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Multiple regulatory elements and phorbol 12-*O*-tetradecanoate 13-acetate responsiveness of the rat placental glutathione transferase gene

(chemical hepatocarcinogenesis/tumor marker/tumor promoter/chloramphenicol acetyltransferase assay/cis-acting element)

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ABSTRACT **We have analyzed the cis-acting regulatory DNA elements of the placental rat glutathione *S*-alkyltransferase (GST-P) gene. Various regions of the 5′ flanking sequence were fused with a bacterial chloramphenicol acetyltransferase gene. The transcriptional activity of each construct was determined by the transient expression assay after introduction into a hepatoma cell line. Multiple regulatory elements were identified. Two enhancing elements were located 2.5 and 2.2 kilobases upstream from the transcription start site and designated GST-P enhancers I and II (GPEI and GPEII, respectively). A consensus sequence of the phorbol 12-*O*-tetradecanoate 13-acetate responsive elements was present in the GPEI and at position −61. GPEII contained two of the simian virus 40 and one of the polyoma enhancer core-like sequences. A silencing element was also found 400 base pairs upstream from the cap site. In accordance with the above observation, endogenous GST-P gene was found to be stimulated when the rat fibroblast line 3Y1 was treated with phorbol 12-*O*-tetradecanoate 13-acetate. Phorbol 12-*O*-tetradecanoate 13-acetate enhanced the expression of the transfected GST-P gene to a much higher degree in HeLa cells than in the hepatoma cells, which constitutively expressed the endogenous GST-P. The results are discussed in terms of the specific derepression of GST-P gene during hepatocarcinogenesis in the rat.**

Glutathione transferases (RX:glutathione R-transferase, EC 2.5.1.18) are a family of enzymes that catalyze the conjugation of glutathione to electrophilic xenobiotics (1–3). One of the isozymes, placental glutathione *S*-alkyltransferase (GST-P), is known to be induced specifically at an early stage of chemical hepatocarinogenesis in the rat (4–8). This induction was shown to be mainly at the transcriptional level (8). The frequency of derepression of GST-P gene in hyperplastic nodules and hepatocellular carcinomas was almost 100% of those examined, irrespective of the carcinogens used (4–7). This high coincidence suggests that the mechanism of specific expression of this gene may be closely related to the process of neoplastic transformation. On the other hand, Cowan and coworkers (9, 10) reported that the GST-Pi (the human enzyme equivalent to rat GST-P) gene was overexpressed only in multidrug-resistant cells and its activity was correlated well with cellular resistance to the drugs, suggesting that GST-Pi may play a key role in cellular multidrug resistance. Under these circumstances, understanding the regulatory mechanism of this gene appears to be increasingly important. To study the molecular mechanisms of GST-P gene expression, we have isolated (8, 11) cDNAs as well as genomic clones of rat GST-P and determined their whole nucleotide sequences. We have further studied the control region of this gene by transfection experiments using the isolated GST-P gene. We now report the localization and characterization of its cis-acting regulatory elements in the 5′ flanking region.† We also describe the phorbol 12-*O*-tetradecanoate 13-acetate (TPA) responsiveness of this gene.

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MATERIALS AND METHODS

Plasmid Constructions. A 3.0-kilobase pair (kb) fragment, between base pair −2900 (*Eco*RI) and base pair +59 (*Acc* I), relative to the cap site of GST-P gene (11) was inserted into the *Hin*dIII site of the pSV0CAT (12) and designated as E-CAT. A series of 5′ deletion mutants were constructed from the E-CAT by using appropriate restriction enzymes as shown in Fig. 1*A* (1- to 5-CAT). Further deletion mutants were obtained by BAL-31 exonuclease digestion of 5-CAT followed by recircularization using the *Sal* I linker. For the construction of an enhancer- or silencer-containing plasmid, an enhancer or silencer fragment was isolated by using the appropriate restriction enzymes as described in the text, and both ends were converted to *Sal* I or *Bam*HI recognition sequences. These fragments were inserted into the *Sal* I site located at the 5′ end of the GST-P sequence of each deletion mutant or at the *Bam*HI site located 0.8 kb downstream from the chloramphenicol acetyltransferase (CAT) gene of the plasmid 5-CAT. The CAT gene with the thymidine kinase promoter (tk-CAT) was described by Klein-Hitpass *et al.* (13).

Cells and CAT Assay. The dRLh84 cell is a rat hepatocellular carcinoma cell line induced by 4-(dimethylamino)-3′-methylazobenzene (14). Either 10^6 dRLh84 cells or 2×10^6 HeLa cells were plated per 100-mm dish in Eagle's minimal essential medium (MEM) containing 10% (vol/vol) newborn calf serum (GIBCO). Ten micrograms of DNA was precipitated in calcium phosphate (15) and left on the cells for 13–16 hr. About 30 hr after washing and medium change, cells were harvested and lysed by several cycles of freezing and thawing. CAT activity of the cell lysate was determined according to Gorman *et al.* (12).

For the determination of TPA responsiveness, cells were treated with 15% (vol/vol) glycerol, 4 hr after the addition of DNA, and the medium was changed to MEM containing 0.5% newborn calf serum. After 24-hr incubation, TPA was added to the medium at a concentration of 60 ng/ml and the cells were harvested after a 15-hr incubation. The 3Y1 cell line, a rat fibroblast line, was used for analysis of the TPA responsiveness of the endogenous GST-P mRNA synthesis. The medium of confluent 3Y1 cells was changed to the MEM containing 0.5% newborn calf serum and left for 24 hr. TPA

Abbreviations: SV40, simian virus 40; GST-P, placental glutathione *S*-alkyltransferase; TPA, phorbol 12-*O*-tetradecanoate 13-acetate; GPE, GST-P enhancer; CAT, chloramphenicol acetyltransferase; TRE, TPA responsive element; AP-1, activator protein 1; nt, nucleotide(s).

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04138).

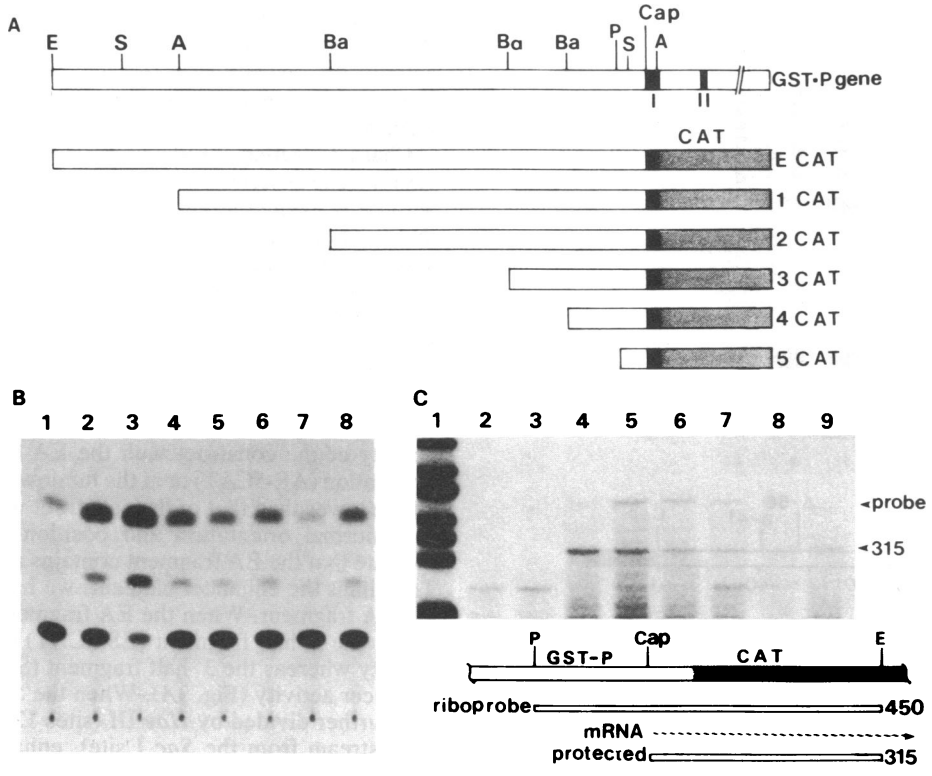


FIG. 1. (*A*) Restriction map of the 5′ flanking region of the rat GST-P gene and 5′ deletion constructs of GST-P–CAT fusion genes. Solid boxes indicate the first exon. Shaded boxes represent the CAT gene. Open boxes represent 5′ flanking and intron regions. A series of deletion mutants were constructed as follows. E-CAT was digested by *Acc* I, *Bam*HI (partially), *Bgl* II, *Bam*HI, and *Pst* I for 1-, 2-, 3-, 4-, and 5-CAT, respectively, and the ends were converted to *Sal* I sites. These deletion fragments were inserted between the *Sal* I (originally the *Acc* I site) and the *Hin*dIII sites of pSV2CAT. (*B*) The CAT assay of the GST-P–CAT fusion genes was done 45 hr after transfection of dRLh84 cells. Lanes: 1, pSV0CAT; 2, pSV2CAT; 3, E-CAT; 4, 1-CAT; 5, 2-CAT; 6, 3-CAT; 7, 4-CAT; 8, 5-CAT. (*C*) RNase protection analysis of GST-P–CAT mRNAs. Total cytoplasmic RNAs were extracted from dRLh84 cells 45 hr after DNA transfection. Twenty micrograms of each RNA was hybridized with labeled riboprobe for 16 hr. RNase-resistant fragments were analyzed on a 5% polyacrylamide gel. *Hin*fI- and *Eco*RI-digested pBR322 DNA was used for size markers (lane 1). RNAs from the cells transfected with pSV0CAT (lane 2), pSV2CAT (lane 3), E-CAT (lane 4), 1-CAT (lane 5), 2-CAT (lane 6), 3-CAT (lane 7), 4-CAT (lane 8), and 5-CAT (lane 9) are shown. Positions of the probe and 315-nt band are indicated. The GST-P–CAT fusion gene, riboprobe, expected mRNA, and the anticipated protected fragment were shown schematically below the autoradiography.

was added to the culture medium and cells were harvested after 8 hr. All of the transfection experiments were done more than three times with at least two preparations of DNA, and similar results were obtained.

RNase Protection Mapping. A 450-nucleotide (nt) fragment between the *Pst* I (at −139) and *Eco*RI (in the CAT structural gene) sites of the GST-P–CAT plasmid was inserted into pSP65 (Promega Biotec) in the opposite direction, and ^{32}P-labeled complementary RNA (riboprobe) was synthesized using bacteriophage SP6 RNA polymerase *in vitro* (16). Total cytoplasmic RNAs from the DNA-transfected hepatoma cells or the TPA-treated 3Y1 cells was extracted as described (17) and hybridized with riboprobe. After RNase treatment of hybridized RNA, protected fragments were analyzed on 5% or 8% polyacrylamide gels containing 8 M urea (16).

RESULTS

Identification of Regulatory Elements in the 5′ Flanking Region of the GST-P Gene. A series of deletion mutants was constructed (Fig. 1*A*) and transfected into the dRLh84 cells, and CAT activities of cell lysates were assayed after 45 hr (Fig. 1*B*). Total RNA was extracted from the cells transfected with each construct and subjected to RNase protection mapping. After hybridization with the ^{32}P-labeled riboprobe and RNase treatment, protected riboprobes were analyzed by polyacrylamide gel electrophoresis (Fig. 1*C*), and the expected 315-nt bands were seen on the gel. The intensities of the bands of various constructs were proportional to their CAT activities, indicating that the CAT activity of each transfectant correlated well with the transcription activity from the correct initiation site. The CAT activity of E-CAT was more than five times higher than that of 1-CAT. This indicates that the DNA region between the *Eco*RI site (−2.9 kb) and the *Acc* I site (−2.2 kb) has transcriptional enhancing activity. Further deletion to −0.8 kb (3-CAT) did not change the CAT activity by more than 2-fold. The activity of the 4-CAT was significantly lower than that of 3-CAT and also lower than that of 5-CAT. In dRLh84 cells, some CAT activity was detected when cells were transfected with SV0CAT, containing a CAT gene without any promoter and enhancer elements (Fig. 1*B*, lane 1). This may be due to some weak read-through phenomenon from unidentified outside sequences. The activity of 4-CAT was almost the same level as that of the SV0CAT, a negative control, and the activity was increased when a further 260 nt were deleted (5-CAT). This result was reproducible and suggests that some element suppressing the transcription activity is located between the *Bam*HI site (−0.4 kb) and the *Pst* I site (−0.14 kb). The analysis of further deletion mutants constructed by using BAL-31 exonuclease showed that a stretch of ≈100 nt from the cap site was sufficient for a low level of transcription (Fig. 2*A*). The nucleotide sequence of the proximal 5′ flanking region is shown in Fig. 2*B*. In addition to the TATA box at position −27, a GC box and a TPA-responsive element (TRE) are found at positions −47 and −61, respectively. This TRE

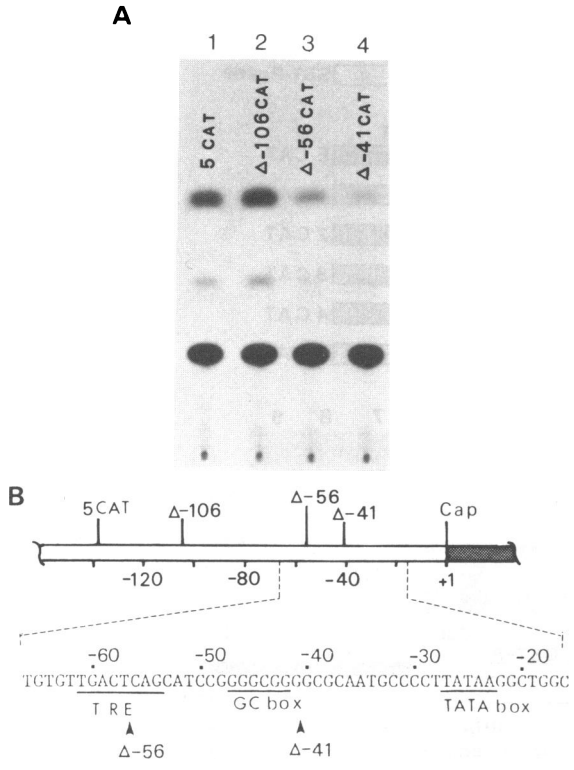
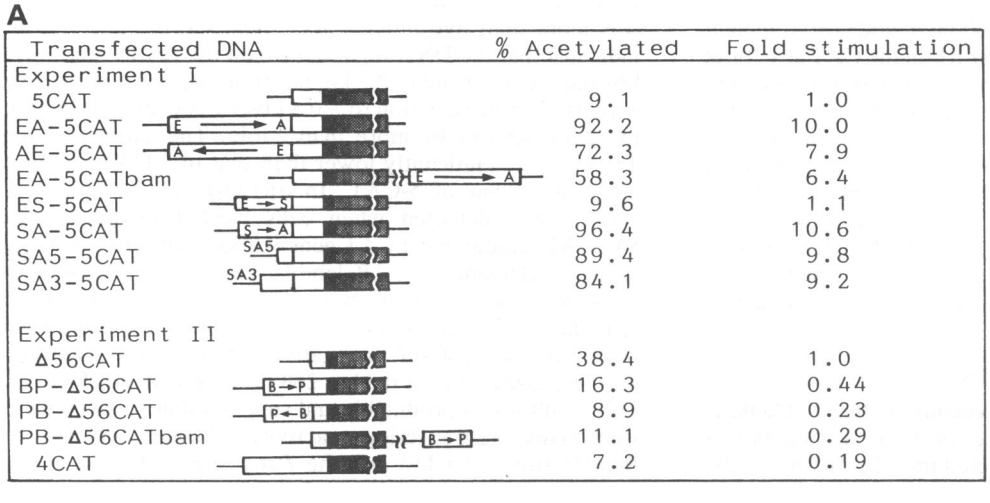


FIG. 2. (*A*) CAT assay of the proximal deletion mutants of GST-P–CAT fusion gene. 5-CAT DNA was digested by exonuclease BAL-31 from the 5′ end. The 5′ end of each mutant is indicated in *B*. (*B*) Schematic representation and nucleotide sequence of proximal 5′ flanking region of GST-P gene.

sequence is identical to the TRE sequence found in the human metallothionein IIA gene (18). Lee *et al.* (18, 19) and Angel *et al.* (20) have reported that the TRE is a binding site for the trans-acting factor activator protein 1 (AP-1), which can be activated by TPA. They also reported that TRE had enhancer activity even without induction by TPA. The activity of the mutant Δ-56, which partially lost its TRE sequence, was significantly lower than that of mutant Δ-106, which had an intact TRE. The mutant Δ-41, which had lost the GC box in addition to the TRE, showed almost no activity. These results suggest that both the TRE and the GC box are functional in hepatoma cells as the cis-acting DNA elements.

Characterization of Enhancers. The deletion analysis of the GST-P gene described above suggested that an enhancer-like activity is located at approximately −2.5 kb. To determine the position and orientation dependency of this element, several constructs were prepared and assayed. An *Eco*RI–Acc I (−2.9 to −2.2 kb) fragment designated as EA fragment was inserted into position −140 in both orientations (EA-5CAT or AE-5CAT) or into the *Bam*HI site of 5-CAT (EA-5CATbam). Fig. 3*A* shows that all the constructs having the above insert exhibited 5- to 10-fold expression compared with the construct without the EA fragment (5-CAT). The activity of the construct with the EA fragment in reversed orientation (AE-5CAT) or at the far downstream position was a little lower than that of the construct with the EA fragment in a normal orientation and position. The above results indicate that the EA fragment contains an enhancer element. To delimit the enhancer element, we have further dissected the EA fragment. When the EA fragment was cut in half by *Sac* I, the 5′ half fragment (ES-5CAT) showed no enhancer activity whereas the 3′ half fragment (SA-5CAT) had strong enhancer activity (Fig. 3*A*). When the *Sac* I–*Acc* I fragment was further divided by *Hae* III (sites 122 and 338 base pairs downstream from the *Sac* I site), enhancer activities were detected on both fragments (SA5-5CAT and SA3-5CAT, Fig. 3*A*). These 5′ and 3′ fragments were, therefore, designated GPEI and GPEII, respectively (for GST-P gene enhancer I and II). The enhancer fragments were almost equally as active as the EA fragment, and these activities were not additive in this experiment. Fig. 4 shows the nucleotide sequence of the *Sac* I–*Acc* I 350-nt fragment. Some of the characteristic features are found in this sequence. First, the tumor promoter TRE is present in GPEI (60 nt from the *Sac* I site, Fig. 4). This sequence is homologous to the TRE sequence present in the 5′ flanking region of the human metallothionein IIA gene (18), with a 1-nt substitution. Further analysis of GPEI indicated that the enhancer element is closely associated with the TRE sequence (unpublished results). Second, two of the enhancer core-like sequences of



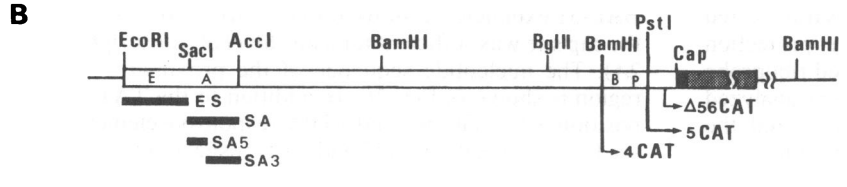


FIG. 3. Characterization of enhancer and silencer elements. (*A*) Each construct was introduced into dRLh84 cells and CAT activity was determined. Acetylated ratios were determined by liquid scintillation counting of each spot. (*B*) Restriction map of the 5′ flanking region and positions of the fragments used for construction are shown as bold bars. Open boxes, solid boxes, and shaded boxes represent 5′ flanking regions, exons, and CAT genes, respectively.

simian virus 40 (SV40) (21) and one of the enhancer core-like sequences of polyoma virus (22) are found in GPEII (238, 279, and 320 nt downstream from the *Sac* I site, respectively; Fig. 4). In addition to the core-like sequences, inverted repeats are found 238 and 256 nt downstream from the *Sac* I site as indicated by dashed arrows in Fig. 4. Which of these sequences or some other sequence in GPEII is really functional remains to be determined.

Characterization of Silencer Element. The *Bam*HI–*Pst* I fragment (BP fragment) located from 400 to 140 nt upstream from the cap site has silencer-like activity as shown in Fig. 1 *B* and *C*. To determine the orientation and position effects of this element, we used constructs having the BP fragment with reversed orientation or at a far downstream position. Fig. 3 shows that all of the constructs decreased the CAT activity by several times. The reversed orientation (PB Δ56CAT) was most effective in repressing CAT activity. This result demonstrates that the *Bam*HI–*Pst* I fragment contains a silencer element that works in a position- and orientation-independent manner in hepatoma cells.

TPA Responsiveness of GST-P Gene. Since at least two TRE sequences exist on the 5′ flanking sequence of the GST-P gene, we asked whether this gene was responsive to TPA. First, TPA responsiveness of the endogenous GST-P gene was tested in 3Y1, a rat fibroblast cell line. The 3Y1 cells were treated with TPA and the GST-P mRNA was quantitated by RNase protection mapping. As shown in Fig. 5*A*, TPA treatment increased GST-P mRNA by up to 5-fold. Next, the response of an exogenously introduced GST-P gene to TPA was examined. E-CAT was transfected into dRLh84 cells and HeLa cells, and CAT activities were determined after 45 hr. When rat hepatoma cells were transfected and treated with TPA, little enhancement was observed (1.2-fold). The SV40 enhancer contains a TRE and its activity is stimulated by TPA (18). When pSV2CAT was transfected as a control, little enhancement was detected in the hepatoma cells (1.1-fold). HeLa cells transfected with E-CAT or pSV2CAT and treated with TPA showed significantly high stimulation (3.4-fold for E-CAT and 2.3-fold for pSV2CAT). Cells transfected with tk-CAT alone and treated with TPA were stimulated a little (for HeLa cells, 1.4-fold; for hepatoma cells, 1.0-fold). These results indicate that the GST-P gene is more responsive to TPA than are the SV40 early genes.

It is well known that the major cellular target of TPA is protein kinase C (23). It may be that the protein kinase C-mediated signal transduction system is fully activated in the hepatoma cells in the absence of TPA but is not yet fully activated in the HeLa cells.

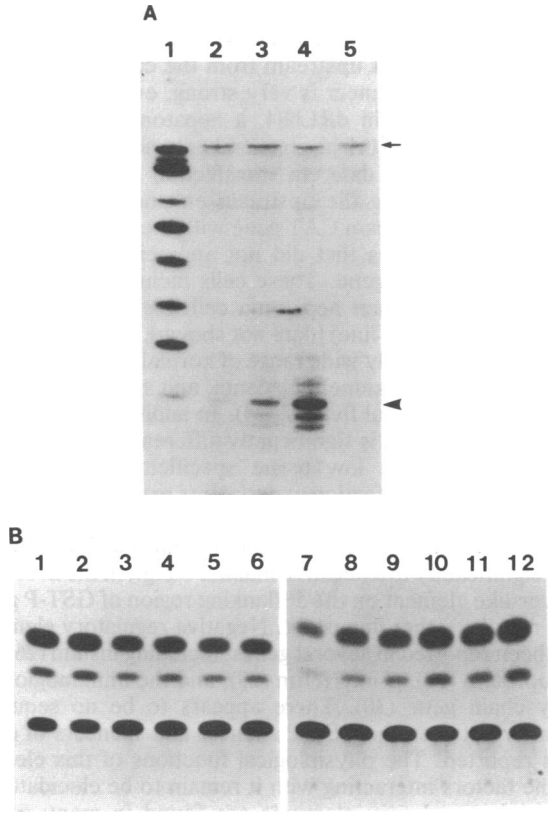


FIG. 5. TPA responsiveness of GST-P gene. (*A*) Endogenous GST-P mRNAs of 3Y1 and Morris hepatoma cells were quantitated by RNase protection mapping. Five micrograms of total cytoplasmic RNA extracted from 3Y1 cells not treated with TPA (lane 2), 3Y1 cells treated with TPA for 8 hr (lane 3), or Morris hepatoma cells (lane 4) was hybridized to the riboprobe, indicated on Fig. 1*C* and analyzed on a 8% polyacrylamide gel. The GST-P mRNA is complementary to only a part of the riboprobe (from the cap site to boundary point of GST-P gene and CAT gene, see Fig. 1*C*), so that the protected bands are seen at approximately position 59. The arrow and the arrowhead indicate the intact probe and protected bands, respectively. Size marker(s) (terminally labeled *Hpa* II-digested pUC19 DNA) and riboprobe hybridized with yeast RNA are in lanes 1 and 5, respectively. (*B*) TPA response of the exogenously transfected GST-P–CAT fusion gene. E-CAT DNA, pSV2CAT DNA, and tk-CAT DNA were transfected into either dRLh84 cells (lanes 1–6) or HeLa cells (lanes 7–12). The amounts used in these transfections were 1 μg, 1 μg, and 5 μg for E-CAT, pSV2CAT, and tk-CAT, respectively. The total amount of DNAs was adjusted to 10 μg by addition of pUC18 DNA. Lanes: 1 and 7, E-CAT without TPA; 2 and 8, E-CAT with TPA; 3 and 9, pSV2CAT without TPA; 4 and 10, pSV2CAT with TPA; 5 and 11, tk-CAT without TPA; 6 and 12, tk-CAT with TPA.

DISCUSSION

Multiple Regulatory Elements. In this report we have identified multiple regulatory elements in the 5′ flanking region of the rat GST-P gene. An upstream enhancer is located between −2.5 and −2.2 kb and consists of at least two domains, one having a TRE and the other having core sequences of the viral enhancers. Another sequence at

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SacI
GAGCTCAGCTGCGCCTCAGCTGGTAAATGGATAAACTGTAGCAAAAGTAGTCAGTCACTATGATTCAGCAACAAAACCCT 80
                                                                  TRE
CACATCCACACATATCCCATCGGGTTTGTCCAGAGAAGCTGGCCCGCCTCCAGCACCCCCTTGCCTCCCCCGCCCCCCTC 160
                                  GPEI          GPEII
CCCCCCCTCCCCTTTTCTTCTTCCCCCTGGGAGTTTCCTGTTTATCTGAGTGTCACAGGCATGAGAACACACAGTTTGTG 240
GAACTGGAGTTCACCTCCCCCTGGGATTGGATTCCTTGGTGGGAAGACAAAATTCTCCAGCCTTCTTCATGACACTTTAG 320
SVE                                SVE
GAAGTCATTTCAGACAGGGCCTCGGTCTAC
PyE                          AccI
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FIG. 4. Nucleotide sequence of the upstream enhancer region of the GST-P gene. The nucleotide sequence of *Sac* I–*Acc* I (−2.5 kb to −2.15 kb) fragment is shown. The numbers on the right indicate the distance from the *Sac* I site. Two enhancer elements (GPEI and GPEII) are separated by opposite arrows. Palindromic sequences are shown by dashed arrows. The TRE, enhancer core-related sequence of the SV40 (SVE), and polyoma (PyE) are underlined.

position −61 is identical to the TRE found in human metallothionein IIA gene. A silencer-like element is located between 400 and 140 nt upstream from the cap site.

The upstream enhancer is very strong, even stronger than the SV40 enhancer in dRLh84, a hepatoma cell line. This element appears to work as a general enhancer, showing little tissue specificity to date. In transfection experiments, the constructs containing the upstream enhancers strongly expressed the downstream CAT gene with the GST-P promoter even in the cell lines that did not appreciably express the endogenous GST-P gene. These cells include rat 3Y1 cells, HepG2 cells (a human hepatoma cell line), and L cells (a mouse fibroblast cell line) (data not shown). GST-P mRNA is detected in a relatively wide range of normal tissues in the rat, such as lung, testis, kidney, placenta, and spleen, though not significantly in normal liver (7, 24). In addition, tissue distribution of this mRNA is significantly different in rats, humans, and mice (25). The low tissue specificity of the GST-P enhancer in the transfection experiments may explain, at least in part, such a wide distribution of the GST-P gene products. These arguments suggest to us that this gene may be regulated more significantly in a negative manner—e.g., in the liver. The silencer-like element on the 5′ flanking region of GST-P gene, then, may be rather important. Negative regulatory elements have been reported in several genes, including insulin (26, 27), α-fetoprotein (28), β-interferon (29), and the immunoglobulin heavy chain gene (30). There appears to be no sequence similarity between the GST-P silencer and silencers of other genes reported. The physiological functions of this element and the factors interacting with it remain to be elucidated.

Multiple regulatory elements are found in many genes, including the metallothionein genes (31, 32), the SV40 early genes (33), and the α-fetoprotein gene (34). In the metallothionein gene, for example, metal-responsive elements, glucocorticoid-responsive elements, and TREs are present in a tandem array, being activated by several kinds of agents. By analogy, the GST-P gene may also be responsive to a number of different stimuli and changes in physiological state.

TPA Responsiveness. The GST-P gene has two TREs at base pairs −2500 and −61 of the 5′ flanking region. The exogenously transfected GST-P gene is activated by TPA treatment as well as the endogenous GST-P gene. It has been speculated that alteration of gene expression by TPA involves a signal transduction cascade, triggered by protein kinase C (23), and, during this process, specific trans-acting factors are modified. It has also been reported that multiple factors are responsive to TPA treatment (35, 36). The TRE in the GST-P gene is homologous to the sequence that binds to AP-1. AP-1 is activated by TPA independent of protein synthesis. Our recent gel-mobility shift analysis shows that an upstream TRE fragment causes a retarded band that can be competed by the DNA fragments containing either a proximal TRE sequence of GST-P gene or a SV40 enhancer sequence (unpublished results). From these observations, one may speculate that one of the causes for the specific expression of GST-P gene during hepatocarcinogenesis is the activation of a protein kinase C-mediated signal cascade followed by AP-1 activation in an early stage of this process. In our preliminary observation, however, primary hepatocyte cells treated with TPA do not express the GST-P gene (unpublished results). This indicates that TPA activation alone is not sufficient for expression of the GST-P gene in rat liver.

In any event, it is certain that multiple DNA elements and factors are involved in GST-P gene expression. Further studies of factors interacting with these cis-acting elements are required for elucidation of the specific expression of this gene during hepatocarcinogenesis.

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