A Protein Binding Specifically to the IgG2b Switch Region

HEINER VÖLK* and MATTHIAS WABL

Department of Microbiology and Immunology, University of California, San Francisco, 94143-0670

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The Abelson-virus-transformed mouse pre-B-cell line 18-81 switches almost exclusively from μ to γ2b. From nuclear extracts of this cell line, we have isolated a factor that specifically binds to Sγ2b. After an eight-step purification scheme, in which different types of DNA-affinity chromatography were used as key elements, we obtained a preparation with two narrowly spaced bands at approximately 69 kD on a silver-stained SDS gel. Binding specificity of main-peak fractions of affinity-purified proteins was analyzed by gel shift assays, in which Sγ2b but not Sμ competes. The results are consistent with this factor being part of the switch recombinase.

Keywords: Immunoglobulin, class switch, nuclear extracts, affinity chromatography, gel shifts, switch recombinase

INTRODUCTION

The earliest antibodies produced during the course of an immune response are of the class IgM. As the response proceeds, antibodies with the same specificity of other classes are produced and IgM production declines. This heavy- (H-) chain class switch is not a population phenomenon; a committed B cell that produces an IgM antibody can switch to the production of another class of immunoglobulin (Wabi et al., 1978). The molecular basis of the H-chain class switch is the deletion of a stretch of DNA from 5′ of Cμ to 5′ of the Cγ1 gene segment to be expressed (Honjo and Kataoka, 1978; Jäck et al., 1988; Iwasato et al., 1990; von Schwedler et al., 1990). The deletion results from a genetic exchange, with the deleted material being excised as a circular DNA molecule, the so-called switch circle (Jäck et al., 1988; Iwasato et al., 1990; von Schwedler et al., 1990; Harriman et al., 1993). Most recombination break points lie within the so-called switch (S) regions. These are repetitive DNA sequences 5′ of all Cγ1 gene segments except Cγ6 (Shimizu and Honjo, 1984). Their overall length (1–10 kb), as well as the length of their repeat units, is variable. However, all switch regions share some homology. By using Sμ as reference sequence, the degree of homology decreases in the following order: Sμ > Sγc > Sγa > Sγβ > Sγλ > Sγ2b > Sγ2a (Nikaido et al., 1982; Stanton and Marcu, 1982).

Little is known about the switch recombinase, the putative enzyme-mediating switch recombination. However, such an enzyme complex must perform the three basic functions of binding, cutting, and ligating. In a current model of class switching, the future Cγ1 gene segment is targeted by transcription and/or demethylation (reviewed in Lin et al., 1992; Siebenkotten and Radbruch, 1995; Stavnezer, 1996). This is thought to be mediated by vari-
ous cytokines produced by T cells (reviewed in Finkelman et al., 1990). Recombination of S regions would then proceed, possibly mediated by the normal recombination and repair machinery. Indeed, evidence has accumulated that so-called germ-line transcripts are necessary for switch recombination (Lennon and Perry, 1985; Stavnezer-Nordgren and Sirlin, 1986; reviewed in Lin et al., 1992; Lorenz et al., 1995). But other loci are being transcribed as well, so switch recombination needs to be restricted to S regions; that is, the transcripts themselves may take part in the reaction (Lorenz et al., 1995), or their translation product (Bachl et al., 1996).

Recombination of an isolated (Borggrefe et al., 1996) or transfected (Daniels and Lieber, 1995) switch substrate now can be assayed in vitro, and this has revealed a dependence of the switch recombination on transcription (Daniels and Lieber, 1995). There has been some progress in defining consensus recognition sites by analysis of DNA sequences around recombination break points (Wuerffel et al., 1992; Chou and Morrison, 1993; Kenter et al., 1993), but a more extensive analysis has failed to find such a sequence (Dunnick et al., 1993). In the last few years, there have been reports of several factors that bind to various switch regions, and sometimes to other loci as well, in humans and mice (Wuerffel et al., 1990; Schultz et al., 1991; Xu et al., 1992; Fukita et al., 1993; Mizuta et al., 1993; Kenter et al., 1993). To our knowledge, no function in switch recombination has been shown for these proteins. To date, only the genes encoding a factor that binds single-stranded DNA related to $S_\mu$ have been cloned from mouse and humans (Fukita et al., 1993; Mizuta et al., 1993). Two other polypeptides were shown to be transcription factors with targets located also outside of switch regions (Waters et al., 1989; Barberis et al., 1990; Schultz et al., 1991; Williams and Maizels, 1991; Liao et al., 1992; Williams et al., 1993; Brys and Maizels, 1994; Liao et al., 1994; Neurath et al., 1994).

We have assumed that specificity during switch recombination is not entirely determined by cytokine specificity, that is, that specificity is also conferred by factors that bind to S regions. Nontransformed pre-B cells do not seem to switch at all. Nevertheless, all mouse Abelson-virus transformed pre-B cells switch almost exclusively from $\mu$ to $\gamma_2b$ (Burrows et al., 1981, 1983; Alt et al., 1982; Akira et al., 1983). If our assumption is correct, nuclear extracts from such transformed cells ought to contain factors that predominantly recognize $S_\gamma_2b$. Here we describe the isolation of one such factor.

### RESULTS AND DISCUSSION

Proteins binding to $S_\gamma_2b$ were purified from nuclear extracts of the Abelson-virus transformed mouse cell line 18–81. Gel shift assays and silver-stained SDS gels were used to screen the fractions at each stage of purification.

#### Purification and Gel Shift Assays

Nuclear extracts (200 mg) were loaded onto a 106-ml DEAE fast-flow Sepharose column, eluted with a 400-ml linear gradient from 100 to 200 mM NaCl, followed by an additional 200 mM wash. On the basis of a gel shift analysis (Fig. 1), fractions 36–96 eluting at 111–195 mM and containing 10.8 mg protein were

![Figure 1](image-url)
pooled, diluted to 100 mM NaCl, and passed over a 50-ml BioRex 70 column. Bound material was released with a linear 300-ml 100–1000 mM linear gradient followed by a 2 M NaCl wash. Fractions 16–40 eluting at 100–310 mM NaCl and containing 1.1-mg protein (NaCl) were pooled, concentrated on a 1-ml DEAE column, and loaded in 50 mM NaCl onto a Streptavidin-agarose-S\(_{20}\)DNA column (SAS). After elution with 250 mM NaCl and adjustment of pooled active fractions to the proper loading conditions, the material was passed a second time over the SAS column under the same conditions. Pooled active fractions were adjusted to 50 mM NaCl and applied to a third affinity column, which was eluted in steps of 100, 150, 200, and 250 mM NaCl. The 100- and 150-mM eluates contained different gel shift activities, which were well separated from each other (not shown); only the former was studied further. Silver-stained SDS gels of the 100 mM NaCl fractions are shown in Fig. 2.

Active samples from 100 mM NaCl eluates were pooled, diluted to 50 mM, and applied to a fourth SAS column, which was successively eluted with buffer containing 65, 75, and 100 mM NaCl. Silver-stained SDS gels are shown in Fig. 3. DNA-binding activity was detectable in the first two steps only after concentration. In the 100 mM NaCl eluates, the activity peak was maximal at fractions 76–78. The activity decreased rapidly toward the inactive fraction 70 and much more slowly toward the inactive fraction 96. There were few proteins left in the shift-positive fractions, which we will call “SAS-purified” fractions hereafter. The major band is about 69 kD.

FIGURE 2 Silver-stained gels of eluates from the third affinity column. Aliquots of 30 \(\mu\)l were analyzed under reducing conditions. Molecular weight standards (kD) are indicated on the left. Lanes 1–15: Fractions 14–42 (100 mM NaCl). + : Shift activity.

FIGURE 3 Silver-stained gels of SAS-purified fractions. Aliquots of 30 \(\mu\)l were analyzed under reducing conditions. Even fractions were tested. (A) Lanes 1–13: Fractions 46–70 (75 mM NaCl). Lanes 14–19: Fractions 72–82 (100 mM NaCl). (B) Lanes 1–12: Fractions 84–106 (100 mM NaCl). Lanes 13–19: Flowthrough fractions 8, 12–32. + : Shift activity.
Molecular-Weight Determination after Dynabead Separation

To confirm that the 69-kD protein is indeed the major binding component in these fractions, we ran an SDS gel with Dynabead-purified material. Dynabeads are paramagnetic particles embedded in polystyrene spheres and can be easily removed from a suspension by means of a strong magnet. Beads to which genomic S\(_{72b}\) had been coupled were incubated with SAS-purified fractions 83–88 and ca. 2% (v/v) of 73–74. Bound material was eluted with basic buffer containing 300 mM NaCl. In pilot experiments, 300 mM NaCl was shown to inhibit gel shifts with the 245-bp S\(_{72b}\) probe. Control Dynabeads without DNA were treated identically. Aliquots were analyzed again by reducing SDS-PAGE and silver staining (Fig. 4). Supernatant from beads without S\(_{72b}\) DNA had two narrow-spaced weak bands of ca. 69 kD (Fig. 4, lane 2), which were not visible in the supernatant incubated with DNA-coated beads (Fig. 4, lane 1). The eluate of the S\(_{72b}\) beads showed a strong narrowly spaced doublet at 69 kD (Fig. 4, lane 3). Although difficult to see on a print, where they appear as a single band, it was easily visible on the original gel that the upper band was slightly weaker. In contrast, the control using beads without S\(_{72b}\) DNA contained no stainable protein (Fig. 4, lane 4).

Specificity for S\(_{72b}\)

For competition experiments, fractions 75–78 from the fourth SAS were incubated with end-labeled 245-bp S\(_{72b}\) fragment and various amounts of various cold DNA fragments in 100 mM NaCl (Fig. 5): There was strong competition with 2.75 ng cold 245-bp S\(_{72b}\) fragment, and 11 ng virtually abolished any binding of protein to a labeled probe (Fig. 5A). There was no competition with 44 ng of pdIdC, a synthetic product that is supposed to soak up unspecific binders to the DNA backbone (Fig. 5B); nor with 44 ng PhiX 174 phage RF DNA digested with Hae III (Fig. 5C). More importantly, 24 ng of a 1.3-kb genomic S\(_{p}\) switch-region fragment did not compete (Fig. 5D). Although a 196-bp pUC piece competed to some extent (Fig. 5A) to reach the same degree of competition as a cold Sy2b fragment, about four times as much DNA was required (11

FIGURE 4 Silver-stained SDS polyacrylamide gel of Dynabead purified material: Aliquots of 100 µl were run under reducing conditions; numbers on the left indicate positions of molecular-weight standards. Lanes 1 and 3: Dynabeads coupled to 3.7-kb genomic S\(_{72b}\) DNA (+). Lanes 2 and 4: Dynabeads without DNA (−). SN: Supernatant (1.4 ml); 300: 300 mM NaCl eluates (120 µl).
FIGURE 5 Autoradiograms of competition gel shift assays with SAS-purified protein. Unless stated otherwise, the labeled probe was 245-bp S_{2b} DNA. (A) Lane 1: No protein. Lane 2: Protein but no competitor. Lanes 3–5: 2.75, 5.5, 11 ng cold 245-bp S_{2b} DNA. Lanes 6–8: 2.75, 5.5, 11 ng cold 196-bp pUC DNA. Lane 9: 196-bp labeled pUC probe without protein. Lane 10: 196-bp labeled pUC probe with protein. (B) Lanes 1–4: 5.5, 11, 22, 44 ng cold pIdlc. (C) Lanes 1–5: 2.75, 5.5, 11, 22, 44 ng cold double-stranded PhiX174 fragments. (D) Lanes 1–6: 4, 8, 12, 16, 20, 24 ng cold 1.3-kb S_{2b}. B: Double-stranded 245-bp S_{2b} probe bound to protein. f: Free double-stranded 245-bp pUC probe.

TABLE I Comparison of Footprint Sequence (TCCCA) and Flanking Sequences with Similar Motifs and Their Flanking Sequences without DNase I Protection

| DNA    | Position | Footprint | Sequence of the top strand |
|--------|----------|-----------|---------------------------|
| 196-bp pUC | B 6–30   | ND        | GCCCA ACTGT TCCGA AGGCC GATCG |
| 196-bp pUC | T 52–76  | ND        | ACCTC GTGAC TCCGA AAGCC CAGCC |
| 57-bp S_{2b} | B 29–53 | ND        | CTGGG GAAGG TGGGA AAGCG |
| 245-bp S_{2b} | B 22–46 | —         | CAGGG ATAGG TGGGA GTATT AGGGA |
| 245-bp S_{2b} | B 120–144 | —        | CTGGG GCAGG TGGGA GTGAG AGGGA |
| 245-bp S_{2b} | B 226–245 | +        | GCTAG GAATG TGGGA GACCA GATCC |

1 T/B: Top/Bottom strand has the motif. Positions always refer to the sequence of the top strand.
2 ND: Not determined.
3 The last 5 bp of the flanking-sequence at the 3' end are part of the pUC multiple cloning site.
FIGURE 6 Autoradiogram of gel shift analysis with single-stranded DNA. The 245-bp S<sub>on</sub> probe was heat-denatured and subsequently incubated in 100 mM NaCl buffer without (lane 1) or with (lane 2) material from SAS-purified fractions 76–77. s: Free single-stranded DNA probe. d: Position where the free double-stranded probe would run.

could have an effect on directing DNA-binding proteins to the one but not the other site. Another reason of why there was no large footprint or no other visible footprints could have been the decreased binding of purified proteins in the presence of divalent cations. But calcium and magnesium ions need to be added to the binding mix together with DNase I in order to promote the nicking reaction. It may be that very high concentrations of purified protein will be necessary to reach a reasonable balance.

Using the data on competition with the pUC fragment, and assuming the 5-bp stretch as the complete and only site in the probe, one can estimate the competitiveness ratio of specific to nonspecific DNA in the gel shift

FIGURE 7 Autoradiogram of a DNase I footprinting experiment with subsequent denaturing polyacrylamide gel electrophoresis: Lanes 1 and 2: Bottom strand. Lanes 3 and 4: Top strand. Positions refer to the complementary sequence on the top strand. Samples were incubated with (lanes 2 and 3) or without (lanes 1 and 4) SAS-purified protein. Subsequent denaturing electrophoresis was done for 4 hr. The arrowhead indicates protection at positions 237–238 (CC) of the bottom strand.
assays to be 110. That is, the specific 5-bp motif is 110 times as good a competitor as nonspecific DNA.

**Does Our 69-kD Factor Correspond to a Published One?**

Kenter et al. (1993) described sites for two factors called SNAP and SNIP/NFκB, which bind to sites A and B, respectively. These sites can be found in the switch regions Sγ3, Sγ1, and Sγ2b. The 245-bp Sγ2b probe used in our experiments has three potential 15-bp spanning A sites at positions 8–22, 57–71, and 204–218. There is only one mismatch, which is located within the first site. SNAP, of unknown molecular weight, is very pH-sensitive and does not seem to bind to DNA above 150 mM NaCl. This seems to distinguish it from the 69-kD factor, although pH dependence and salt concentrations for elutions are difficult to compare. Attempts to identify SNAP activity in cell line 18–81 were not successful (Amy Kenter, personal communication). For the 69-kD factor reported here, binding at pH 7.4 was only little less than binding at pH 7.9. In addition, our protein-DNA complexes were shown to be more stable in salt; they disappear only between 250 and 300 mM NaCl. Finally, the DNase I protected positions were 236–240, a region well separated from the nearest possible A site at positions 204–218. Thus, our 69-kD protein does not seem to be SNAP.

The B site, which is recognized by NFκB, spans 11 bp. Based on competition gel shifts and a supershift induced by polyclonal anti-p50 serum, it was proposed that NFκB is involved in binding to various switch regions. NFκB consists of a p50 and p65 subunit with molecular weights of 50 and 65 kD, respectively. Heterodimers consisting of p50 and p65, as well as p50 homodimers, can bind to DNA (Urban et al., 1991). The Sγ2b probe used in our experiments has two potential B sites at positions 42–52 and 140–150 (one mismatch each). However, there are several reasons why we think the purified shift activities from our work are different from NFκB. In competition gel shift assays, a 57-bp DNA containing three complete NFκB sites should have been a very strong competitor, but it did not compete. The 196-bp pUC fragment contains one complete NFκB site, but used as a probe, it hardly bound anything. Further, our foot-printing experiments show a protected site at position 236–240, which is far away from the two potential B sites at positions 42–52 and 140–150. Thus, our 69-kD protein does not seem to be SNIP/NFκB. Nor is it likely that it is the LR-1 factor, which has been described to be a 106-kD protein (Williams et al., 1993).

We conclude that the 69-kD factor that specifically binds to Sγ2b is likely to be a novel protein.

**MATERIALS AND METHODS**

**Nuclear Extracts**

Cells of the Abelson-virus-transformed mouse pre-B-cell line 18–81 were grown to 0.5 to 2 × 10⁶/ml. Nuclear extracts (200 mg) were prepared from 261 of cells according to Ausubel et al. (1987) with the following modifications: All buffers contained 1 mM PMSF, 1 μg/ml Leupeptin, Pepstatin, and Aprotinin. Hypotonic buffer for swelling as well as all subsequently used buffers were supplemented with 0.1% NP-40. The final NaCl concentration during the 45-min extraction was 405 mM. Before freezing, samples were adjusted to 100 mM NaCl and 20% glycerol by dilution rather than by dialysis.

**Column Chromatography**

All steps were performed at 4°C. The basic buffer was 20 mM HEPES, pH 7.9, 20% glycerol, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, as well as 1 μg/ml E-64, Leupeptin, Pepstatin, and Aprotinin. Flowthrough, washes, and eluted fractions were screened for DNA-binding activity by gel shift assays. Determination of protein concentrations was done with Bradford assays.

To prepare the SAS affinity column, 5.1 ml Streptavidin agarose was washed with 2 M NaCl, TE, 0.1% NP-40 followed by PBS. Biotinylated (double-stranded) 57-bp Sγ2b DNA (300 μg) in 3 ml fill-in mix with 0.1% NP-40 was passed over the column four times before washing with PBS, 0.1% NP-40, 1 mM EDTA followed by 2 M NaCl, TE, 0.1% NP-40, then 100 mM
NaCl, TE at pH 8.0. An equilibration step was performed with basic buffer supplemented with 50 mM NaCl, NP-40, BSA, DTT, and Bestatin. Before loading, samples were adjusted to 50 mM NaCl, 0.1% NP-40, 0.1 mg/ml BSA, and 40 μg/ml Bestatin, unless otherwise specified. Washes and elution were performed without BSA. Bound material was eluted with step gradients.

Purification with Dynabeads Coupled to Genomic Sγb DNA

The 3.7-kb insert of the plasmid p245 was released by digest with the restriction enzymes Eco RI and Hind III. Biotinylation of the Eco RI end was accomplished by partial Sequenase fill-in with dATP and Biotin-16-11-UTP. DNA (72 μg) was coupled to 1 ml of Dynabeads suspension (6–7 × 10⁸/ml) in TE, 1 M NaCl for 2 hr. Before and after coupling, beads were washed extensively with the same buffer used for immobilization of the DNA. Ca. 1.4 ml SAS-purified protein, mostly fractions 83–88 and only about 2% (v/v) 73–74, were incubated with the beads for 7 hr on ice with occasional agitation. Elution was performed for 20 min on ice with 120 μl basic buffer containing 300 mM NaCl.

Gel Shift Assays

The gel shift assay was modified from Strauss and Varshavsky (1984). Protein was incubated with 10,000 cpm of 3' end-labeled probe and various amounts of pdIdC for 20 min at RT. The final volume was 20 μl in 20 mM HEPES pH 7.9, 10–14% glycerol, 1 mM DTT, and 100 mM NaCl. Samples were electrophoresed for 10 min at 200 V at RT through a 4% TAE polyacrylamide gel and then at 180 V for 1.5–2.5 hr at 4°C.

DNAs and DNA Sequencing

Plasmids

pUC/Sμ (A), a gift from Richard Scheuermann (Dallas), is a derivative of pUC 19 with the 3.5-kb Xba I fragment 5' to the Cμ exons (Stanton and Marcu, 1982). The insert with the switch μ region was modified with linkers and cloned into the Cla I site. pSγb is a pUC 19 with the insert of the plasmid pSL1 (Lang et al., 1982) with a ca. 400-bp deletion at the 3' end of the insert. p245 is a pUC 19 containing the 245-bp Bgl II subfragment from the pSγb insert cloned into the Bam HI site.

17-mer Primer mcs1
17-mer Primer mcs2
57-mer Sγb (Nikaido et al., 1982)
57-mer Sα (Nikaido et al., 1982)
57-mer Sμ (Nikaido et al., 1982)
57-mer NFκB (Urban et al., 1991)
57-mer Cμ (Schreier et al., 1986)
8-mer Primer (Bio)-TAATCGCG synthesized with Biotin-ON phosphoramidite (Clontech).
pdIdC. 1.2–1.4 kb average length (Pharmacia).
PhiX 174 RF (Sanger et al., 1978).
DNase I Footprinting and AG Chemical Sequencing. These were done according to Ausubel et al. (1987). Strand scission was done in 10 μl 100 mM NaOH, 1 mM EDTA for 30 min at 90°C. After precipitation, samples were run on a 6%, 7 M urea sequencing gel.
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