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Isolation of cDNA encoding a binding protein specific to 5′-phosphorylated single-stranded DNA with G-rich sequences

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ABSTRACT

We have isolated the cDNA encoding a binding protein to the sequence motif of the immunoglobulin Sμ region by the southwestern method. The binding protein designated Sμbp-2 specifically binds to 5′-phosphorylated single-stranded DNA containing 5′-G and GGGG stretches. The amino acid sequence deduced from the the cDNA sequence showed that the Sμbp-2 belongs to the putative helicase superfamily which is involved in replication, recombination and repair. Expression of Sμbp-2 mRNA is ubiquitous and augmented in spleen cells stimulated with lipopolysaccharide and interleukin 4 which also induce class switching. The Sμbp-2 gene is conserved among vertebrates. Possible involvement of Sμbp-2 in class switching is discussed.

INTRODUCTION

Genetic recombination events play important roles in the regulation of turning-on and -off of genes. Recombination of the MAT locus in yeast (1), and VDJ and S-S recombination in the immune system are examples (2, 3). In order to understand the regulatory mechanism of such recombinations, two general strategies have been adopted. One is the search for *cis*-acting DNA segments that promote recombination in surrounding DNA. Such DNA segments as the Chi sequence of *Escherichia coli* (4), hypervariable minisatellites (HVM) (5,6) and immunoglobulin switch (S) regions (7) are called recombinogenic sequences or hot spots, and known to enhance the rate of recombination in the genomes of many different organisms (8). These three sequences are guanine (G) rich and mutually similar. G-rich repeats, and probably higher-order structures such as G4-DNA have been proposed to play an important role as recombination hot spots (9). HVM and S sequences are similar in their tandem repeat structure as well. The other strategy is to search for proteins which are capable of interacting with recombination hot spots. The identification of such a protein and its molecular characterization should provide the clue to understand the recombination mechanism. The RecBC enzyme interacting with Chi (4) and four proteins binding to HVM have so far been identified (6, 10, 11). There are some reports of S region binding proteins (12, 13) but none of them have been characterized at the molecular level.

We have been interested in the molecular mechanism of immunoglobulin class switching, by which the progeny of a single cell switches the isotype of the produced Ig from IgM to IgG, IgE, or IgA, while maintaining the same V_H region during the ontogeny of B lymphocytes. Class switching is accomplished by a DNA rearrangement event called S-S recombination which occurs between the S regions located in front of each C_H gene except for the C_δ gene (14). S-S recombination can juxtapose an assembled V_H gene to a newly expressed C_H gene and delete the intervening C_H genes (15). This deletion model has been supported by numerous experiments using myelomas, hybridomas, and normal B cells (16–19). Recent studies have provided a direct evidence for the looping-out deletion mechanism by isolating circular DNAs composed of the deleted sequences as counter products of S-S recombination (20–22). Class switching is regulated by lymphokines. Lipopolysaccharide (LPS) alone induces switching to IgG_3 and IgG_{2b}, whereas the combination of LPS and interleukin-4 (IL-4) promotes switching to IgG_1 and IgE (20, 21, 23–26). Transforming growth factor-β (TGF-β) promotes switching to IgA (21, 27, 28).

Since the S regions are the targets of S-S recombination, it is likely that some proteins involved in S-S recombination interact with S regions. Using the expression cDNA library of LPS/IL-4-stimulated spleen cells, we have cloned a mouse cDNA encoding the Sμbp-2 protein which specifically binds to 5′-phosphorylated single-stranded DNA with G-rich sequences similar to the Sμ region motif. Although the Sμbp-2 mRNA is expressed ubiquitously, its expression in spleen cells was strongly augmented by LPS. The amino acid sequence deduced from the

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cDNA sequence showed that the Sμbp-2 protein belongs to the putative helicase superfamily which is involved in replication, recombination and repair.

MATERIALS AND METHODS

Preparation and screening of the cDNA library

RNA was extracted from mouse spleen cells cultured with LPS (30 μg/ml) and recombinant mouse IL-4 (20 U/ml) for 3 days (29). Complementary DNA library was constructed using λgt11 cDNA kit (Amersham). Screening was done according to the southwestern method (30), using 6M guanidine-hydrochloride to denature proteins on nitrocellulose filters (BA85, Schleicher and Schuell). The binding buffer was 25mM NaCl, 0.5mM DTT, 25mM HEPES (pH 7.9), and 0.25% milk powder. The screening probe was generated by concatemerization of the two complementary oligonucleotides shown below:

5′ GAGCTGGGGTGAGCTGAGCT 3′
3′ CCCACTCGACTCGACTCGA 5′

The sequence comprises the mouse immunoglobulin Sμ consensus sequence (31). The oligonucleotides were labelled by nick translation to a specific activity of $1-2\times10^8$ cpm per μg DNA. As a control probe we used labeled pUC vector. Approximately 2×10^6 plaques were screened.

RNA isolation and Northern blot hybridization

Total RNAs were prepared by the guanidine thiocyanate method (32) from various tissues of BALB/c mouse (6 weeks old) and mouse cell lines such as 38B9 (pre B cell), 2B4 (T cell hybridoma), PN (natural killer) and L (fibroblast). Total RNAs were prepared also from BALB/c spleen cells cultured with LPS (30 μg/ml) or LPS and IL-4 (20 U/ml). Poly (A) RNAs were purified with oligo (dT)-latex (Takara Shuzo Ltd.). 3 mg of mRNAs were fractionated by electrophoresis and transferred to Nylon membranes (Hybond, Amersham) as described by Thomas (33). The filters were hybridized with the 5′ *Bam*H I fragment of clone 21 or a β-actin probe (34). The probe DNAs were labelled by the random oligonucleotide priming method (35). Autoradiograms were analyzed by a Bio-image analyzer (Fujix BA2000, Fuji Film Co. Ltd.).

DNA isolation and zoo blot hybridization

High molecular weight DNA was extracted from yeast, salmon sperm, *Drosophila*, livers from *Xenopus*, chicken and mouse, and human placenta. DNAs were digested with *Eco*RI *or Bam*HI, electrophoresed and transferred to a nitrocellulose filter (BA85, Scheicher and Schuell) as described by Southern (36). Hybridization was carried out in 1M NaCl at 60°C as previously described (37). The filter was thereafter washed in 2×SSC (1×SSC is 0.15M NaCl and 0.015M Na citrate) and 0.1% sodium dodecyl sulfate at 50°C and analyzed as Northern blot.

Sμbp-2 protein production in *E.coli*

Y1089 lysogens harboring λ*gt*11, λ21 and λ22 were isolated and induced to express high levels of cDNA products (38). A crude extract of the induced lysogen was prepared by the method of Miyamoto *et al.* (39). Briefly, IPTG (10mM) was added during the last 2hrs of lysogen culture. Cells were pelleted and suspended in a lysis buffer (50mM Tris HCl, pH8.0, 1mM EDTA, 100mM NaCl). Cell suspensions were subjected to three rapid freeze-thaw cycles and subsequently centrifuged at 30,000 rpm for 1 hr at 4°C using a Hitachi RP 50−2 rotor. The supernatant was used for binding and competition assay. Crude extracts were concentrated by using Centricon-10 (Amicon) to a final protein concentration of 10 mg/ml.

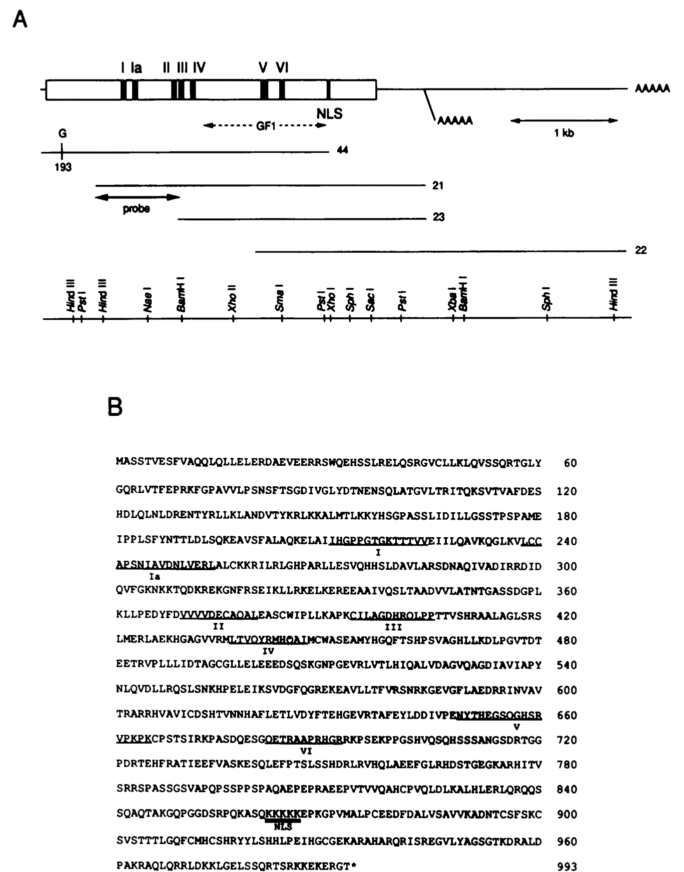

Figure 1. Organization of the Sμbp-2 cDNA and the nucleotide sequence. (A) Restriction site map and organization of the mouse Sμbp-2 cDNA. The rectangular box indicates the coding region. Helicase motifs (I–VI), and nuclear localizing signal (NLS) are shown. The dotted arrow corresponds to the region homologous to human GF_1 cDNA reported by Kerr and Khalili (46). The bold arrow shows the probe used in Northern and Southern blot analyses. A one base (G) deletion was found at position 193 of clone 44. Restriction sites are shown at the bottom. (B) Amino acid sequence of Sμbp-2. The consensus sequences of the putative helicase-domains (I–VI) and nuclear localizing signal are underlined. GenBank accession number of the nucleotide sequence of Sμbp-2 cDNA is L10075.

To obtain a purified preparation, we also expressed the fusion protein of Sμbp-2 with the maltose-binding protein (MBP). The system kit for MBP was purchased from New England Biolabs. The *Hin*d III-*Xba* I fragment (nucleotide positions 529−3825) of clone 21 was purified and ligated with oligonucleotides containing restriction enzyme sits at both ends, *Eco*R I and *Eco*R V at the 5′ end and *Sal* I at the 3′ end. The resulting *Eco*R I-*Sal* I fragment was inserted into the *Eco*R I-*Sal* I site in the polylinker region of the MBP expression vector, pMAL-c. Expression and purification were carried out as described in the protocol of the producer. The crude extract was applied on an amylose column and eluted with maltose. The eluate, containing the MBP-fusion protein and MBP, was adjusted to a final concentration of 1 mg/ml, and 1 μl was used in the competition assay.

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Motif             I                  Ia                   II               III               IV                   V                  VI

rep       19-VLAGAGSGKTRVIT-17-AAVTF-TNKAAREMKERV-141-YLLVDEYQDTN-16-FTVVGDDDQSIY-24-LEQNYRSSGRI-271-LMTLHA-SKGL-EFPYVYMVG-22-LAYVGITRAQK

PIF      255-YTGSAGTGKSILLR-14-VAVTASTGLAACNIGGTI- 34-ALVVDEISMLD-25-LIFCGDFFQLPP-27-LQKVFRQRGDV-219-MQTIHQNSAGKRRLPLVRFKA-63-CAYVALSRAVS

CONSENSUS     + G AG GKS            +T  T  AA N    +     ++++DE o       +++GD oQ       +ooo R          +T+  oSQG  o   V +          VA+ RAoo
                A P   T            A      T E                                                          K
              : : :: :::            :   .  :  :    :     ::::::         :: :: ::        :: : :          ::   ::::  :   :            :  : ::

Sμbp-2   210-IHGPPGTGKTTTVV-13-LCCAP-SNIAVDNLVERL-115-VVVVDECAQAL-13-CILAGDHRQLPP-31-LTVQYRMHQAI-201-NYT-HEGSQGH-S--RVPKPK-16-QETRAAPRHGR
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Figure 2. Alignment of conserved motifs of helicase and integrase. Two representatives (rep and PIF_1) and consensus sequences of the helicase family are compared with that of Sμbp-2. The number of residues between each motif is shown. Colons highlight complete concordance between the consensus and Sμbp-2 sequences, and dots indicate partial concordance. +, hydrophobic residues (I, L, V, M, F, Y and W); o, charged or polar residues (S, T, D, E, N, Q, K and R).

Oligonucleotide binding and competition assays

One μl each of concentrated protein preparations was spotted onto nitrocellulose filters and bound with [^{32}P] labeled single-stranded DNA (oligonucleotide) probes using the southwestern method as described for the screening of cDNA clones. Probe DNAs and competitors were phosphorylated at the 5′-termini with γ-[^{32}P] ATP and cold ATP, respectively, using T4 polynucleotide kinase. In experiments using lysogen extracts, 0.1 pmole of probe was incubated with or without the indicated amounts of competitors in a 1-ml reaction mixture. In experiments using the MBP-fusion proteins 1 pmole of Sμ-F probe was incubated with 25 pmole of competitors in a 50-μl reaction mixture. Filters were incubated for 2 hours at 4°C, washed 3 times for 20 minutes, and exposed on imaging plates for 30 minutes for analysis with Fujix BAS2000 (Fuji Photo Film Co., Ltd.). Alternatively, the radioactivity was counted with a scintillation analyzer (model 2000CA, Packard). Oligonucleotides were synthesized with an automated DNA synthesizer (model 381A, Applied Biosystems Inc.).

RESULTS AND DISCUSSION

Cloning and structure of Sμbp-2

To isolate proteins which can bind S regions, we prepared an expression cDNA library of mRNA isolated from LPS/IL-4-stimulated spleen cells using the λ*gt*11 vector. Concatenated oligonucleotides of the Sμ motif sequence were used as probe for screening 2×10^6 plaques of the cDNA library. Ten clones were isolated and classified into two groups (Smbp-1 and Smbp-2) by restriction mapping. The Smbp-1 clones showed DNA binding activity without obvious sequence specificity and bound also to the pUC vector. However, the Sμbp-2 clones (clones 21, 22 and 23) did not bind to the pUC vector and had a strong preference for the concatemer Sμ probe (see below). We therefore analyzed the Sμbp-2 clones further.

Nucleotide sequence determination of the cDNA clones revealed an open reading frame (700 residues) without initiation codon. To obtain 5′ cDNA clones we screened the cDNA library using a 5′ end probe (the 5′ *Bam*H I fragment of clone 21) and isolated clone 44 (Fig. 1A). By comparison of the sequences of clone 44, the human cDNA, and murine genomic DNA we found one base deletion (position 193) in clone 44. A 993-residue sequence was thus deduced from the full length cDNA (Fig. 1B). A nuclear localizing signal (residues 862 −866) was located in the C-terminal region. Two poly (A) signals (positions 3587−3594 and 5444−5449) were seen in the 3′-untranslated region.

Figure 3. Expression of mRNA in various mouse tissues and cell lines. RNA blotting analysis was carried out by using the 5′ *Bam*H I fragment of clone 21 as probe. Each lane received 3 μg of mRNA. Origins of mRNAs are; lane 1, brain; lane 2, thymus; lane 3, heart; lane 4, liver; lane 5, kidney; lane 6, spleen; lane 7, LPS-stimulated spleen cells; lane 8, LPS and IL-4-stimulated spleen cells; lane 9, 38B9; lane 10, 2B4; lane 11, PN; lane 12, L cells.

As shown in Fig. 2, the Sμbp-2 protein contains the seven motifs shared by the helicase family such as Rep helicase and PIF_1 (40−42). Rep helicase encoded by the *rep* gene of *E. coli* is a single-stranded DNA-dependent ATPase required by some phages for their replication (43). Rep was reported to participate in repair and recombination of DNA. The PIF_1 protein is a DNA helicase involved in repair and recombination of yeast mitochondrial DNA (44). Motif I contains a hydrophobic stretch—G(A)xxGxGKS(T) which is known as an NTP binding domain, and motif II, a hydrophobic stretch—DE(D) is responsible for Mg^{2+}NTP binding (42). Motif III is homologous to the conserved sequence of viral DNA polymerase (45). The functions of the motifs IV, V and VI are not known. The Sμbp-2 protein contains all these motifs, suggesting that the Sμbp-2 protein may be involved in replication or recombination. Unfortunately, however, we were unable to demonstrate that extracts of Cos cells transfected with Sμbp-2 expression plasmid possess either ATPase or helicase activity although the extracts contain a binding activity to the specific DNA to be described below. Obviously these negative results do not rule out a final helicase activity which might be undetectable with the present assay.

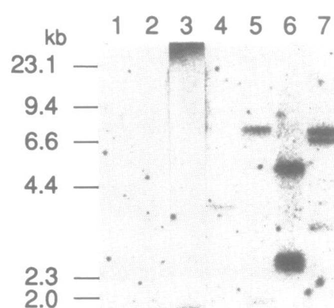

Figure 4. Zoo blot analysis of the Sμbp-2 gene. High molecular weight DNAs were digested with *Bam*H I (salmon) or *Eco*RI (the others), analyzed by the method of Southern (36) using the same probe as for Northern blot analysis. The amounts of DNA used were one μg for yeast and *Drosophila*, and 5 μg for the others. Lane 1, yeast; lane 2, *Drosophila*; lane 3, salmon; lane 4, *Xenopus*; lane 5, chicken; lane 6, mouse; lane 7, human.

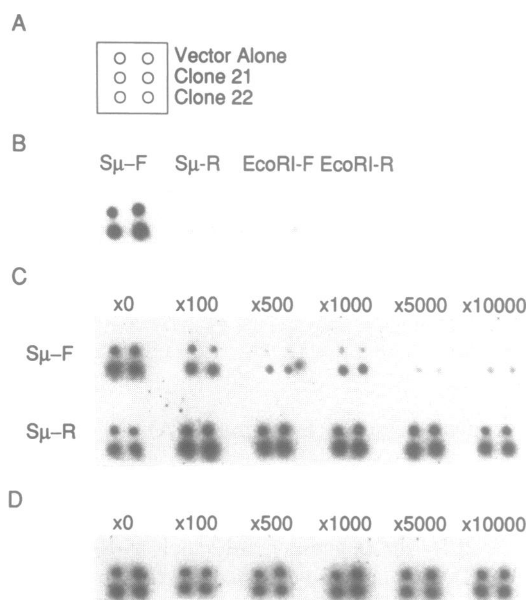

Figure 5. Binding and competition assay of the Sμbp-2 protein using the Southwestern method. **(A)** Arrangement of dot blot filters of the Sμ-2 protein used in B-D. One ml (10 μg) of crude lysate of each lysogen harboring λ*gt*11 vector alone, l21 or l22 was loaded as two spots onto a nitrocellulose filter. **(B)** Single-stranded DNA indicated was kinase-labeled and bound to the dot blot filters indicated in A. Sequences of the oligonucleotides are; EcoRI-F, 5′-AATTCGAGGATCCGGGTAC CATGG-3′; EcoRI-R, 5′-CCATGGTACC-CGGATCCTCG-3′. Sequences of Sμ-F and Sμ-R are shown in Fig. 6. **(C)** Labeled Sμ-F probe was made to compete with indicated amounts (in excess fold) of 5′ phosphorylated Sμ-F (upper) or Sμ-R(lower). **(D)** Labeled Sμ-F probe was bound to dot blot filters in the presence of indicated amounts (in excess fold) of 5′ dephosphorylated Sμ-F.

Expression and conservation of Sμbp-2 gene

We studied the expression of Sμbp-2 mRNA using the 5′ *Bam*H I fragment of clone 21 as probe (Fig. 1A) and detected two bands of 3.7 kb (major) and 5.5 kb (minor) which seemed to be due to the alternative poly (A) addition (Fig. 3). The expression of Sμbp-2 mRNA was induced in spleen cells stimulated with LPS+IL-4 (lane 8), or LPS alone (lane 7) as compared to non-stimulated spleen cells (lane 6) or IL-4 stimulated spleen cells (data not shown). We checked the expression pattern of Sμbp-2 mRNA in various tissues and cell-lines. All tissues examined contained at least small amounts of Sμbp-2 mRNA. Thymus contained a relatively large amount of the Sμbp-2 transcript but its content in brain was not particularly high. Many cell lines contained the same level of Sμbp-2 mRNA as stimulated spleen cells.

If the Sμbp-2 gene is of functional importance, it may be conserved in other vertebrates. The zoo blot analysis was performed using *Eco*R I or *Bam*H I-digested high molecular weight DNAs of several species (Fig. 4). DNAs of vertebrates including salmon contained positive signals, whereas those of yeast and *Drosophila* did not.

	Competition	Sequence
Sμ-F	2+	5'-GAGCTGGGGTGAGCTGAGCT-3'
5'(OH)Sμ-F	-	5'(OH)-GAGCTGGGGTGAGCTGAGCT-3'
Sμ-R	+/-	3'-CCCCACTCGACTCGACTCGA-5'
M1-F	-	5'-GAGCTGAGCTGAGCTGAGCT-3'
Chi-F	1+	5'-GAGCTGGTGGGAGCTGAGCT-3'
HVM-F	+/-	5'-AGGTGGGCAGGTGGAG-3'
HVM-R	-	3'-CGTCCACCTCTCCACC-5'
SS 1	2+	5'-GAGCTGGGGT-3'
SS 2	1+	5'-AGCTGGGGTG-3'
SS 3	2+	5'-GCTGGGGTGA-3'
SS 4	1+	5'-CTGGGGTGAG-3'
SS 5	+/-	5'-TGGGGTGAGC-3'
SS11	2+	5'-GGGGTGAGCT-3'
SS12	1+	5'-GGGTGAGCTG-3'
SS13	1+	5'-GGTGAGCTGG-3'
SS14	1+	5'-GTGAGCTGGG-3'
SS15	1+	5'-TGAGCTGGGG-3'
SS16	1+	5'-GGGGGGGGGG-3'
SS17	-	5'-TTTTTTTTTT-3'
SS18	+/-	5'-AAAAAAAAAA-3'
SS19	+/-	5'-CCCCCCCCCC-3'
5A	2+	5'-GAGCAGGGGT-3'
5G	2+	----G-----
5C	2+	----C-----
6A	+/-	-----A----
6T	-	-----T----
6C	-	-----C----
7A	1+	------A---
7T	-	------T---
7C	+/-	------C---

Figure 6. Summary of oligonucleotide binding and competition assays. Competition assays were performed by using one pmole of kinase-labeled Sμ−F probe and 25 pmole each of phosphorylated non labeled oligomer. 5′(OH)Sμ−F is the 5′-dephosphorylated Sμ−F. One μg of the MBP-fusion protein was blotted onto a filter. Level of the complex formation was quantitated by Cherenkov counts of the filters. Competition activities were categorized roughly into 4 groups; 2+, more than 90% competition; 1+, 56−80% competition; +/− 35−46% competition; − less than 26% competition. The 4 groups formed reasonable clusters and no intermediates were found. In series 5, 6 and 7 of probes, identical residues with SS1 are noted by dashes.

Sμbp-2 protein binds to G-rich single-stranded DNA

The Sμbp-2 clones were isolated originally by binding to concatemers of mutually complementary oligonucleotides with a consensus Sμ motif. Unexpectedly, however, we found that the Sμbp-2 protein did not bind to the Sμ probe which was prepared from plasmid DNA by restriction enzyme digestion. It can be assumed that the catenated Sμ probes contained many

single-stranded ends. We therefore examined the binding ability of the Sμbp-2 protein to single-stranded oligomer DNA of various sequences. First, we synthesized four oligomers Sμ-F, Sμ-R, EcoRI-F and EcoRI-R (Fig. 5 and 6) and checked their binding ability to freeze-thaw lyzates of *E.coli* lysogens of clones 21 and 22. EcoRI-F and EcoRI-R were chosen as controls consisting of unrelated sequences. As shown in Fig. 5B, the Sμbp-2 protein bound well to Sμ-F which consisted of two consensus motifs contained in all S sequences (GAGCT and TGGGG) but not to the other probes (Sμ-R, EcoRI-F and EcoRI-R) which did not contain such motifs. We also carried out a competition experiment using derivatives of Sμ-F probes. The labeled Sμ-F probe competed well with nonradioactive Sμ-F, but not with Sμ-R (Fig. 5C). Since the labeled Sμ-F did not compete with the dephosphorylated Sμ-F oligomer (Fig. 5D), the 5′ end phosphorylation appears to be essential for binding to the Sμbp-2 protein. We checked the binding ability of other probes by this competition assay. The G-rich strand of the Chi sequence competed weakly (Fig. 6). The M1-F oligomer which differs from Sμ-F by the absence of the G cluster did not compete with the Sμ-F probe at all. We also checked decanucleotide probes derived from Sμ-F. From the SS-series experiment we found that the 5′ end G contributed to the strong competition, because SS2, SS4, SS5 or SS15 which did not have any 5′-G could not compete with the Sμ-F probe. SS12 and SS13 which contained 5′ G but no GGGG stretch could not compete with Sμ-F. GAGCT does not appear to be essential. Experiments with modifications at positions 5, 6 and 7 showed a G-stretch longer than 3 is essential. However, poly G alone was not effective for Sμbp-2 binding (SS 16). Taken together, the Sμbp-2 protein appears to recognize a 5′-phosphorylated G, and a single stranded stretch of GGGG.

Is Sμbp-2 involved in S-S recombination?

The Sμbp-2 sequence was found to have homology with the human GF_1 cDNA that encodes a protein capable of binding to the JC virus enhancer and enhancing transcription of the viral late and early promoters in glial cells (46). From the strong conservation of nucleotide and amino acid sequences we suspect that the GF_1 cDNA encodes part of the human homolog of Sμbp-2. In fact the sequence of the human homologue of Sμbp-2 matched the GF_1 sequence completely (our unpublished data). GF_1 corresponds to the polypeptide between residues 490 and 864, and does not contain any N-terminal helicase motifs (I, Ia, II, III and IV) and the C-terminal nuclear localizing signal. Although the DNA binding domain of the Sμbp-2 protein was not precisely mapped it should be in the C-terminal part because clone 22 consisting of only the C-terminal half can bind DNA. The GF_1 protein should include the DNA binding domain. The probe used for isolation of GF_1 contains 5′-GAGCT-3′ and 5′-TGGCTGGC-3′ which are related to the human Sμ motif (47). Therefore, the function of the GF_1 protein as a transcription factor should be reevaluated with the full-length protein. Although the GF_1 transcript was reported to be most abundant in mouse brain, we could not confirm such a tissue distribution (Fig. 3). Sμbp-2 mRNA was abundant in rapidly growing cells such as thymus, stimulated spleen cells and various cell lines.

Several well characterized single-strand DNA-binding proteins have been shown to play important roles in recombination processes, including the bacterial proteins SSB and Rec A of *E.coli*, and the gene 32 protein of T4 phage (48,49). Msbp-1 binds to single-stranded minisatellite DNA sequences, especially to the kinase-labeled G-rich strand and may promote recombination by stabilizing minisatellite DNA in a single stranded conformation (11). The Chi and HVM sequences are known to mediate or stimulate recombination. The immunoglobulin S regions are responsible for class-switch recombination (7, 50, 51). It is interesting to note that all of the hree recombination related sequences (Chi, HVM and S) are rich in G. The G-rich repeats have a possible role as structural elements facilitating DNA-DNA interaction such as G4-DNA (9). This special structure might be responsible for synapsing and rearranging the chromosomal structure. The S regions are composed of tandem repeats of unit sequences which contain consensus motifs like GAGCT and TGGGG. The Sμ sequence is the most typical S region. Several characteristics of Sμbp-2 such as helicase motif and binding to 5′-phosphorylated single-stranded G cluster suggest that this protein might be involved in some aspects of DNA metabolism including replication, repair and recombination. Sμbp-2 recognizes the Sμ motif sequence for binding. In addition, human Sμbp-2 prefers the conserved human Sμ motif (GAGCTGGGC̲T) to the murine Sμ motif (GAGCTGGGG̲T) (our unpublished data). It is, therefore, an interesting possibility that the Sμbp-2 protein may be involved in class switching although there is no direct evidence for that.

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