A Protein Binding Specifically to the IgG2b Switch Region

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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 (Wabl 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 > Sγ > Sα > Sγ3 > Sγ1 > 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 (Dunick 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 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 μ to γ2b (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 Sγ2b. Here we describe the isolation of one such factor.

RESULTS AND DISCUSSION

Proteins binding to Sγ2b 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-mM 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 Autoradiograms of gel shift assays with eluates from the 106-ml DEAE column. Every fourth fraction was probed with a 57-bp Sγ2b fragment. In each gel, lane 1 had no protein; lane 2 had 13.4 μg crude nuclear extract. (A) Lanes 3–19: Fractions 54–118. (B) Lanes 3–18: Fractions 122–182. Fractions 36–96 (pool 1) were processed further.]
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 fol-
lowed by a 2 M NaCl wash. Fractions 16–40 eluting at 
100–310 mM NaCl and containing 1.1-ng protein 
(NaCl) were pooled, concentrated on a 1-ml DEAE 
column, and loaded in 50 mM NaCl onto a Strepta-
vidin-agarose-500-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 ad-
justed 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 con-
tained 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 con-
taining 65, 75, and 100 mM NaCl. Silver-stained SDS 
gels are shown in Fig. 3. DNA-binding activity was de-
tectable 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-puri-
fied” fractions hereafter. The major band is about 69 kD.
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 $S_{2b}$ 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 $S_{2b}$ 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 $S_{2b}$ 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 $S_{2b}$ 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

\[
\begin{array}{cccc}
SN & + & - & 300 \\
1 & 2 & 3 & 4 \\
\hline
SN & 200 & 97.4 & 69 & 46 \\
300 & & & & \\
\end{array}
\]
vs. 2.75 ng). Competition by the pUC piece was somewhat puzzling, especially because when the same pUC fragment was used as a labeled probe, almost no binding activity was detectable (Fig. 5A, lanes 9 and 10). There is some, albeit quite limited, homology between S₂ᵇ and pUC sequences (Table 1).

In another experiment with the 245-bp S₂ᵇ probe in 80 mM NaCl, up to 18 ng of five 57-bp DNAs in combination with 12-ng pdIdC were used. S₂ᵇ, S₂, S₄, and NFkB consensus sequences did not compete, nor did part of a C₄ exon (not shown).

We also tested binding of the 69-kD complex to single-stranded DNA. The 245-bp S₂ᵇ probe was heat-denatured and incubated with protein from fractions 76–77 of the fourth SAS. Little, if any, protein/single-stranded DNA complex was detected (Fig. 6, Lane 2).

To exclude a possible formation of bands by binding of protein to residual nucleotides from labeling reactions, radioactive dCTP or ddATP was incubated with fractionated material in the presence or absence of unlabeled DNA. None of the samples showed any trace of complex formation (not shown).

SAS-purified fractions 74, 79, and 80 were used for DNase I footprinting. Only one very small site was protected at positions 237–238 (CC) of the bottom strand (Fig. 7, lanes 1 and 2). Because the flanking positions were insensitive to DNase I cleavage, even without added fraction material (Fig. 7, lane 1), the actual contact site most likely stretches from positions 236 to 240 (TCCCA). Two other sites with the TCCCA motif were found within the 245-bp S₂ᵇ probe (Table 1), but no protection could be demonstrated under the conditions tested. However, their neighboring sequences showed some diversity, and some of these surrounding bases

<table>
<thead>
<tr>
<th>DNA</th>
<th>Position</th>
<th>Footprint</th>
<th>Sequence of the top strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>196-bp pUC</td>
<td>B 6–30</td>
<td>ND</td>
<td>GCGCA ACGTC TCGCC AGGCC GATCG</td>
</tr>
<tr>
<td>196-bp pUC</td>
<td>T 52–76</td>
<td>ND</td>
<td>AGGTC GTGAC TCGCC AAAAC CGTGCC</td>
</tr>
<tr>
<td>57-bp S₂ᵇ</td>
<td>B 29–53</td>
<td>ND</td>
<td>CTGGG GAGTC TCGCC AATGT ACGCC</td>
</tr>
<tr>
<td>245-bp S₂ᵇ</td>
<td>B 22–46</td>
<td>—</td>
<td>CAGGG ATAGG TGGCC GTATT AGGGA</td>
</tr>
<tr>
<td>245-bp S₂ᵇ</td>
<td>B 120–144</td>
<td>—</td>
<td>CTGGG GCAGG TGGCC GTTGAG AGGGA</td>
</tr>
<tr>
<td>245-bp S₂ᵇ</td>
<td>B 226–245</td>
<td>+</td>
<td>GGGAG GAGTC TGGCC GACCA GATCC</td>
</tr>
</tbody>
</table>

1 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.
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 experiment.
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/NFkB, which bind to sites A and B, respectively. These sites can be found in the switch regions $S_{p3r}$, $S_{p1r}$, and $S_{r2b}$. The 245-bp $S_{r2b}$ 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 NFkB, spans 11 bp. Based on competition gel shifts and a supershift induced by polyclonal anti-p50 serum, it was proposed that NFkB is involved in binding to various switch regions. NFkB 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 $S_{r2b}$ 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 NFkB. In competition gel shift assays, a 57-bp DNA containing three complete NFkB sites should have been a very strong competitor, but it did not compete. The 196-bp pUC fragment contains one complete NFkB site, but used as a probe, it hardly bound anything. Further, our footprinting 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/NFkB. 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 $S_{r2b}$ 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 $\times 10^6$ 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 $S_{r2b}$ 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 S_y2b 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-ddUTP. DNA (72 μg) was coupled to 1 ml of Dynabeads suspension (6-7 x 10^8/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.

DNA and DNA Sequencing

Plasmids

pUC/S_yt (A), a gift from Richard Scheuermann (Dallas), is a derivative of pUC 19 with the 3.5-kb Xba I fragment 5' to the C_y exons (Stanton and Marcu, 1982). The insert with the switch y region was modified with linkers and cloned into the Cla I site. PS_yb 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 pS_y2b insert cloned into the Bam HI site.

17-mer Primer mcs1
CGAGCTCGGTACCGCGG

17-mer Primer mcs2
TGCAATGCCAGCGTGCG

57-mer S_y2b
GGGACCAGWCCATACGACGCTPTGGGGGAGCTGGGGAWGGTPGAGTPGAGCGCGATTA
(Nikaido et al., 1982)

57-mer S_y
ATGAGCTGGGATGAGCTGAGCTAGGCTGGAATAGGCTGGGCTGGGCTGGCGCGATTA
(Nikaido et al., 1982)

57-mer S_y
GAGCTAGCTGGGATGAGCTGAGCTAGGCTGGAATAGGCTGGGCTGGGCTGGCGCGATTA
(Nikaido et al. 1982)

57-mer NFeB
GAGGGGACTTTCGGAGAGGGGACTTTCGAGAGGGGACTTTCGGAGAGGGGACTTTCGAGAGGGGACTTTCGAGAGGGGACTTTCG
(Urban et al., 1991)

57-mer C_y
GGGAACCTTCGGCGCCAGCCACCCATTTCTTCTACGGAACACTACCAGAAACAGCAGCGATTA
(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.
Acknowledgements

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References


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