

# Silencer binding proteins function on multiple *cis*-elements in the glutathione transferase P gene

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Received October 26, 1990; Revised and Accepted November 28, 1990

## ABSTRACT

The glutathione transferase P (GST-P) gene is specifically expressed during chemical hepatocarcinogenesis of the rat, whereas mRNA of this gene is virtually undetectable in normal liver. We have previously identified a stretch of DNA, that acted negatively in transcription, at 400 bp upstream from the cap site of the rat GST-P gene. Further characterization has revealed that this negative fragment functions in an orientation and position independent manner, suggesting that it is acting as a silencer. This silencer consists of multiple negative elements to which nuclear factors bind. This silencer is active not only in rat non-hepatoma and hepatoma cells but also in human and mouse cell lines, suggesting that these elements function as general regulators of basal gene expression. At least two proteins bind to this silencer fragment, one of which, designated SF-A (Silencer Factor A), has been partially purified. SF-A binds to several regions in this silencer, and likely plays an important role on negative regulation of this gene.

## INTRODUCTION

The rat glutathione transferase P (GST-P) gene is strongly and specifically expressed during chemical hepatocarcinogenesis and is considered to be an excellent tumor marker (1). To understand the regulatory mechanisms of this gene, we have characterized the 5' flanking region of GST-P gene. At -2.5Kb, an enhancer, termed GPEI, has been identified (2,3). This enhancer consists of two phorbol 12-*o*-tetradecanoate 13-acetate responsive element (TRE)-like sequences, and GST-P gene expression is mediated mainly by these two TRE-like elements (3). The GST-P gene also has a TRE and a GC box in the promoter region (-60 to -40) (2). In the human metallothionein IIA gene, the TRE and the GC box function as a basal level enhancer and contribute to basal activity of gene expression (4). The TRE and the GC box of the GST-P gene promoter also show high activities in

several cell lines (3,5). Nevertheless, mRNA of the GST-P gene as well as the protein is undetectable in normal liver (6,7). These observations prompted us to investigate the *cis*-acting negative element that had been identified just upstream of the TRE and the GC box of the promoter (5). In this report, we describe the structure of the negative elements of the GST-P gene and their binding factors. The results indicate that multiple elements contribute to the repression of gene expression, and at least two different proteins bind to these multiple elements. In this manuscript we demonstrate that one of these factors, SF-A (Silencer Factor A), binds to six regions of the promoter, including a region between the TATA box and the cap site. SF-A probably functions as a general negative regulator.

## MATERIALS AND METHODS

### Plasmid constructions

$\Delta$ -106CAT contains the GST-P promoter (-106 to +59) and the chloramphenicol acetyl transferase (CAT) structural gene as described previously (5). The silencer fragment, -396 to -140 in the promoter region of GST-P gene, was inserted into the Sall site located at the 5' end of GST-P promoter sequence (-106) or into the BamHI site located 0.8Kb downstream from the CAT gene in  $\Delta$ -106CAT according to the standard protocol (8). This fragment was also inserted into Sall site (originally AccI site) (5) or downstream BamHI site in pSV2CAT, and polylinker site in pBLCAT2 (tkCAT) in which CAT gene were regulated by Herpes simplex virus thymidine kinase promoter from -105 to +51 (9). For *in vivo* competition experiments, oligonucleotides were synthesized and purified as described (3), multimerized using T4 DNA ligase after kination, and subcloned into pUC18. Internal deletion mutants of 4CAT which contained up to -396 in the promoter region of GST-P gene (5) were constructed by using polymerase chain reaction techniques (10). All deletion mutants generated by polymerase chain reaction were checked by sequencing by the dideoxy method, using denatured plasmid templates (11).

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### Cell culture and DNA transfection

The 3Y1 cell, a rat fibroblast cell line, was grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The dRLh84 cell, a rat hepatocellular carcinoma cell line, was maintained in minimal essential medium (MEM) supplemented with 10% FBS. Mouse embryonic carcinoma F9 and human hepatoma HepG2 cells were cultured in MEM-alpha (alpha modified) and DMEM, respectively, both supplemented with 10% FBS. Cells were transfected by the calcium phosphate co-precipitation techniques described by Chen and Okayama (12) for 3Y1 cell, and Graham and Eb (13) for other cells, respectively. CAT activity was determined by the method of Gorman et al., (14). All the transfection experiments were performed at least three times by using two or three different preparations of DNA, and the mean values are shown in the results. In some experiments, the transfection efficiency was checked by co-transfection with pRSVGAL, an eukaryotic expression vector which contained the *Escherichia coli*- $\beta$ -galactosidase (lacZ) structural gene controlled by Rous sarcoma virus LTR.  $\beta$ -Galactosidase activity was assayed as described (15). It was confirmed that the variation of transfection efficiency was less than 20%.

### Nuclear extract and purification of a silencer binding protein

Nuclear extract from rat liver was prepared according to the method of Lichtsteiner et al., (16). In brief, after homogenization of the liver, nuclei were purified by ultracentrifugation in sucrose cushion. Nuclear proteins were extracted in 10mM Hepes, (pH 7.6) containing 0.55M KCl, 0.1mM EDTA, 10% glycerol, 3mM MgCl<sub>2</sub>, 1mM DTT, 0.1mM PMSF and 1% aprotinin. After centrifugation, nuclear extract was precipitated by ammonium sulfate solution (0.3g/ml). The buffer used in later steps was Buffer A, which consisted of 25mM Hepes (pH7.8), 12.5mM MgCl<sub>2</sub>, 1mM DTT, 20% glycerol and KCl as indicated. The precipitate was dialyzed against 0.1M Buffer A and stored at -80°C. Nuclear extract obtained was loaded on a column of UltogelAcA44 equilibrated with 0.1M Buffer A containing 0.1% NP-40. DNA affinity column was prepared as described by Kadonaga and Tjian (17). The oligonucleotide sequences for purification of SF-A, which was a GPS4 binding factor, were as follows:

5' GATCTTTCTTGGAGCAGGACCCAAAAAT 3'  
3' AAAGAACCTCGTCTGGGTTTTACTAG 5'

Binding activity was monitored by DNase I footprinting, according to the method of Jones et al., (18). Chemical footprinting by methidiumpropyl-EDTA (MPE) was performed as described previously (19).

## RESULTS

### Silencing effect of negative fragment in GST-P gene

Previous experiments with successive upstream deletion mutants revealed the presence of a DNA region which appeared to act negatively in the transcription of the GST-P gene (5). To test the silencing effect of the negative fragment in this gene, the BP(BamHI-PstI) fragment located from -396 to -140 was connected to heterologous promoters. When these constructs were transfected into the rat non-hepatoma cell line 3Y1 or the hepatoma cell line dRLh84, BP fragment functioned as a negative element and functioned in an orientation and position independent manner, irrespective of the promoter used (Table 1). This

**Table 1.** Silencing effects of negative fragment in GST-P gene on activities of heterologous promoters and enhancer in non-hepatoma and hepatoma cells.

Construct	Relative CAT Activity	
	Non-hepatoma (3Y1)	Hepatoma (dRLh84)
<b>A. GST-P Promoter</b>		
$\Delta$ -106CAT	1.00	1.00
BPA-106CAT	0.45	0.44
PBA-106CAT	0.17	0.23
$\Delta$ -106CATBP	0.27	0.29
<b>B. SV40 Promoter and Enhancer</b>		
pSV2CAT	1.00	1.00
BPpSV2CAT	0.44	0.35
PBpSV2CAT	0.38	0.35
<b>C. TK Promoter</b>		
TKCAT	1.00	1.00
BPTKCAT	0.33	0.43

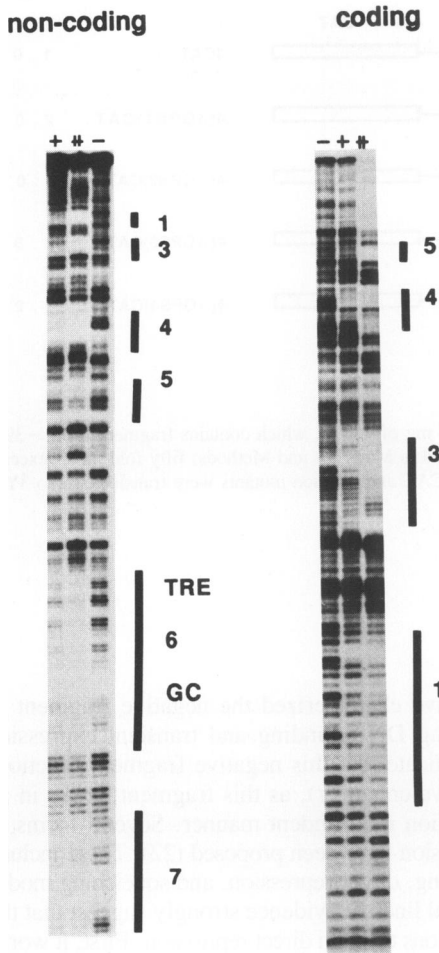
BP (BamHI-PstI) fragment located from -396 to -140 in GST-P 5' flanking region was introduced just upstream of heterologous promoters including the GST-P promoter itself in normal orientation (BP) or reverse orientation (PB). In the case of  $\Delta$ -106CATBP, BP fragment was joined to BamHI site located 0.8 Kb downstream from the end of CAT structural gene, and was 2 Kb away from cap site. Each construct was transfected into 3Y1 and dRLh84 cells and CAT activities were determined.

fragment showed a repressive effect on the activities of the GST-P promoter and the SV40 promoter-enhancer in HepG2 cells (a human hepatoma cell line) and F9 cells (a mouse embryonic carcinoma cell line) (data not shown). These observations suggested that the BP fragment might function as a general silencer (or negative enhancer) among different species, and not in a cell type specific manner. Here we refer to the BP fragment as the silencer fragment of GST-P gene.

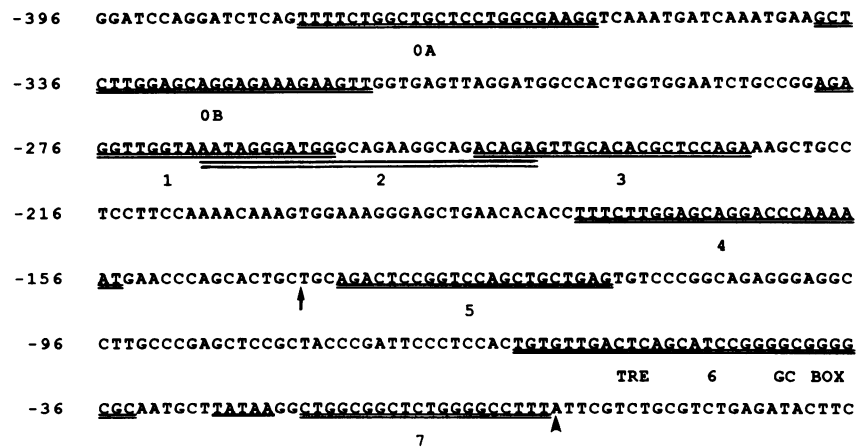
### Functional analyses of GST-P silencer

To further characterize this silencer fragment, we performed DNase I footprint analysis using a rat liver nuclear extract. Because this extract contained some exo- and endo-nuclease activities endogenously, and because many *trans*-acting factors were heat-stable [such as ATF (20), AP1 (20) and C/EBP (21)], the extract was heated at 55°C for 5 min, centrifuged and the supernatant used for the footprint analysis. Three regions in the silencer fragment, designated as binding sites, 1, 3 and 4, were observed to be protected (Fig.1-A). In the region just downstream of the PstI site (-140), the 3' end of the silencer fragment, another site was observed to be protected (binding site 5). Additionally, footprint analysis revealed two other binding sites (binding sites 6 and 7). Protection region in binding site 6 corresponding to the TRE and the GC box is likely the results of AP1 and SP1 binding, respectively. Binding site 7 was present between TATA box and the cap site. In some experiments, using the rat liver nuclear extract frozen and thawed five times, additional binding site (binding site 2) was observed between sites 1 and 3, although the binding was rather weak and the protected region was partially overlapped in binding sites 1 and 3, precluding our defining the exact boundary in detail (data not shown). The protected regions of the various factors are summarized in the sequence shown in Fig.1-B. We refer to these binding sites as GPS 1-5. (GST-P silencer 1-5). Although binding site 5 is present just outside of silencer fragment (BP fragment), we still refer to it as GPS, since this site has some silencing effect, as will be shown later.

A



B

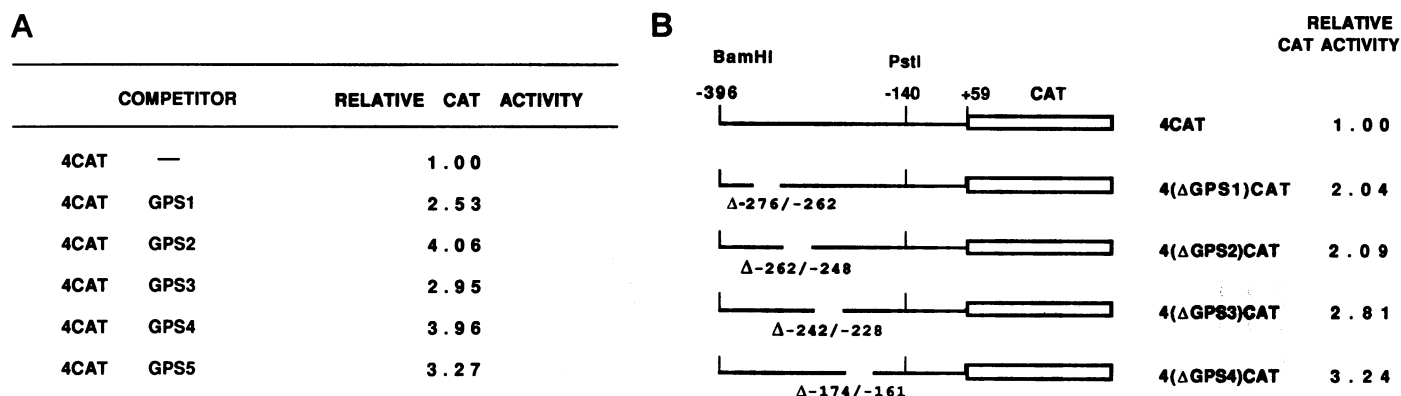


**Figure 1.** DNase I footprint analysis of rat liver nuclear extract on promoter and silencer of GST-P gene. (A) DNase I footprint analysis. The DNA probes consist of a BamHI-HindIII fragment (-396 to +59) labeled at the HindIII end of the non-coding strand and a Sall-HindIII fragment (-305 to +59) labeled at the Sall end of the coding strand by Klenow fragment. The probe was incubated without (-) or with 12  $\mu$ g (+) or 24  $\mu$ g (++) of nuclear extract heated at 55°C for 5 min. The boundaries of several protected regions are indicated by vertical bars. TRE and GC box are indicated. (B) Binding sites of nuclear factors in GST-P promoter and silencer. Protected sequences are double underlined. Protected regions by SF-A found in Fig.3. are also shown as 0A and 0B. Arrow and arrowhead indicate the PstI site (-140), 3' end of BP fragment, and cap site (+1), respectively. TATA box is underlined. TRE and GC box are indicated.

Footprint analysis of the nuclear proteins to these regions suggests that the GPS4 binding protein is either present in high abundance or may have a higher affinity than the other factors, as indicated by the complete protection afforded the GPS4 site by very low amounts of the nuclear protein fraction (Fig.1-A). GPS1 binding protein appears to be distinct from GPS4 binding protein. When the nuclear extract was applied to an Ultrogel Aca44 column, GPS1 binding protein eluted immediately following the void volume, whereas the proteins which bound to GPS3, 4 and 5 eluted together and much more slowly than the GPS1 binding protein (data not shown). These observations indicate that at least two binding proteins, and possibly three proteins (including the GPS2 binding protein) bind to the GST-P silencer.

To test whether these binding *cis*-elements were functional as silencers or not, we next carried out *in vivo* competition

experiments. As shown in Fig. 2-A, when one of the GPS1-sequences was used as a competitor, silencing effect disappeared suggesting that each element, including GPS5 which is located downstream of the silencer, was a functional negative element. To confirm this, we next made internal deletion mutants which lacked one of the GPS1-4 sequences. Silencing effect disappeared in all internal deletion mutants, indicating that GPS1-4 were active as negative elements (Fig.2B). In both cases, GPS4 had a stronger negative effect than the other elements. Taken together with footprint analysis (Fig.1), it is likely that GPS4 and the GPS4 binding protein are playing a major role in the function of this silencer. We next analyzed each GPS element on silencing activity. Interestingly, however, the individual elements did not show any silencing activity, even when connected to the GST-P promoter in a tandem multimer form (4 mer or 5 mer) of synthesized oligonucleotides (data not



**Figure 2.** Functional analyses of GST-P gene silencer in 3Y1 cells. (A) *In vivo* competition experiment. Two mg of 4CAT, which contains fragment from -396 to +59 in promoter region of GST-P gene, was used as a reporter plasmid. Competitor plasmids were described in Materials and Methods; fifty-fold molar excess (five fold amount excess, 10 mg) of plasmids were used for each experiment. (B) Internal deletion analysis. 4CAT and deletion mutants were transfected into 3Y1 cells. Positions deleted corresponded to the binding sites in Fig.1.(B).

shown). Only one fragment, a fragment that contained both GPS4 and GPS5 in the native arrangement, demonstrated silencing activity.

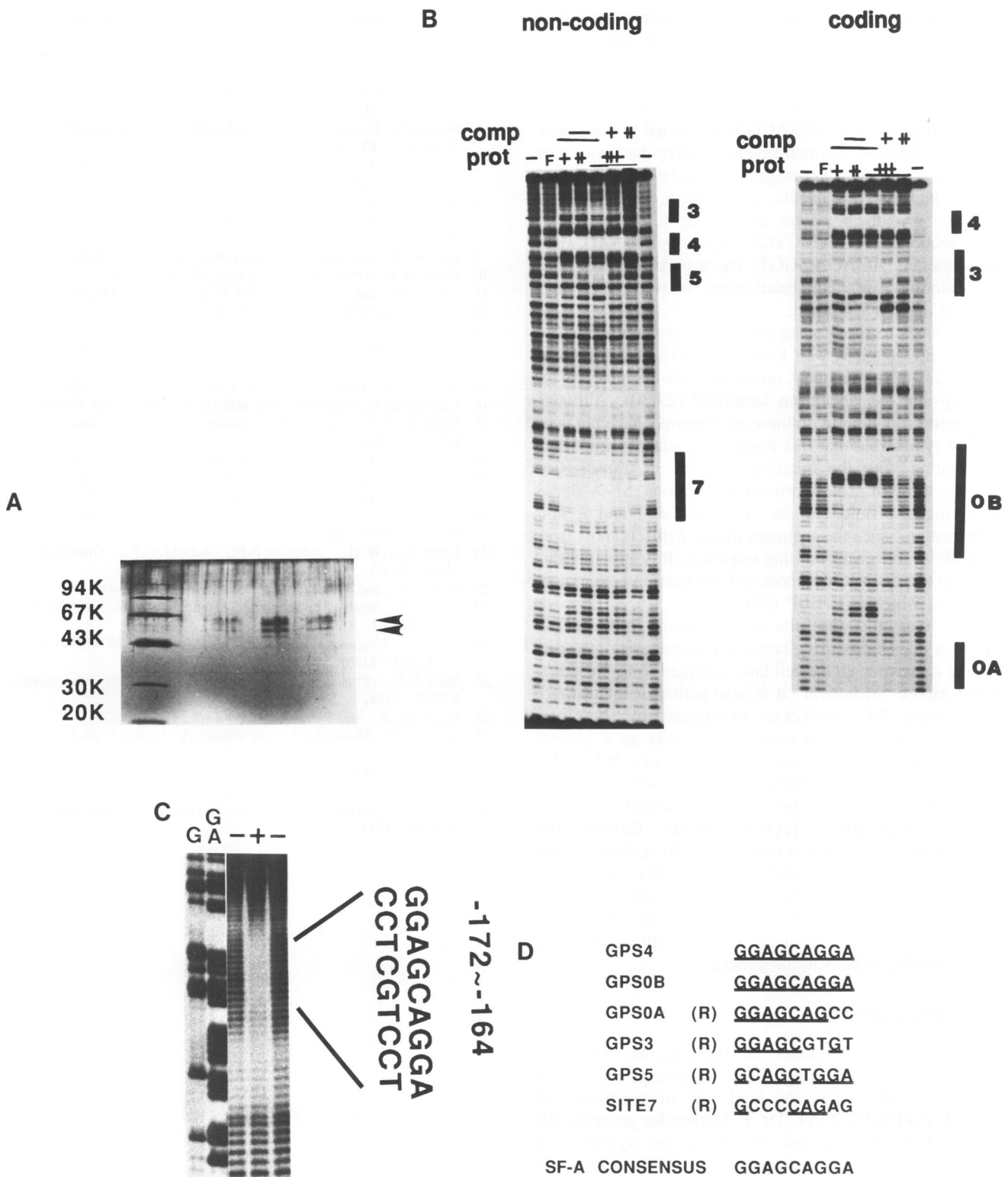
#### Partial purification of SF-A (Silencer Factor A)

Functional analyses demonstrated the multiple *cis*-elements and multiple *trans*-acting factors in the silencer region of the GST-P gene. Since GPS4 seemed to be the more dominant element of this region, we proceeded to purify the GPS4 binding protein, which we have called SF-A (Silencer Factor A). Rat liver nuclear extract was applied to an Ultrogel Aca44 column and SF-A binding activity was monitored by DNase I footprinting (data not shown). The enriched SF-A fraction was then applied to a multimerized GPS4-affinity column and eluted with the buffer containing 0.6M KCl. The eluates numbered 2, 3 and 4 revealed two main bands and two minor bands of Mr of 50–55Kd, as determined by silver staining in SDS-polyacrylamide gels (Fig.3-A). To determine the binding region of SF-A in this silencer, DNase I footprint analysis was performed using purified fraction number 3 (Fig. 3-B). Surprisingly, several regions besides GPS4 were protected by this fraction. These sites include GPS3 and 5 and other regions in the upstream of the silencer designated as GPS0A and 0B, and site 7 near the TATA box. However, SF-A binds to GPS4 more strongly than to other binding sites, as judged by the competition assay with poly (dI-dC) (Fig. 1-C & 3-B). To ascertain the core binding sequence of SF-A, we carried out the MPE chemical footprint analysis (19). As shown in Fig.3-C, using affinity purified SF-A, we identified a 9 bp core binding sequence (GGAGCAGGA) in the GPS4 site. This sequence was then compared with the sequences of the other SF-A binding sites to determine if we could identify a consensus among the various binding sites. As shown in Fig.3-D, binding sequences of GPS0B, 0A, 3 and 5 were quite similar to the that of GPS4 with GPS0B being identical. In contrast to this, the sequence of site 7 demonstrated very little homology, although the binding affinity of SF-A to site 7 was higher than that of GPS3 or 5, as determined by footprint analysis using the poly (dI-dC) competitor (Fig. 3-B).

#### DISCUSSION

In this work, we have characterized the negative fragment of GST-P gene by using DNA binding and transient expression assays. The data indicate that this negative fragment functions as a silencer (negative enhancer), as this fragment works in an orientation and position independent manner. Several forms of transcriptional repression have been proposed (22). These include competition, quenching, direct repression, and squelching modes of repression. Several lines of evidence strongly suggest that the GST-P silencer functions through direct repression. First, it works irrespective of its position and orientation. Second, this silencer also works on enhancer-less promoters, like the GST-P and TK promoters (Table 1), although repressors interact with enhancers or activators in other models (22). Finally, the nucleotide sequence of the negative elements which we have identified does not show any similarity to the sequences of GST-P promoter, TK promoter, or SV40 promoter-enhancer.

The GST-P silencer fragment consists of several *cis*-elements. Deletion or *in vivo* competition of each *cis*-element resulted in a decrease in activity. Thus each element apparently plays an important role on silencing activity. However, each element itself is not sufficient for full silencing activity. These findings indicate a cooperative function, including a spacing effect, between the silencer elements in the repression of promoter activity, as has been observed for numerous elements including the glucocorticoid responsive element (23), TRE (24), and serum responsive element (25). To analyze the mechanism of cooperativity, we have partially purified SF-A, the dominant silencer binding protein of the GST-P gene. Footprint analysis indicates that SF-A binds to several regions, including a region outside of what we have defined as the silencer region. This observation strongly suggests a coordinate action of the SF-A protein on multiple sites of the GST-P silencer. However, it is also apparent that SF-A related proteins bind to GPS0A, 0B, 3, 5, and binding site 7, and that these proteins act in concert with SF-A to repress transcriptional activity. Indeed, the SF-A binding sequence in GPS0B is exactly the same as that in GPS4, while its binding affinity is different (Fig.3).



**Figure 3.** Partial purification of SF-A (Silencer Factor A) and its binding to the GST-P silencer region. (A) SDS-polyacrylamide gel electrophoresis of affinity purified SF-A. SF-A was affinity-purified as described in Materials and Methods, electrophoresed in 10% gel and stained with silver. The main protein bands are shown by the arrowheads. M; standard marker protein, 2-4; tube number from affinity column with buffer containing 0.6M KCl. (B) DNase I footprint analysis of purified SF-A. The DNA probes consist of a BamHI-HindIII fragment (-396 to +59), labeled at the HindIII end of non-coding strand and a SalI-SacI (-396 to -90) fragment labeled at the SalI end of coding strand. PROT (-); BSA instead of SF-A, PROT (+ to +++); increasing amounts of SF-A, PROT (F); flow through fraction of enriched SF-A fraction on multimerized GPS4-affinity column chromatography, COMP (-); without competitor poly (dI-dC), COMP (+, + +); 100 ng and 500 ng of competitor poly (dI-dC), respectively. Several region protected from DNase I digestion by purified SF-A binding are indicated by vertical bars. These region are also shown in Fig. 1-B. (C) MPE chemical footprint analysis of purified SF-A. G,G+A; Maxam and Gilbert sequence ladder, (-); BSA instead of SF-A, (+); affinity-purified SF-A. (D) Sequence comparison of SF-A binding sites. R indicates the sequences in reversed orientation.

Several proteins that bind to negative elements have been described recently. GCF, whose cDNA has recently been cloned, binds to GC-rich sequences and acts as a negative regulator (26). It is unlikely that GCF is similar or identical to SF-A, since the GCF consensus sequence, GCGGGGC, is very different from the SF-A binding site, GGAGCAGGA, identified in this paper. Other negative elements and their binding proteins have also been demonstrated. Among the negative regulatory sequences identified in the lysozyme, insulin,  $\beta$ -interferon, immunoglobulin heavy chain, growth hormone and collagen II genes, the consensus sequence, ACCCTCTCT, was reported (27). This inverted sequence, AGAGAGGGT, has a partial similarity to the SF-A binding site. The relationship between these two sequences, if any, is unknown.

SF-A also binds to site 7, a region located between the TATA box and the cap site. It is well known that TFIID binds to the TATA box (28). More recently, an initiator *cis*-element present near the cap site has also been identified (29,30). Although binding proteins functioning on these *cis*-elements have not been identified, it is possible that this region may play an important role cooperating with the initiation complex in transcription regulation by through protein-protein interactions. SF-A shows a relatively higher affinity for site 7 than for GPS3 and 5. This is very interesting, since the sequence of site 7 differs most from that of the SF-A consensus binding sequence. It is apparent that SF-A binds to two distinct sequences and functions in both region, an example of which is C/EBP (20).

What is the real function of the silencer, found in the GST-P gene and what does it do during hepatocarcinogenesis of the rat? This element is active in all the cell lines analyzed so far including human and mouse. Moreover, it is also active in rat primary hepatocyte cultures (Morimura et al., in preparation). Therefore, it is quite likely that this silencer element acts as a general regulator to modify the basal expression of genes. While this silencer seems to be active through the hepatocarcinogenesis according to the data as shown here, it is also possible that some changes in regulatory factors occur during the hepatocarcinogenesis. Characterization of the action of this silencer in hyperplastic nodules and newly developed hepatocellular carcinomas, and the cloning of the genes of silencer binding proteins including SF-A, are required for further elucidation of the mechanism of the specific expression of the GST-P gene during hepatocarcinogenesis.

## ACKNOWLEDGEMENTS

We thank Drs. M. B. Diccianni, H. Hamada, M. Sakai and S. Morimura for critical reading of this manuscript and helpful discussions, Dr. M. Sakai for kind gift of constructs of BPpSV2CAT and PBpSV2CAT, Dr. P. Dervan for generous gift of MPE, and Y. Maeda and M. Noda for synthesizing oligonucleotides. This work was supported in part by grants from the Ministry of Education, Sciences and Culture, Japan, from the Foundation for Promotion of Cancer Research backed by the Japan Shipbuilding Industry Foundation, and from the Life Science Research Project of Institute of Physical and Chemical Research (RIKEN).

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