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SF-B THAT BINDS TO A NEGATIVE ELEMENT IN GLUTATHIONE TRANSFERASE P GENE IS SIMILAR OR IDENTICAL TO *TRANS*-ACTIVATOR LAP/IL6-DBP[1]

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Summary: We have previously identified a silencer (negative enhancer) in glutathione transferase P (GST-P) gene which is strongly and specifically induced during hepatocarcinogenesis of the rat. At least three trans-acting factors bind to multiple *cis*-elements located in this silencer. One of these factors, SF-B (Silencer Factor B) specifically binds to GPS1 (GST-P Silencer 1) and has been cloned by a Southwestern protocol. Analysis of DNA and deduced amino acid sequence reveals that SF-B clone is most likely identical to an IL-6 inducible *trans*-activator LAP/IL6-DBP. Binding efficiency of SF-B to GPS1 is indistinguishable from that to IL6-responsive element found in C-reactive protein gene. The possibility that SF-B/LAP/IL6-DBP functions as a dual positive and negative regulator is discussed.

Many mammalian genes are regulated both positively and negatively at the transcriptional level (1). More than a dozen *cis*-elements and their respective specifically binding *trans*-acting factors have been identified (2,3). While a number of positive regulators have been cloned and are well characterized, little is known on negative regulators. In a previous work, we have characterized the 5' region of rat glutathione transferase P (GST-P) gene, which is virtually silent in normal liver, but is specifically and strongly expressed during hepatocarcinogenesis (4-8). We identified multiple *cis*-elements in GST-P gene including two enhancers, GPEI and GPEII (7,8) and a silencer (4). At least three *trans*-acting factors bind to this silencer (5), and SF-A (Silencer Factor A) binds to GPS4 (GST-P Silencer 4) with a strong

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affinity and to several other *cis*-elements in silencer region with a weaker affinity (5). While SF-A is a dominant negative regulator, SF-B (Silencer Factor B), which binds to GPS1 (GST-P Silencer 1), also contributes to silencer function (5). It is likely that there is some cooperativity among silencer factors, including SF-C (Silencer Factor C), which binds to GPS2 (GST-P Silencer 2), since internal deletion of any of these binding sites has effect on silencer activity (5). For this reason, we are attempting to clone all the *trans*-acting factors which bind to the GST-P silencer region. We report here the molecular cloning of the SF-B cDNA by a Southwestern protocol. We show that SF-B is identical to or a variant form of IL6-inducible *trans*-activator LAP/IL6-DBP (9,10) and binds to GPS1 as well as to CRPα that is the IL6-responsive element in C-reactive protein gene (11).

MATERIALS AND METHODS

Plasmid constructions: 4CAT and 4(ΔGPS1)CAT were constructed as described previously (5). For constructing 5xGPS1-tkCAT, synthesized GPS1 was multimerized to a 5 mer and cloned into the BamHI site of pUC18 (4), and SmaI-HincII fragment containing 5xGPS1 was ligated at blunt-ended SalI site of tkCAT (pBLCAT2) (12). Cell culture, DNA transfection and CAT assay were performed as described previously (5).

Synthesized oligonucleotides: Oligonucleotides (24-26 mer) having Sau3AI cohesive end for screening and binding analyses are as follows (only upper strands are shown):

GPS1: SF-B binding site; 5'GATCAGAGGTTGGTAAATAGGGATGG3'
GPS2: SF-C binding site; 5'GATCAATAGGGATGGGCAGAAGGCAG3'
GPS4: SF-A binding site; 5'GATCTTTCTTGGAGCAGGACCCAAAAAT3'
GPS5: SF-A binding site; 5'GATCAGACTCCGGTCCAGCTGCTGAG3'
GPEI: GST-P enhancer I; 5'GATCTAGTCAGTCACTATGATTCAGCAA3'
AP-2: Activator Protein 2; 5'GATCGAACTGACCGCCCGCGGCCCGT3'
MT-CAP: human MTIIA cap site 1; 5'GATCACCACGCCTCCTCCAAGTCC3'
CRPα: IL-6 responsive element; 5'GATCCATAGTGGCGCAAACTCCCTTA3'

Southwestern screenig of SF-B clone and sequence analysis: Normal rat liver λgt11 cDNA library (0.34-3.4 Kb, average size: 1.1 Kb) was purchased from Clontech. Cloning of SF-B was accomplished by the method of Vinson et. al (13) using a ^{32}P-labeled multimerized GPS1 as a probe. Plasmid cDNA libraries of a normal rat kidney cell line NRK and a rat fibroblast cell line 3Y1 for rescreening are the generous gift from Drs. H. Okayama and H. Nojima (Institute for Microbial Diseases, Osaka University). Sequence reaction was performed by the dideoxy method (14) using Sequenase (United States Biochemical Corp.). The regions compressed were sequenced by using SSB (single strand binding protein) or Tth polymerase at 70°C (United States Biochemical Corp.).

Production of fusion protein: Y1089 lysogen containing λ27 was prepared and a fusion protein with β-galactosidase was produced by the method of Kageyama et al. (15) with a slight modification as follows. In brief, harvested cells were disrupted by sonication. After spinning down, the precipitate containing fusion protein was solubilized with buffer A (30 mM Tris (pH7.5), 30 mM NaCl, 1 mM DTT) containing 8 M urea; soubilized protein was dialyzed against buffer A containing 4 M urea followed by buffer A alone 3 times. The resultant supernatant was used for DNA binding analysis.

Gel mobility shift assay: Protein fraction (6.25μl) was mixed with 6.25μl of 20 mM Tris (pH7.5), 10 % glycerol, 2 mM DTT, 20 mM EDTA, 0.2 μg of poly (dI-dC) and radio-labeled probe (10,000 cpm/10 f mol). Binding reaction was continued at room temperature for 30 min. Each reaction was loaded on a 4 % non-denaturing polyacrylamide gel, electrophoresed at 150 V for 1 hr, fixed with 10 % methanol and 10 % acetic acid, and autoradiographed overnight at -80°C.

RESULTS

Silencer effect of GPS1 element: In a previous work, we identified several *cis*-elements in the silencer fragment of the GST-P gene. As shown in Fig. 1, 4(ΔGPS1)CAT lacking the SF-B binding site has a higher CAT activity than 4CAT, the natural GST-P promoter having the whole silencer when expressed in the rat hepatoma cell line dRLh84. Furthermore, thymidine kinase promoter attached with 5 mer of GPS1 showed a significantly lower activity than the tkCAT itself. Similar results were obtained using the human hepatoma cell line HepG2, HeLa cells and a mouse embryonic carcinoma cell line F9 (data not shown). These data strongly suggest that SF-B (GPS1 binding protein) has a silencer activity in various cell lines and functions as a negative regulator like SF-A.

Molecular cloning of the cDNA encoding SF-B: By direct screening of λgt11 expression library, a clone which bound specifically to GPS1 was isolated. Out of

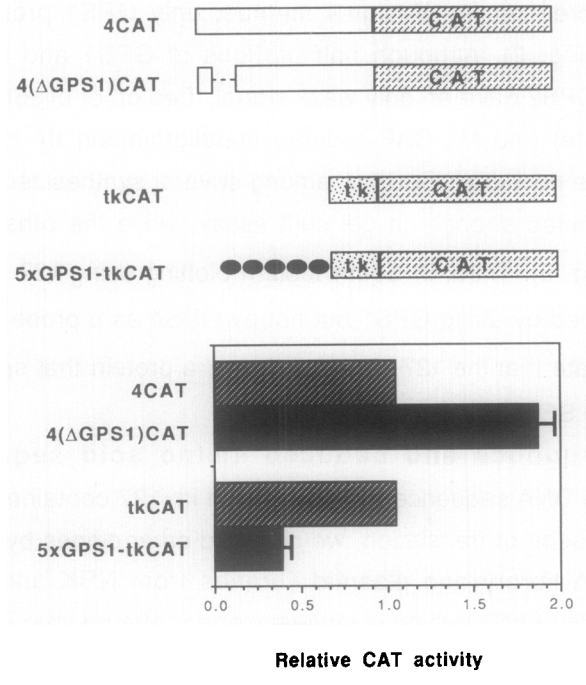

Figure 1. Analyses of GPS1 on silencer activity by CAT assay in a rat hepatoma cell line, dRLh84. Upper panel shows the constructs used. Lower panel shows the relative CAT activity with standard deviation.

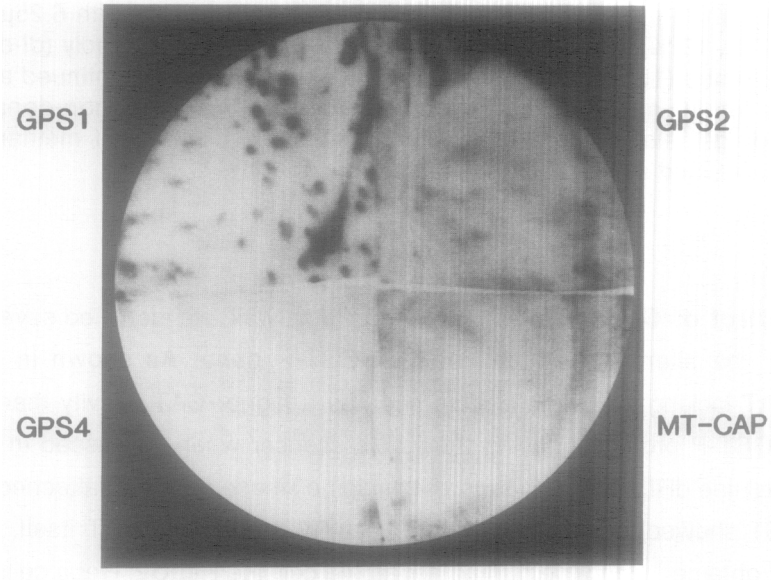

Figure 2. Specificity of isolated SF-B clone (λ27). SF-B clone (λ27) was plated and transferred to nitrocellulose filter, which was incubated with either one of labeled-GPS1, GPS2, GPS4, or MT-CAP.

1.5×10^6 clones, only one clone (λ27) was obtained by the method of Vinson et al. (13). Several lines of evidence indicate that thr λ27 codes for SF-B. First, in Southwestern screening by Vinson's method, only GPS1 probe gave a strong positive signal (Fig. 2). Although half portions of GPS1 and GPS2 sequences overlapped (5), GPS2 gave an only weak signal. Two other oligonucleotides, GPS4 (SF-A binding site) and MT-CAP (human metallothionein IIA cap site 1 binding region (16)), gave no signal. Second, among several synthesized oligonucleotides, only GPS1 competed strongly in gel shift assay, while the other oligonucleotides had no effect (Fig. 3). Third, in Southwestern blotting using λ27 protein, a positive signal was obtained by using GPS1 but not by GPS4 as a probe (data not shown). These data indicate that the λ27 clone codes for a protein that specifically binds to GPS1 and hence SF-B.

Nucleotide sequence and deduced amino acid sequence of SF-B cDNA: Since the DNA sequence of 618bp insert in λ27 contained neither initiation codon nor stop codon of translation, we obtained other clones by rescreening a rat liver λgt11 cDNA library and plasmid libraries from NRK and 3Y1 cells. DNA sequence analysis of pN1, which is longest clone (1194 bp long including 48 bases of poly (A) tail) isolated from NRK cell library showed a typical "leucine zipper" structure (data not shown). To our surprise, this 1146 bp DNA sequence was almost identical to IL-6 inducible *trans*-activator LAP and IL-6DBP reported by Schibler's

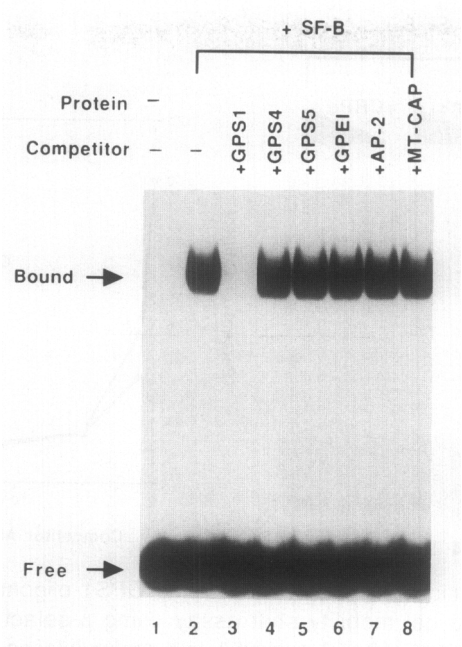

Figure 3. Gel mobility shift assay of β-galactosidase/SF-B fusion protein. Lane 1: no protein, Lane2-8: fusion protein, Lane3-8: 250 fold molar excess of indicated oligonucleotides were added as competitor as indicated.

(9) and Cortese's (10) groups, respectively. That is, the sequence of SF-B is same as the sequence 355 to 1500 of LAP genomic clone except that another G is inserted at 1104 and C at 1334 is G. This also corresponds to the sequence 229 to 1350 of IL6-DBP cDNA clone, although N-terminal portion of SF-B clone was not yet isolated, and polyadenylation signal and poly (A) tail at 3' end of IL6-DBP cDNA sequence has not been described. All DNA sequences of seven other clones obtained from three kinds of libraries by rescreening described above shared the part of pN1 DNA sequence (data not shown).

β-Galactosidase-SF-B fusion protein specifically binds to both GPS1 (negative element) and CRPα (IL6-responsive positive element): As described above, fusion proteins of β-galactosidase-SF-B bound to GPS1 specifically and DNA sequence analysis indicated that the SF-B clone is at least partially identical to LAP/IL6-DBP [LAP and IL6-DBP were identified as a liver-enriched transcriptional activator protein (9) and as an IL-6 dependent DNA binding protein (10), respectively]. Therefore we determined the difference of binding affinity of SF-B to GPS1 and CRPα, one of the IL-6 responsive elements. First, gel shift assay was performed using GPS1 as a probe. Specific binding of SF-B to GPS1 was competed out by the addition of either non-labeled GPS1 or CRPα (Fig. 4. left panel). Right panel in Fig. 4 shows the quantitation of competition data which indicates that CRPα and GPS1 inhibit the SF-B-GPS1 binding with an

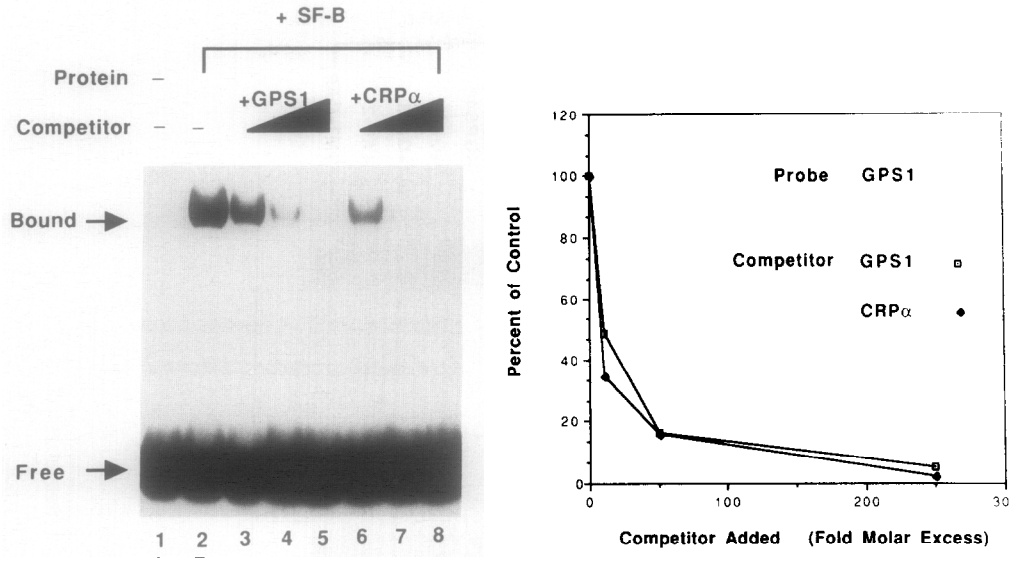

Figure 4. Competition analysis of SF-B using GPS1 oligonucleotide as a probe. Left panel shows a gel mobility shift assay using β-galactosidase/SF-B fusion protein. As competitors, 10, 50 and 250 fold molar excess of GPS1 and CRPα oligonucleotides were used. The dried gel was cut and counted by liquid scintillation counter, and competition ratio (% of control) is shown in right panel.

indistinguishable efficiency. The gel shift assay was then carried out using CRPα as a probe. SF-B binds to CRPα as specifically as it does to GPS1, and both GPS1 and CRPα can inhibit the specific binding with the same efficiency (Fig. 5). These results

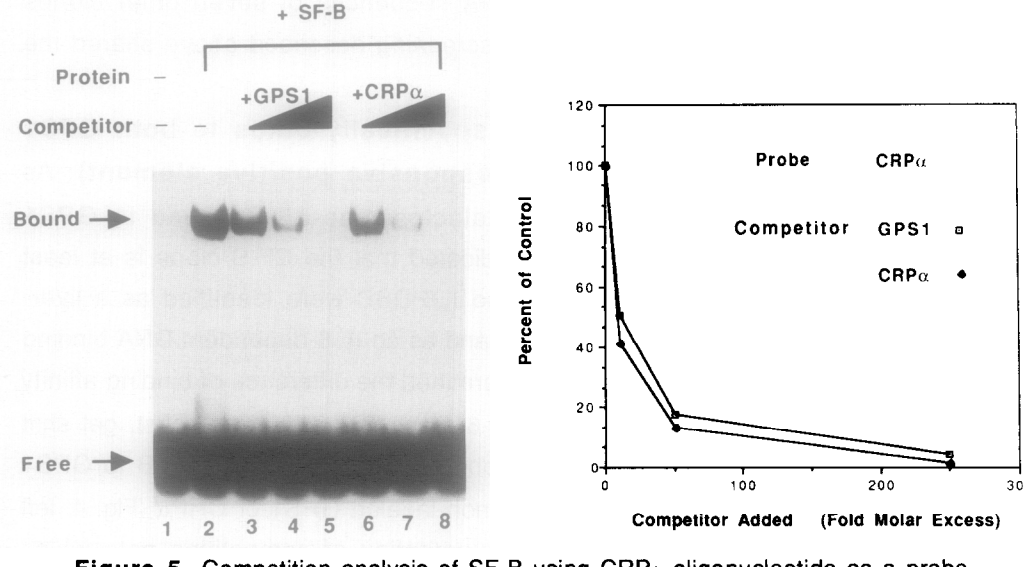

Figure 5. Competition analysis of SF-B using CRPα oligonucleotide as a probe. The data are shown in the same way as in Fig. 4.

indicate that SF-B binds to both GPS1 and CRPα specifically and efficiently, and binding affinities of SF-B to GPS1 and CRPα are essentially the same.

DISCUSSION

In this study, we have cloned a factor SF-B that binds to a silencer element GPS1 specifically. It is of particular interest that the cDNA of the negative regulator SF-B has a partial sequence that is identical to activator LAP/IL6-DBP. There are several possibilities on the relationship between SF-B and LAP/IL6-DBP. The possibility that SF-B is a LAP/IL6-DBP related but distinct gene acting as a repressor like IRF-2 versus IRF-1 (17) is unlikely because the sequence of SF-B is exactly the same as LAP/IL6-DBP at the nucleotide level, and Southern blot analysis gave only a single strong band (data not shown). Second possibility is the existence of different forms of mRNA by alternative splicing like ΔfosB versus fos-B (18). Although this is quite possible, we do not have any evidence for that so far. Rather, there is only one band (1.55 Kb long) by Northern blotting (10) and our polymerase chain reaction failed to obtain a cDNA with different lengths by using several primers corresponding to 5' region of LAP/IL6-DBP cDNA (data not shown). Furthermore sequences of several clones obtained by rescreening cDNA libraries were the same as LAP/IL6-DBP. The third but most likely possibility is that the SF-B and LAP/IL6-DBP are the same molecule. In this case, some *trans*-modulator that changes protein-protein interactions from positive to negative effect may exist and the complexes of *trans*-modulator and SF-B/LAP/IL6-DBP may act as either an activator or a repressor. Similar mechanisms have been demonstrated as selector function of c-Jun/c-Fos complex to composite glucocorticoid response element in positive and negative glucocorticoid regulation (19). It is also possible that a single protein has dual function as an activator or a repressor. This phenomenon may be caused by the binding sequence which forms a different tertiary structure of DNA-protein complexes. This may also be caused by modifications of *trans*-acting factors as shown in CREB, whose dual fuction is modulated by phosphorylation (20). Using SF-B expression vector as an effector plasmid and 5xGPS1-tkCAT as a reporter plasmid, SF-B acts as a negative regulator not as a positive regulator (unpublished observations). However, since the activity of tkCAT itself as a control reporter plasmid also decreased slightly, detailed analyses including use of different reporter plasmids are required.

Further search for related genes, alternatively spliced form and *trans*-modulators/selectors, and further characterization of *trans*-activation/*trans*-repression domain of LAP/IL6-DBP/SF-B will help solve this question. Cloning of other silencer factors, SF-A and SF-C that bind to *cis*-elements existing near the GPS1 site and appear to work cooperatively with SF-B, is also needed and this is now in progress.

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