen receptor recombinational signal sequences by NBP and postulate that this conformational change in the RSS DNA may play a role in V(D)J recombination.

Little is known about the mechanism of V(D)J recombination. The substrates and products of the rearrangement event are well defined from cloning and sequence analysis of immunoglobulin and T-cell receptor gene segments both before and after recombination has taken place. The recombination process is directed by RSS elements that have either a 12 ± 1 or 23 ± 1 bp spacer region. In non-aberrant recombination events of endogenous gene segments, recombination takes place between gene segments with different spacer lengths, i.e.. between RSS with 12 and 23 bp spacer elements. This 'spacer rule' has been thought to be a reflection of the physical arrangement of proteins that carry out the recombination process on the DNA, and that the 12 and 23 bp spacer regions correspond to one or two turns of the DNA helix. This idea has been re-enforced by studies in which gene segments with altered spacer lengths were introduced into lymphoid cells and found to recombine at a significantly lower frequency than gene segments with normal spacer lengths. The finding that the RSS has an intrinsic bend and that an greater bend can be induced by the binding of the RSS by NBP will be of importance in understanding the mechanism of V(D)J recombination as more information about the proteins involved in the process becomes available.

References

1. Alt, F.W., Oltz, E.M., Young, F., Gorman, J., Taccioli, G., and Chen, J. (1992) Immunol Today 13, 306-314.
2. Lewis, S. and Gellert, M. (1989) Cell 59, 585-588.
3. Lieber, M.R. (1991) FASEB J. 5, 2934-2944.

4. Schatz, D.G., Oettinger, M.A., and Schlissel, M.S. (1992) Annu. Rev. Immunol. 10, 359-383.
5. Hesse, J.E., Lieber, M.R., Mizuuchi, K., and Gellert, M. (1989) Genes Dev. 3, 1053-1061.
6. Aguilera, R.J., Akira, S., Okazaki, K., and Sakano, H. (1987) Cell 51, 909-917.
7. Matsunami, N., Hamaguchi, Y., Yamamoto, Y., Kuze, K., Kangawa, K., Matsuo, H., Kawaichi, M., and Honjo, T. (1989) Nature 342, 934-937.
8. Halligan, B.D. and Deisderio, S.V. (1987) Proc. Natl. Acad. Sci. USA 84, 7029-7023.
9. Li, M., Morzycka-Wroblewska, E., and Desiderio, S.V. (1989) Genes and Devl. 3, 1801-1813.
10. Wu, H.-M. and Crothers, D.M. (1984) Nature 308, 509-513.
11. Travers, A.A. (1989) Annu. Rev. Biochem. 58, 427-452.
12. Jin, R., Breslauer, K.J., Jones, R.A., and Gaffney, B.L. (1990) Science 250, 543-546.
13. Robertson, C.A. and Nash, H.A. (1988) J. Biol. Chem.
14. Thompson, J.F. and Landy, A. (1988) Nuc. Acids Res. 16, 9687-9705.
15. Craig, N.L. (1988) Ann. Rev. Genet. 22, 77-105.
16. Snyder, U.K., Thompson, J.F., and Landy, A. (1989) Nature 341, 255-257.
17. Moitoso de Vargas, L. and Landy, A. (1991) Proc. Natl. Acad. Sci., USA 88, 588-592.
18. Schwartz, C.J.E. and Sadowski, P.D. (1990) J. Mol. Biol. 216, 289-298.
19. Chen, J.W., Evans, B., Rosenfelt, H., and Jayaram, M. (1992) Gene 119, 37-48.
20. Goodman, S.D. and Nash, H.A. (1989) Nature 341, 251-254.