

Switch Recombination Breakpoints Are Strictly Correlated with DNA Recognition Motifs for Immunoglobulin S γ 3 DNA-binding Proteins

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Summary

The deletion looping out model of switch (S) recombination predicts that the intervening DNA between switch regions will be excised as a circle. Circular excision products of immunoglobulin switch recombination have been recently isolated from lipopolysaccharide (LPS)-stimulated spleen cells. The recombination breakpoints in these large circles were found to fall within switch regions. Since switch recombination is clearly focused on switch regions, we hypothesized that some DNA-binding protein factor might be involved in specifically recognizing and facilitating the alignment of switch regions before recombination. Two DNA-binding proteins that specifically interact with two discrete regions of the S γ 3 tandem repeat have been identified in crude and partially purified nuclear extracts derived from LPS- and dextran sulfate (DxS)-activated splenic B cells. The first factor has been found indistinguishable from NF- κ B by mobility shift assays, methylation interference, competition binding studies, and supershift analysis using an antiserum specific for the p50 component. The second appears to be composed of two closely traveling mobilities that do not separate upon partial purification. This second complex is unique and specific for S γ 3 by methylation interference assays and competition-binding analysis. The sites at which recombination occurs in the S γ 3 switch region have been analyzed and found to strictly correlate with the binding sites of the S γ 3 switch binding proteins.

The immunoglobulin (IgH) heavy chain class switch permits expression of a mature V region with C regions other than C μ (1). Switch (S)¹ recombination occurs by a DNA rearrangement that brings one of six downstream C $_H$ genes proximal to the V gene (2). This results in the deletion of the C μ gene and the intervening genomic material (1, 3, 4). The recombination event focuses on S DNA, located upstream of each C $_H$ gene, and produces a new hybrid DNA combination (5–7). The deletion looping out model for switch recombination predicts that the intervening DNA between S regions will be excised as a circle (4). Circular excision products of Ig switch recombination have recently been isolated from LPS-stimulated spleen cells (8–10). This finding confirms that deletion looping out is the mechanism that produces a large number, if not the vast majority, of successful switch recombination events.

Recombination requires that the two recombining DNA

helices be in synapsis. The precise spatial arrangement of the juxtaposed DNA sequences will determine the product of the recombination event (11–14). A common feature of such processes as transcription, site-specific recombination, and the initiation of DNA replication is formation of DNA–multiprotein complexes (15). Ig switch recombination involves DNA rearrangements between sequences distanced by 50–100 kb of genetic material, and is clearly focused on switch regions (2, 4). We reasoned, based on these considerations, that synapsis of switch regions before recombination may be stabilized by sequence-specific DNA-binding proteins. We have detected an S μ sequence-specific DNA-binding protein, termed NF-S μ , that is induced in mitogen-activated B cells and recognizes a portion of the S μ tandem repeat, GAGCTGGGGTGAGCT (16). The kinetics of expression of NF-S μ in mitogen-stimulated splenic B cells parallels the induction of recombinational activity at S μ in these cells (16). LPS/DxS activation of normal splenic B cells produces switching from IgM expression to production of a high level of IgG3, some IgG2b, and no IgG1 (17). We hypothesized that another sequence-specific DNA-binding protein might recognize the

¹ Abbreviations used in this paper: DMS, dimethyl sulfate; DxS, dextran sulfate; EMSA, electrophoretic mobility shift assay; S, switch; SNAP, switch nuclear A protein; SNIP, switch nuclear protein.

S γ 3 switch regions and facilitate S μ to S γ 3 switch recombination. Reported here is the identification of two DNA binding proteins that specifically and individually interact with two discrete portions of the S γ 3 tandem repeat. One of these S γ 3 switch-binding proteins has been identified as NF- κ B, suggesting that this transcription factor plays an important role in switch recombination. Significantly, the DNA sequences at which recombination occurs in the S γ 3 switch region are highly correlated with the binding sites of these two switch-binding proteins.

Materials and Methods

Preparation of Plasmids, Probes, and Oligonucleotides. The genomic clone, pS γ 3 0.8 (18), containing \sim 850 bp of the γ 3 switch region, was a gift from K. Marcu (State University of New York, Stony Brook, NY). The EcoRI/PvuII fragment of pS γ 3 0.8 was subcloned into pUC19. Probe pl.S γ 3⁺, comprising 1.5 49-bp repeats, was derived from this subclone by cutting with Avall/BamHI. Plasmid pS γ 3.A was generated by cloning the 30-bp A oligo into the HincII site of pUC19. The probe pl.S γ 3.A was produced by XbaI/HindIII digestion of this recombinant plasmid. These probes were gel purified after labeling with α [³²P]deoxyribonucleotides to a specific activity of 5×10^7 cpm/ μ g using the Klenow fill-in reaction. The B2 and κ B probes were prepared by labeling the double-stranded B2 and κ B synthetic oligomers with γ -[³²P]ATP using T4 polynucleotide kinase. Unincorporated nucleotides were separated from these probes using Sephadex G50-300 (Sigma Chemical Co., St. Louis, MO) mini-spin columns. Radioactivity of probes was measured by Cerenkov counting. Oligonucleotides were chemically synthesized by Operon Technologies, Inc. (Alameda, CA) or the Macromolecular Analysis Facility (Loyola University, Chicago, IL) and we purified them either by Nensorb cartridge (DuPont NEN, Boston, MA) or HPLC. Double-stranded oligomers were annealed by mixing equimolar amounts of complementary strands, heating to 95°C for 10 min, followed by slow cooling. Concentrations were determined spectrophotometrically and by ethidium bromide staining.

Cell Culture and Nuclear Extracts. Single-cell suspensions prepared from the spleens of 8–12-wk-old female BALB/c mice were stimulated in culture with LPS and D \times S as previously described (19). A20 cells and 70Z/3 cells were grown in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 4 mM glutamine. Also included in the 70Z/3 cell cultures were 10 μ g/ml LPS (*Salmonella typhimurium*, phenol extract; Sigma Chemical Co.) and 50 μ M 2-ME. The cells were maintained in culture at densities of 0.5 – 2.0×10^6 cells/ml. Nuclear extracts were prepared by a modification of the Dignam procedure (20) as described (16).

Partial Purification of Switch-Binding Proteins by Column Chromatography. Nuclear extract from LPS/D \times S-stimulated B cells (9 mg) was applied to a 1 ml MonoS fast protein liquid chromatography (FPLC) column equilibrated with buffer D (20 mM Hepes pH 7.9, 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 μ g/ml leupeptin, 0.5 mM dithiothreitol [DTT]). The column was run at a flow rate of 0.5 ml/min, and proteins were eluted with a 22.5 ml linear gradient of buffer D containing 50–750 mM KCl. A20 nuclear extract (80 mg) was applied to heparin-agarose (14 ml in a 1 \times 18 cm column) equilibrated with buffer D. A flow rate of 10 ml/h was maintained. The column was washed with four column volumes of buffer D and step eluted with buffer D

containing 0.1, 0.2, 0.5, and 1.0 M KCl, successively. Fractions identified as having A-site-binding activity were pooled and then concentrated on a 1 ml heparin-agarose column. LPS/D \times S-stimulated B cell nuclear extract (5 mg) was fractionated by stepwise elution (100 mM step increases in KCl concentration) from a 2 ml heparin-agarose column. Fractions were dialyzed against buffer D and analyzed for switch-binding proteins by EMSA.

Electrophoretic Mobility Shift (EMSA), Supershift, and Methylation Interference Assays. Specific DNA-protein interactions were detected as described (21). Binding reactions (10 mM Tris-HCl pH 7.6, 50 mM NaCl, 5% glycerol, 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 1.5 μ g poly(dI.dC), 2 μ g BSA) were carried out at room temperature in final volumes of 20 μ l. Nuclear extracts (0.5–8 μ g total protein) were preincubated with the binding mixture for 10 min. In the case of competition experiments, competitor DNA was added next and incubated for 10 min. In all cases, the DNA probe (10,000 cpm) was added last. The complete reaction mixes were incubated for an additional 15 min and then loaded on low ionic strength (6.7 mM Tris-HCl pH 7.5, 3.3 mM Na-acetate, 1.0 mM EDTA) polyacrylamide gels (acrylamide/bisacrylamide weight ratio of 30:1) that had been prerun for 1 h. Electrophoresis was carried out at \sim 11 V/cm (1.5 h for 4% gels; 2.5 h for 6% gels) with recirculation of fresh running buffer. Autoradiography of the dried gels was carried out using intensifying screens at -80°C .

The anti-p50 antibody is directed against a synthetic peptide encompassing amino acids 394–404 of the murine p107 protein (22). The peptide, NH₂-STGSPGPGYGYC-COOH, was crosslinked to BSA (Sigma Chemical Co.) with MBS (m-maleimidebenzoic acid-N-hydroxysuccinimide ester, Pharmacia Fine Chemicals, Piscataway, NJ) and injected into female New Zealand white rabbits. Antisera from the second boost was characterized in ELISA and in super-gel shift assays. Anti-p50 antiserum could specifically shift in vitro-translated and affinity-purified p50 protein, as well as p50 and NF- κ B complexes from nuclear extracts of A20, murine B cell and D5H3, murine T cell hybridoma. Specificity of antibody supershift was verified by comparison to preimmune serum, and by competition with specific and nonspecific peptides (Jamieson, C. A. M. and Sen, R., manuscript in preparation). For supershift assays, nuclear extracts were first incubated with either immune or preimmune serum for 3 h at 0°C. The additional, premixed components of the binding reaction were subsequently added according to the procedure for standard mobility shift assays. Methylation interference assays were performed as described (16). Probes labeled on one strand were partially methylated with dimethyl sulfate (DMS) and then used in scaled-up mobility shift assays. The bound and free DNAs were electroeluted from the gel, extracted and precipitated, and treated with piperidine. The cleavage products were resolved on 15–20% denaturing gels.

Results

NF- κ B Binds to a Discrete Site within the S γ 3 Tandem Repeat. We set out to determine whether an S γ 3-specific DNA binding protein(s) is present in the nuclear extracts of mitogen-stimulated splenic B cells. The probe, pl.S γ 3⁺, contains a fragment of genomic S γ 3 DNA representing 1.5 repeats of the repetitive switch motif (Fig. 1). When nuclear extracts, prepared from normal splenic B cells stimulated with LPS/D \times S for 44 h were incubated with pl.S γ 3⁺, a complex pattern of five distinguishable retarded bands are visualized by EMSA (Fig. 2 A). To begin assignment of DNA-binding proteins with specific S γ 3 DNA recognition sequences, crude nuclear

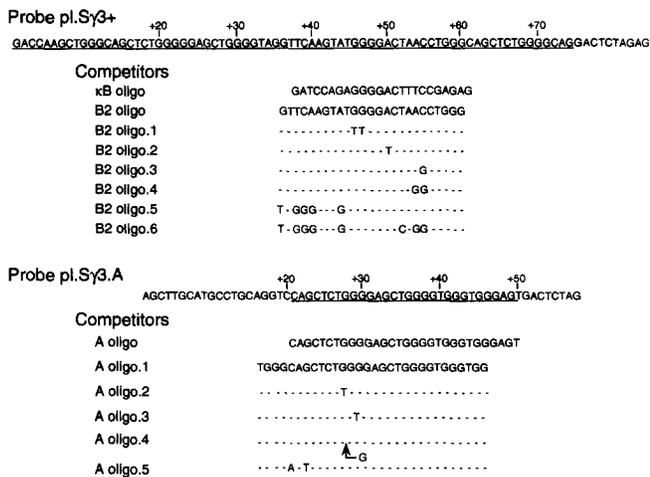


Figure 1. List of probes and competitors. Probes pl.Sy3⁺ and pl.Sy3.A were prepared as described in Materials and Methods. The Sy3 sequences are underlined. Four bases of the pUC polylinker at the 3' end of pl.Sy3⁺ are not shown. For simplicity, only the coding strands are shown. Numbering for the noncoding strands proceeds in the 5'–3' direction using negative numbers. The κ B oligo sequence is from the κ B site of the intron enhancer of the κ L chain gene (23). The κ B and B2 series of oligos are aligned under the B2 site in pl.Sy3⁺. (Dashes) Positions of identity with the B2 oligo. The A series of competitors are aligned under the A site in the pl. Sy3.A probe. (Dashes) Positions of identity with A oligo.1. (Arrow) Additional G inserted in A oligo.4.

extract was fractionated by MonoS FPLC. This fractionation resulted in the successful separation of an Sy3-specific DNA-binding protein that migrates coincident with Sy3.B2 found in crude extract as analyzed by EMSA (Fig. 2 B and data not shown). This fraction, referred to as MonoS.B2, was taken for analysis by methylation interference.

The methylation interference procedure was used to precisely map the guanine residues involved in essential protein contacts. Using the probe pl.Sy3⁺, we found that methylation of G residues at positions +46–+49 on the coding strand, –33, –34, and –38 on the noncoding strand markedly interfere with binding (Fig. 3). The binding site focuses on a run of guanine residues on the coding strand and spans ~ one turn of the helix (Fig. 3). It is striking that other similar runs of purines within the Sy3 repeat sequence are not a target for this DNA-binding protein. We used the MonoS.B2 protein fraction and the pl.Sy3⁺ probe in a competition-binding assay. The 25-bp B2 oligomer was chemically synthesized to represent the binding site defined in the methylation interference assay for MonoS.B2 (Fig. 1). The B2 oligomer successfully competes with pl.Sy3⁺ for binding the fraction MonoS.B2 (Fig. 2 B). In contrast, no competition was found when B2 oligo.1 was used as a competitor (Fig. 2 B). This oligomer carries two mutations, at positions 12 and 13, corresponding to positions +47 and +48 in the probe, which interrupts the string of four guanines with thymidines. These results confirm the methylation interference data which suggests that these G residues are critical protein-DNA contact sites.

The specificity of the B2 protein–DNA interaction was further tested by competition-binding assays using a series of mutant B2 oligomers and crude nuclear extracts from normal splenic B cells stimulated with LPS/DxS for 44 h. The B2 oligomer successfully competes with pl.Sy3⁺ for binding the B2 band (Fig. 2 A). B2 oligo.1 was unable to compete for binding even at the highest concentrations tested (Fig. 2 A). A single point mutation at position 20, corresponding to position –34 in the probe, reduced the capacity of B2 oligo.3 to compete for binding by fourfold (Fig. 2, lanes 14–16). This mutation affects a single contact residue as defined by the methylation interference assay. B2 oligo.4, contains two mutations of which only one is predicted to be a protein contact site and has the same capacity to compete for binding as B2 oligo.3. Radical upstream mutations that fall outside the protein binding motif, as found in B2 oligo.5, have no effect on binding, as expected.

Upon inspection, the B2 site in the pl.Sy3⁺ genomic probe was found to deviate somewhat from the most common sequence found in the Sy3 tandem repeat. To determine whether the B2-binding protein was capable of specifically binding the most common motif, oligo.6 was synthesized to represent the consensus DNA sequence from this region of the tandem repeat. B2 oligo.6 contains eight point mutations, of which only position 20 is a protein contact site. This consensus oligomer is able to specifically compete for binding though it is about 16-fold weaker than the B2 oligo (Fig. 2, lanes 23–25). Indeed, oligo.3–.6 represent stepwise changes toward a consensus B2 site, and confirm that this most prevalent sequence is capable of forming the B2 complex, though with apparently lower affinity.

We observed a striking identity between the B2 DNA-binding motif and the DNA recognition motif for 3' κ enhancer site of NF- κ B (23 and Fig. 1). A number of different DNA-binding proteins are capable of interacting with the NF- κ B-binding motif (24). To test whether NF- κ B itself binds the Sy3 switch region, a 25-bp oligomer containing an NF- κ B-binding site derived from the κ intron enhancer was prepared and used in a competition-binding assay (Figs. 1 and 2, lanes 5–7). The κ B oligo was equally able to compete for the B2 binding-complex when compared with the B2 oligo. These results demonstrate that the B2 binding site is also an NF- κ B recognition motif.

To more directly determine whether bona fide NF- κ B protein binds to the B2 binding site, an antiserum was made to a synthetic peptide from the p50 polypeptide of NF- κ B (anti-p50). The sequence of the immunogenic peptide is not found in other members of the rel family of proteins (21). In this experiment, the NF- κ B and B2 oligos were used as probes in a binding assay with either nuclear extract derived from 70Z/3 stimulated with LPS or mitogen-activated B cells (Fig. 4). The mobility and specificity of protein-DNA binding was checked using direct and reciprocal competition binding assays and found to be essentially identical (data not shown). In an EMSA, the anti-p50 specific antiserum altered the migration of the lower complex formed with κ B probe, and the single complex formed with B2 probe when used with the 70Z/3 cells and activated B cell nuclear extracts, as indicated

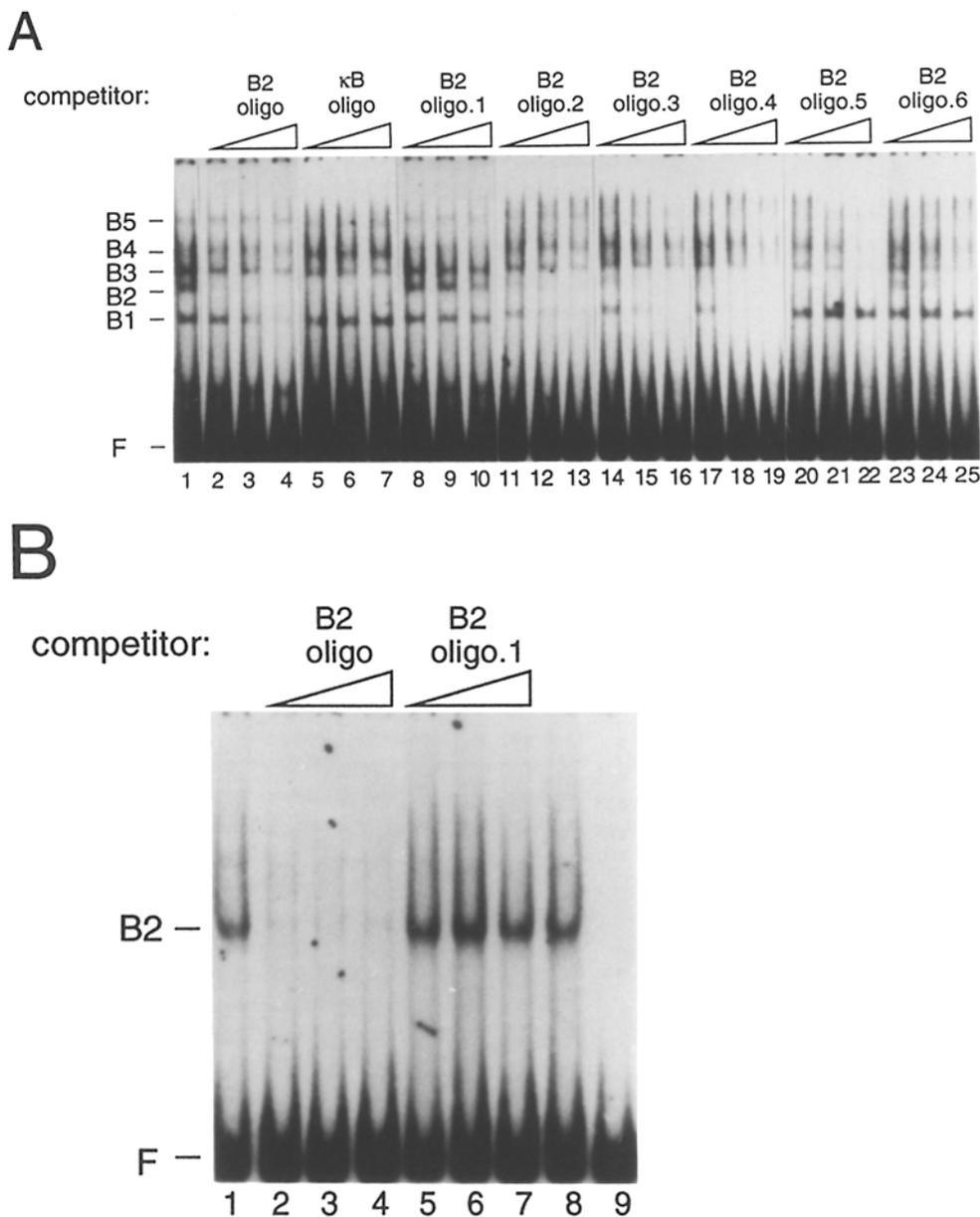


Figure 2. Mobility shift analysis of $Sy3$ -specific proteins from nuclear extract of mitogen-stimulated B cells. (A) Crude nuclear extract ($3.4 \mu\text{g}$) from mitogen-stimulated B cells was used in binding reactions with probe $pl.Sy3^+$ ($10,000 \text{ cpm}$) and resolved on 4% low ionic strength polyacrylamide gels. The specificities of the complexes were analyzed in the absence of competitor (lane 1) and by including increasing amounts (5.6, 22.5, and 90 ng) of different competitor DNAs in the binding reactions, as indicated (lanes 2–25). (B) Mobility shift analysis of the MonoS B2 complex. MonoSB2 complex ($2 \mu\text{g}$) was used in a binding reaction with probe $pl.Sy3^+$ either without competitor DNA (lanes 1 and 8) or with increasing amounts (22.5, 90, or 360 ng) of B2 oligo (lanes 2–4) or B2 oligo.1 (lanes 5–7). (Lane 9) Probe in the absence of extract. (F) Position of free probe.

by the arrow (Fig. 4). Preimmune serum had no effect on the mobility of this complex (Fig. 4). These results confirm that the $\text{NF-}\kappa\text{B}$ p50 or a closely related protein is involved in protein–DNA complex formation within the $Sy3$ switch region. $\text{NF-}\kappa\text{B}$ is found in the cytoplasm of quiescent B cells in an inactive form (25). Upon mitogenic stimulation of B cells, $\text{NF-}\kappa\text{B}$ is modified, activated, and transferred to the nucleus (25). Similarly, the B2 complex is not found in nuclear extracts of resting splenocytes, but is induced by treatment with LPS/DxS (Fig. 4 and data not shown).

Analysis of Other Complexes that Interact with $pl.Sy3^+$. It was interesting to determine the precise binding sites of the four other retarded complexes that interact with the $Sy3$ repeat motif (Fig. 2 A). We used unfractionated nuclear extract from mitogen-activated B cells as a source of $Sy3$ -binding proteins, the $pl.Sy3^+$ probe, and the methylation interference procedure to map the guanine residues involved in critical protein contacts (Fig. 5). To begin, the B2 pattern of methylation interference derived from crude extract is identical to that obtained from MonoS.B2 (Figs. 3 and 5). Again,

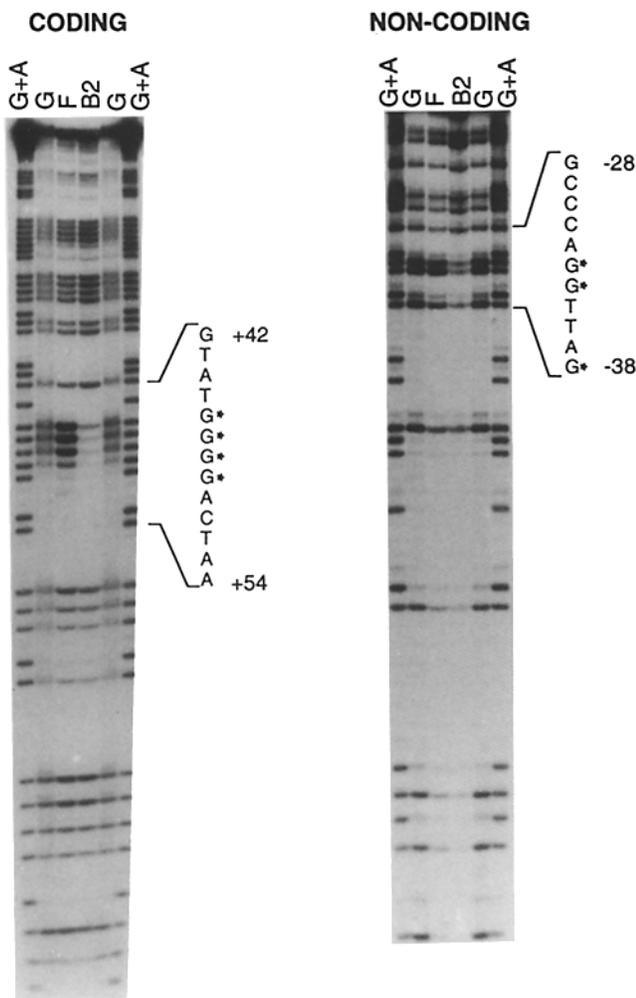


Figure 3. Methylation interference analysis of the MonoS B2 complex from LPS/DxS-stimulated B cells. Partially methylated pl.Sy3⁺ probe, labeled on either the coding or noncoding strand, was incubated with a MonoS B2 fraction (as shown in Fig. 2 B), and treated as described in Materials and Methods. Maxam and Gilbert G + A and G reactions were coelectrophoresed with the cleavage products from the free (F) and bound (B2) DNA. *Residues involved in binding.

the interference pattern is centered on a string of four G residues beginning at position 46 on the coding strand (Fig. 5). This supports the B2 pattern of guanine contact residues and that these results are reliable, even though derived from crude nuclear extract. The B1 and B2 mobilities appear to bind overlapping recognition motifs in the Sy3 repeat sequence where the B1 interference pattern begins at position 42 and extends through the run of guanines at positions 46–49 on the coding strand (Fig. 5). No contacted residues were detected on the noncoding strand for complex B1. The B3 moiety did not produce an interference pattern, and by these criteria is judged to be nonspecific. The B4 complex focused on a string of G residues beginning at position 69 and extending to position 75 on the coding strand (Fig. 5). The specificity of the B4 protein–DNA interaction will be examined in detail below.

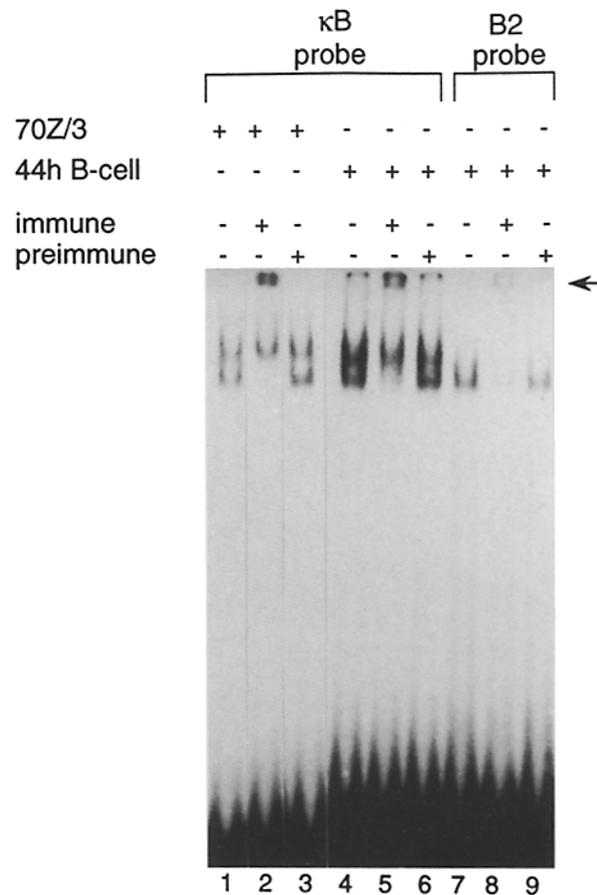


Figure 4. Presence of the NF-κB p50 subunit in Sy3 B2 binding analyzed using anti-p50 antiserum and the supershift assay. Mobility shift assays were performed in which nuclear extracts from LPS-stimulated 70Z/3 cells or mitogen-stimulated B cells were incubated with either the κB oligo or the B2 oligo probe. Extracts were preincubated with antiserum specific for the p50 subunit of NF-κB (lanes 2, 5, and 8), preincubated with preimmune serum (lanes 3, 6, and 9), or added without pretreatment (lanes 1, 4, and 7). (Arrow) Position of the supershifted complexes.



Figure 5. Summary of methylation interference analyses of the five complexes from crude nuclear extract of mitogen-stimulated B cells and probe, pl.Sy3⁺. Methylation interference was carried out as described in the text. (Bracketed) Regions of marked interference. *Residues whose methylation inhibits binding.

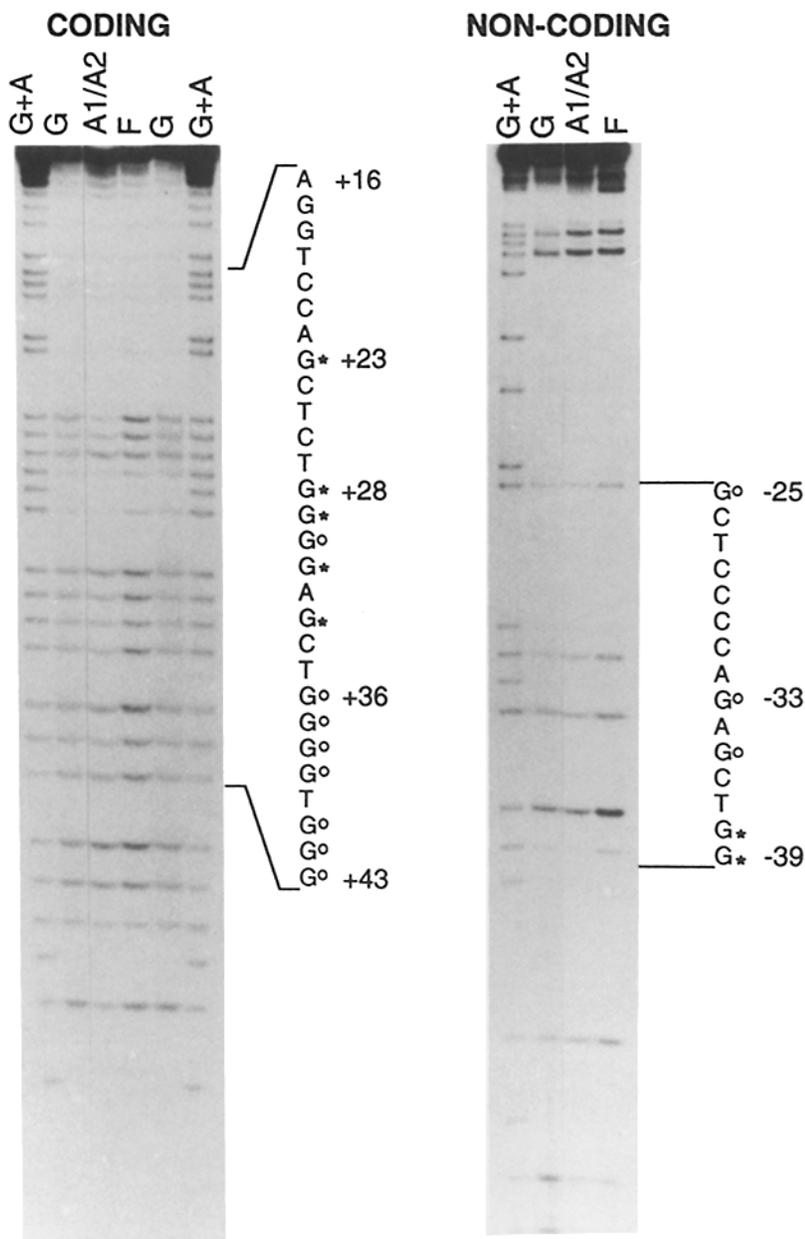


Figure 6. Methylation interference analysis of the A1/A2 nuclear factors on Sy3 DNA. The A1/A2 Sy3-binding proteins from A20 nuclei were partially purified by heparin-agarose chromatography and used in methylation interference analyses with probe pl.Sy3.A. Maxam and Gilbert G + A and G reactions are shown with the cleavage products of the free (F) and bound (A1/A2) DNA. Residues whose methylation either strongly (*) or moderately (o) interferes with binding are indicated.

The B5-bound moiety overlapped the junction of the Sy3 sequence and the pUC flanking sequence (Fig. 5). This suggests that this binding specificity is a result of the juxtaposition of Sy3 and plasmid DNA in the probe and is not dependent on Sy3 DNA alone. In summary, the DNA sequence-recognition motifs have been identified for the B1, B2, and B4 moieties where B1/B2 bind to overlapping recognition motifs and B4 binds to a downstream purine-rich site. The specificity of the B1 complex was further examined by competition-binding assays (Fig. 2). The methylation interference studies suggested that the B1 and B2 complexes recognize the same string of G residues, and that B1 extends further upstream than does B2 (Fig. 5). We find that the B2 oligomer successfully competes with pl.Sy3⁺ for binding

both the B1 and B2 moieties (Fig. 2 A). It appears that B2 has a relatively higher affinity for this competitor than B1, since it requires only 6 ng of competitor to completely ablate B2 binding, whereas B1 requires 90 ng of competitor to abolish binding. In contrast, κ B oligo, B2 oligo.1, and B2 oligo.5 were incompetent competitors for B1 binding (Fig. 2 A). The fact that B2 oligo.1 does not compete for the B1 complex demonstrates that this DNA-binding protein is dependent on an intact run of guanines beginning at position 46, as is found for the B2 complex. The κ B oligo and B2 oligo.5 both contain intact B2-binding sites, but are altered with respect to the upstream sequences. This finding supports the importance of the B1 contact residues at position +42, as suggested by the methylation interference analysis. The B2

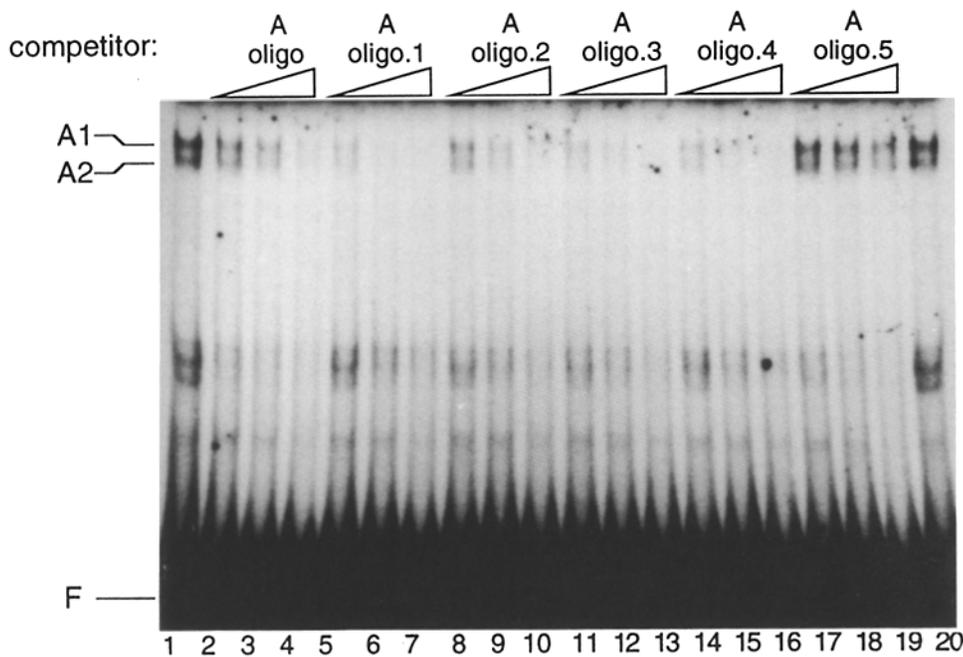


Figure 7. Competition-binding assays were used to test the specificity of the A1/A2 binding proteins for $S\gamma 3$ DNA. Binding assays were carried out using mitogen-stimulated B cell heparin-agarose A1/A2 fraction (0.5 μg /binding reaction) with the pl. $S\gamma 3$.A probe, either in the absence (lanes 1 and 20) or presence of increasing amounts (5.6, 22.5, and 90 ng) of the indicated competitor DNAs (lanes 2–19). The complexes were resolved on 6% low ionic strength mobility shift gels.

oligos.2–4 are mutated at positions downstream of the string of G residues, and are competent competitors of the B1 complex. This analysis confirms that the B1 DNA-binding site overlaps that of B2, but extends in the upstream direction. These results further confirm the sequence-specific nature of the B1 protein– $S\gamma 3$ DNA interaction, and differentiate it from the B2 complex. However, comparison of the B1 binding-

site sequence in the genomic probe with the sequence commonly found in the $S\gamma 3$ tandem repeat reveals substantial differences. Compare B2 oligo with B2 oligo.6 (Fig. 1). B2 oligo.6 was unable to compete for B1 complex binding to pl. $S\gamma 3^+$ in a competition-binding assay (Fig. 2A). These results demonstrate that the B1 complex is specific for a single site in the $S\gamma 3$ region, but does not recognize the $S\gamma 3$ canonical repeat.

A Unique DNA-binding Protein Interacts with a Second Discrete Site in the $S\gamma 3$ 49 mer. We noticed that in the region of B4 binding, pl. $S\gamma 3^+$ contains an additional C at position 72 relative to many other $S\gamma 3$ repeats. The A oligo, representing a 30-bp portion of the $S\gamma 3$ tandem repeat with a corrected sequence in the region of B4 binding, was synthesized and used in competition binding assays (Fig. 1). The A oligomer successfully competes with pl. $S\gamma 3^+$ for binding the B4 band (data not shown). These results directly demonstrate the specificity of the B4 complex for a discrete portion of the $S\gamma 3$ tandem repeat.

There is some complexity in analyzing the results of the methylation interference assay using crude nuclear extracts. This is especially so when multiple factors with different binding requirements compete for partially methylated probe. To refine the analysis of the B4 complex, the methylation interference assay was repeated using a partially purified preparation of B4-complex derived from the A20 cell line and cloned A oligomer, pl. $S\gamma 3$.A, as probe. B4-complexes were fractionated by heparin agarose chromatography and are referred to as H.A.A1/A2. The precise sequence requirements of H.A.A1/A2 for $S\gamma 3$ DNA were analyzed by methylation interference (Fig. 6). On the coding strand, G residues were strongly contacted at positions 23, 28, 29, 31, and 33, and weakly contacted at positions 30, 36–39, and 41–43. On the noncoding strand, strong guanine contacts occurred at posi-

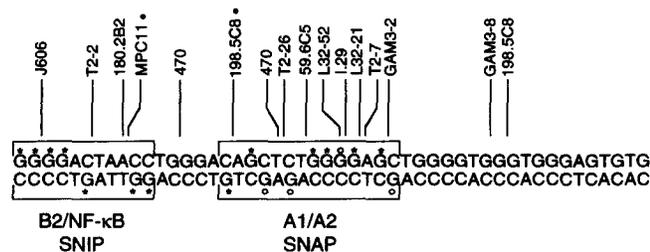


Figure 8. Summary of switch recombination breakpoints in $S\gamma 3$ DNA and position of NF- $S\gamma 3$ -binding sites. A representative 49-bp $S\gamma 3$ repeat is presented. The sequence is the consensus as reported by T. Nikaido et al. (7) except for changes at three positions in the SNIP site. These changes reflect the differences between the SNIP site as it appears in probe pl. $S\gamma 3^+$ and the consensus. Compare pl. $S\gamma 3^+$ and B2 oligo.6 in Fig. 1. (Boxed) Recognition motifs for SNIP/NF- κB and SNAP/A1/A2. The G residues, which contact proteins as determined by methylation interference, are indicated. (Vertical lines) Sequenced breakpoints of $S\gamma 3$ switch recombination events. The breakpoints were identified in the following cells: Myeloma J606 (7) and hybridomas 180.2B2 and 59.6C6 (31) and lymphoma I.29 (30) are $S\mu/S\gamma 3$ recombinants; 198.5C8 is a $S\gamma 3/S\gamma 3$ recombinant (30), and 407 is a $S\mu/S\gamma 3$ and $S\gamma 3/S\gamma 1$ recombinant (29); LPS blasts GAM3-2 and GAM3-8 are $S\mu/S\gamma 3$ recombinants (34); switch circles T2-26 (35) and L32-21 are $S\gamma 3/S\mu$ crossovers, L32-52 is a $S\gamma 2b/S\gamma 3$ recombinant, T2-2 and T2-7 recombined at $S\alpha/S\gamma 3$ (10). Myeloma, MPC11 is a $S\gamma 3/S\gamma 2b$ recombinant (6). (●) Sequences surrounding the breakpoints for MPC11 and 198.5C8, were particularly degenerate when compared with the consensus.

tions -38 and -39 , and weak interactions were detected at positions -25 , -33 , and -35 . A very similar pattern of protein-DNA contact sites was obtained using the pl.S γ 3.A probe and crude nuclear extract derived from mitogen-stimulated B cells by methylation interference analysis (data not shown). Based on these findings, we set the 5' boundary of the binding site at position -38 on the noncoding strand (Fig. 6). The contact position -39 was omitted since it fell in the pUC sequence and was not present in intact switch region. The 3' boundary of the binding site is set at position 33 on the coding strand or -25 on the noncoding strand. The 3' boundary assignment is based on competition-binding studies presented below.

When analyzed by mobility shift electrophoresis, the A oligo-binding complexes give rise to a prominent band (A1) and a weak secondary band (A2) and had exactly the same mobility and sequence-specificity profile in A20 extracts as in activated B cell extracts (Fig. 7, and data not shown). A series of competitors related to the A oligo were used in competition binding assays using H.A.A1/A2 derived from mitogen-activated B cells (Fig. 7). The methylation interference data indicated that position -38 was a strong contact. The competitor oligomers were therefore synthesized such that the binding site was better centered within the oligomer. A oligo.1 contained an unmutated centered binding site and was about a 16-fold better competitor than the original A oligo (Fig. 7). The A oligo.2 is mutated at position $+28$, a strong contact G residue. This single change causes a greater than fourfold reduction in the ability of this oligomer, as compared with A oligo.1, to compete for H.A.A1/A2 complexes and the B cell complex B4 (Fig. 7, and data not shown). The G at position 30 is shown by methylation interference to be only weakly involved in protein binding. This is confirmed by the competition assays which show that A oligo.3 containing a T substitution at position 30, competes almost as efficiently as A oligo.1 (Fig. 7). A common feature of the S γ 3 49-bp repeat is a string of five guanine residues instead of the four found in pl.S γ 3.A starting at position 28 (1). Since this was a region of multiple strong protein-DNA contacts we wished to test whether the presence of an additional G residue in this string of purines would perturb binding. The A oligo.4 was synthesized to contain a string of 5 G residues at this position and was found to be slightly less efficient than A oligo.1 for competing complexes (Fig. 7). Taken together, these results confirm the methylation interference findings that center binding on the string of guanines beginning at position 28.

The methylation interference data suggested a series of weak protein-DNA contacts along two contiguous strings of guanine residues starting at position 36 (Fig. 6). This pattern may suggest two overlapping binding sites, one of which is weaker than the other. To test for this, A oligo.5 was synthesized to contain two substituted sites at strongly contacted positions, 21 and 23. These mutations should abolish binding only for that protein which is dependent on the 5' end of the DNA recognition site. We found that A oligo.5 was unable to compete for either the B4 complex or H.A.A1/A2

(Fig. 6, and data not shown). This strongly suggests that these complexes bind a recognition motif at positions 21–33 on the coding strand and that the downstream guanine strings may be involved in stabilization of the complex but are not sufficient for sequence-specific binding. We have not determined whether the 3' boundary of the binding site ends at position 33 on the coding strand or -25 on the noncoding strand.

Switch Recombination Breakpoints for S γ 3 DNA Are Strongly Correlated with B2 and A Binding Sites. The canonical S γ 3 repeat sequence is a 49 mer that is imperfectly reiterated through the switch region. Recombination breakpoints have been found to occur at apparently random positions throughout the entire switch region. It is important to ascertain the functional significance of NF-S γ 3.B2 and NF-S γ 3.A1/A2 in switch recombination. To approach this question, the known switch recombination breakpoints for S γ 3 have been examined. The points of recombination between S μ /S γ 3 and S γ 3/S γ 2b in 16 myelomas, hybridomas, or the large circular reciprocal recombination products of mitogen-activated B cells were available from the literature (Fig. 8). Astoundingly, nine recombination breakpoints fell within the A binding site and four others fell within the B2 recognition site. Only three recombination breakpoints out of 16 examples fell outside the DNA recognition motifs for these binding proteins. The correlation of these binding protein recognition sites with recombination breakpoints strongly suggests that these proteins are active participants in the switch recombination process.

Discussion

Recombination requires the apposition of two recombining DNA helices. We have searched for DNA binding factors that might be involved in specifically recognizing and facilitating the alignment of switch regions during recombination. We have detected three switch sequence-specific DNA-binding proteins, B1, B2/NF- κ B, and A1/A2 (B4 in crude extract) that interact with two discrete portions of the S γ 3 49-bp tandem repeat. The DNA recognition motif for B1 overlapped that of B2/NF- κ B, but was only found once in the S γ 3 switch region. For this reason, it is unlikely that this protein plays a major role in interaction with the tandem repeats during recombination. The B2-binding protein is so far indistinguishable from NF- κ B with respect to its mobility in a band shift assay, its affinity for related binding sequences, contact residues in cognate DNA, and its reactivity with p50 antiserum. It is still possible that the B2/p50 is a different but highly related protein. This consideration notwithstanding, we designate the B2-binding protein, switch nuclear protein or SNIP/NF- κ B. The H.A.A1/A2 partially purified protein(s) gave rise to two closely migrating complexes. Nevertheless, these complexes produced a clear interference pattern when analyzed by methylation interference (Fig. 7). These complexes may represent two distinct proteins with identical protein-DNA contact residues or identical but differentially processed proteins. Other scenarios that could give rise to such complexes with very similar or iden-

tical cognate DNA contacts are also possible. For the sake of simplicity, we refer to the A1/A2 complexes (the B4 complex in crude extracts) as switch nuclear A protein (SNAP). SNAP bears no resemblance to the $S\mu$ switch-binding protein we previously described since it has a unique mobility in a shift assay and has a different pattern of DNA contact residues (16). LR1 is a binding activity derived from splenic B cells activated with LPS plus IL-4 and specific for a distinct portion of the $S\gamma 1$ switch region (26). We found no such binding activity in extracts derived from primary B cell cultures activated with mitogen alone. Furthermore, LR1 binds to a sequence that is unrelated to the SNAP recognition motif and has radically different cognate DNA contacts (26). We therefore conclude that SNAP is a unique switch-binding complex, specific for $S\gamma 3$ DNA and distinct from LR1. It will be interesting to determine whether purified SNAP has the capacity to bind to other $S\gamma$ switch regions.

Sequence analyses of the switch recombination joints of switched IgH genes have revealed that the $S\mu$ (donor) breakpoints fall within or upstream of the tandem repeats (9, 10, 27, 28). In acceptor S regions, the recombination breakpoints fall within the tandemly repeated sequence (7, 9, 10, 27–31). No obvious consensus recombination signal sequences were previously identified, though the pentamers, GAGCT, GGGGT, and GGTGG, components of all the switch regions, are often found at or close to recombination joints (30). Sequence comparison of S DNAs has shown that although all are highly repetitive, there has been significant sequence divergence between them (6, 7). Two views on the mechanism of switch recombination have been articulated. The first emphasizes that there may be switch region-specific recombinases that are responsible for the recombination event, and that the sequence divergence of S regions contributes specificity (5). The second suggests that switch recombination is due to homologous recombination and highlights the short nucleotide repeats which are common amongst all the switch regions (6, 7). Our studies demonstrate that 56% of $S\gamma 3$ recombination breakpoints occur within the SNAP-binding site and 25% of breakpoints within the SNIP/NF- κ B recognition motif. These findings strongly suggest that switch recombination is a form of specialized recombination, dependent upon sequence-specific DNA binding proteins to orient the switch DNA within a nucleoprotein complex and to facilitate recombination.

Site-specific recombination is mediated by specialized proteins that recognize specific DNA sequences and catalyze reciprocal exchange between those sites. The fact that $S\gamma 3$ recombination breakpoints fall across the SNIP/NF- κ B and SNAP-binding motifs implies that the process employs a customized form of site-specific recombination. Homologous recombination, with its requirement of sequence homology at the site of rearrangement, and illegitimate recombination, are very unlikely mechanisms given these results.

Identification of an NF- κ B recognition motif in the $S\gamma 3$ region suggests a role for this well-characterized transcription factor in switch recombination (23, 24). This may occur by NF- κ B-mediated transcriptional activation of the $S\gamma 3$ region leading to the production of sterile switch transcripts and increased accessibility of the switch recombinase to this locus. Alternatively, NF- κ B and SNAP may be involved in generating a higher order chromatin configuration upon full occupancy of their binding sites. It has been demonstrated that such processes as transcription, site-specific recombination, and the initiation of DNA replication require formation of DNA-multiprotein complexes that are dependent upon protein-protein interactions (13–15). NF- κ B is a member of the rel family of proteins for which the rel domain has been shown to function as a dimerization motif. NF- κ B is therefore capable of the protein-protein interactions that may be required for formation of nucleoprotein complexes involved in synapsis of switch DNA during recombination. We favor the latter interpretation because of the coincidence of the NF- κ B- and SNAP-binding sites with characterized switch recombination breakpoints.

It was fortuitous that the genomic $S\gamma 3$ probe used in these studies contained a high affinity binding site for SNIP/NF- κ B (Fig. 1). However, the most common sequence in this domain of the tandem repeat contains a binding site with about 16-fold lower affinity for SNIP/NF- κ B (Fig. 2A). Multimerization of the NF- κ B-binding site upstream of a heterologous promoter facilitates enhanced expression of the receptor gene (32). In contrast, a single NF- κ B-binding site at this position in the construct has no effect on transcription (32). Thus, regular repetition of closely spaced NF- κ B recognition motifs leads to a functional synergy of NF- κ B-dependent transcription activation. This suggests that the highly repeated low affinity SNIP/NF- κ B-binding sites in the natural $S\gamma 3$ switch region may lead to high affinity SNIP/NF- κ B binding. Though the $S\gamma 3$ switch region is highly repetitive, local sequence permutation occurs at a high frequency and is likely to influence binding affinities at individual binding sites. The higher order switch region configuration is likely dictated by the combined affinities of the nonidentical endogenous binding sites.

SNIP/NF- κ B was found to bind NF- κ B recognition sites by competition-binding assays, methylation interference, and supershift analysis. A large family of factors has been shown to bind the NF- κ B-binding site, NF- κ B/KBF1, H2TF1, EBP1, MBP1 or PRDIIIBF1, TC-II, HIVEN 86, and v-rel (33). This indicates that factors with different types of DNA-binding domains can bind to the same site and have similar affinities to variants of this site. More work is needed to determine the relevance of SNIP/NF- κ B and SNAP to switch recombination *in vivo*.

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