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GLUTATHIONE TRANSFERASE π
ITS MINIMAL PROMOTER AND DOWNSTREAM CIS-ACTING ELEMENT

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Fragments of the human glutathione S-transferase π gene and 15 kb of its 5' flanking region have been fused to the chloramphenicol acetyl transferase (CAT) reporter gene. Transfection into a number of human cell lines (Hela, HepG2, MCF7 and EJ) has demonstrated that the AP1 binding site, located between nucleotides -58 and -65 (Cowell et al. 1988, Biochem. J. 255, 79-83), is essential for basal level promoter activity. We have also identified a positive cis-acting DNA element between nucleotides +8 and +72 which seems to be part of the promoter. No other regulatory activity was identified within the 17 kb analyzed.

Glutathione S-transferases (GSTs) (EC. 2.5.1.18) are a group of enzymes concerned with the detoxication of electrophilic cytotoxins and genotoxins (1). The soluble GSTs of human and rat can be grouped into four families, alpha, mu, pi and theta, on the basis of the primary structure of their component subunits (1,2). The pi-class isozymes, namely GST π in human and GST 7-7 in rat, are particularly interesting because they are associated with malignancy (3,4) and also with cancer chemotherapeutic drug resistance (5,6). The gene encoding GST subunit 7 has been studied by Muramatsu and colleagues (7-9) who have identified multiple regulatory elements. In particular, there are two enhancer elements, GPEI and GPEII, which are localized 2.5 and 2.2 kilobases upstream from the transcription start site respectively. GPEI contains an AP1 binding site (TRE) and GPEII contains two enhancer sequences similar to those of Simian virus 40 and one similar to that of polyoma virus. The promoter region of the GST subunit 7 gene contains a G/C box, a TATA box and an additional consensus AP1 binding site.

The gene encoding GST π has also been sequenced (10,11). Its promoter region also contains a consensus AP1 binding site together with two G/C boxes and a TATA box. Studies have shown that the consensus AP1 binding site fails to respond to the phorbol ester, 12-O-tetradecanoyl-phorbol 13-acetate (TPA) and additional regulatory sequences upstream

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have yet to be characterized (12,13). In this paper, we have demonstrated a function for the consensus AP1 binding site. We have also identified a sequence downstream from the transcription start site which appears to be part of its promoter.

MATERIALS AND METHODS

Cell Culture and CAT Assays The cell lines HepG2 (derived from a human hepatocellular carcinoma) and EJ (a human bladder carcinoma) (12) were grown in DMEM. The human cervical carcinoma cell line, Hela was maintained in MEM and the human breast carcinoma cell line, MCF7 was cultured in RPMI 1640. All media were supplemented with 10% foetal calf serum and 10 ug/ml gentamycin sulphate. Transfection and chloramphenicol acetyl transferase (CAT) assays were carried out as described (14). Plasmid DNA used for transfection experiments was purified by two successive CsCl density gradient centrifugations. 30 pmoles plasmid DNA were used in each transfection together with pUC 13 DNA to make the total amount of DNA up to 10 ug. In cotransfection experiments, 15 pmoles of each plasmid were used. In the case of MCF7, transfection was carried out in DMEM, and the medium was changed back to RPMI 1640, four hours after transfection.

Plasmid Constructs Plasmids pSS0.2CAT and pSS0.1CAT have been described by Dixon et al.(12). pCR0.15CAT was constructed by the polymerase chain reaction (PCR) using pSS0.2CAT as template. The reaction conditions were as described in (15). The PCR primers used were as follows: Primer 1, 5'-GCGGAGCTCCAGCGAAGGCCTCGC-3', was made complementary to nucleotides -11 to +13 of the GST π gene with two nucleotides altered in order to generate an Sst I site at +5 to +10. Primer 2, 5'-CCGCATATGGTGCACTCTCA-3', was complementary to nucleotides 542 to 562, at the 5' end of the polylinker of pICCAT (12). The fragment generated by PCR was cleaved at its Hind III and Sst I sites, ligated into the multicloning site of pICCAT, thereby yielding pCR0.15CAT.
pCR0.25CAT was also constructed by PCR using pSS0.1CAT as template. Primer a, TGGAAGCTTTGAGCCCCA, was made complementary to nucleotides +63 and +80 with two nucleotides altered to generate a Hind III site at +72 to +77. Primer b was the same as primer 2 used for constructing pCR0.15CAT. The fragment generated by PCR was digested at its two Hind III sites and ligated into the Hind III site of pCR0.15CAT.

pBS-CATs were obtained by Bal 31 exonuclease digestion of Hind III restricted pSS0.2CAT, then blunt-ended with Klenow DNA polymerase and digested with Sst I. The small Sst I / blunt-end fragments were purified and ligated into Sma I / Sst I digested pICCAT. Appropriate constructs were selected by double-strand sequencing across the insert.

A 3.5 kb Hind III fragment (residues -4800 to -8300) was ligated into the Hind III site of pSS0.2CAT to generate pUH3.5CAT. A 5.2 kb Pst I fragment (residues -8300 to -13500) was blunt-ended, ligated with Hind III linkers and inserted into the Hind III site of pSS0.2CAT to construct pUP5.2CAT. A 1.8 kb Stu I / Hind III fragment (residues -8 to +1800) was ligated with Hind III linkers and cloned into the Hind III site of pSS0.2CAT to produce pDSH1.8CAT.

RESULTS

A Function for the Consensus AP1 Binding Sequence Previous work has shown that the promoter region of the GST π gene contains a TATA box, two G/C boxes (SP1 transcription factor binding sites) and a consensus AP1 binding site (-58 to -65) (12,13). Unlike rat GST subunit 7, GST π

failed to respond to the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (12,13). In order to investigate the function of the consensus AP1 binding site, the effect of its deletion on basal promoter activity was determined. A series of deletion mutants was constructed (Fig. 1) and tested by transient transfection into EJ, MCF7 and HepG2 cells, which differ in their endogenous GST π expression (12, G. Fraser, personal communication). pSV2CAT which contains the SV40 promoter, and pICCAT which has no promoter were used as positive and negative controls respectively. The patterns of CAT expression from pBS-CAT constructs were very similar in all three cell lines (Fig. 1) indicating the absence of cell-specific cis-acting elements. When pBS60CAT (containing only part of the consensus AP1 binding site), pBS46CAT (lacking one G/C box in addition to the consensus AP1 binding site) and pBS43CAT (lacking both G/C boxes as well as the consensus AP1 binding site) were transfected into cells, the CAT expression in each

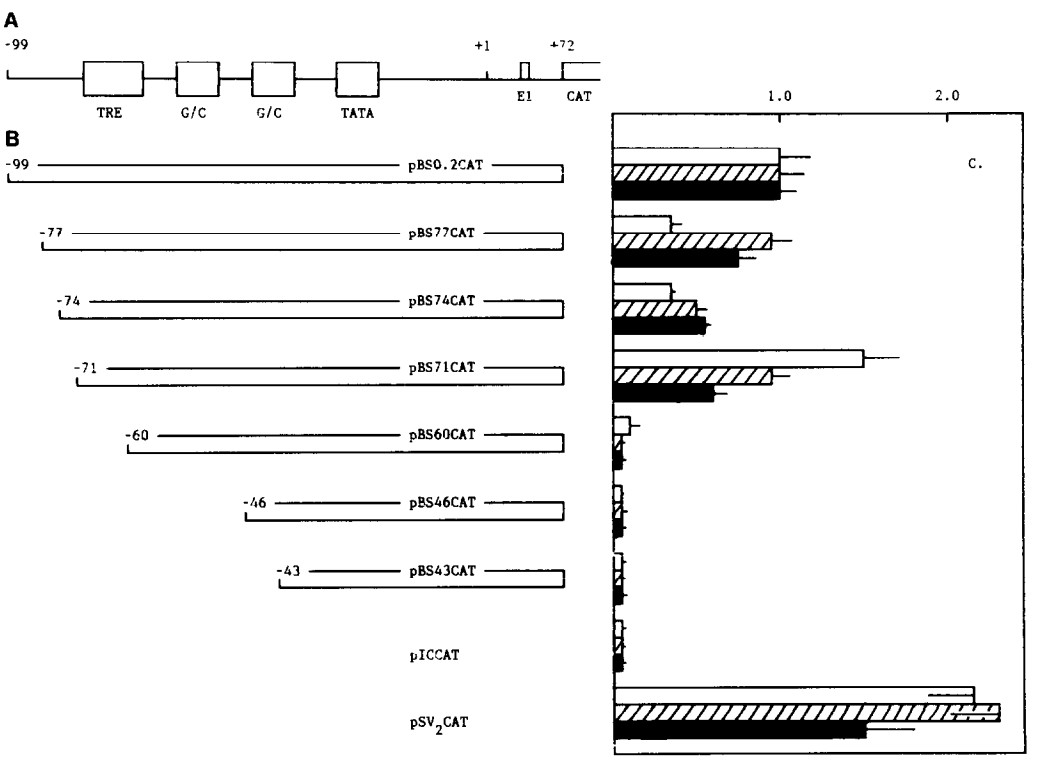
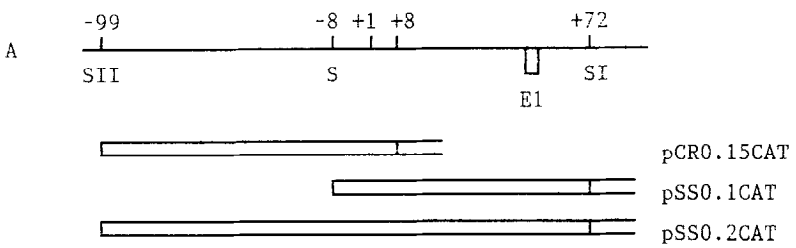


Fig. 1. Transfection of pBS-CATs into EJ, MCF7 and HepG2 cells. A) The map of GST π promoter region. Consensus TRE sequence, G/C boxes and TATA box are indicated. Exon 1 is indicated as box E1. **B)** The schematic representation of 5' deletion constructs which were generated by Bal31 digestion and cloned into the multicloning site of pICCAT directionally. **C)** The relative CAT expression of pBS-CATs in EJ cells (depicted by open bar), MCF7 cells (depicted by striped bar) and HepG2 cells (depicted by filled bar). For every construct, each bar represents the mean of nine to twelve determinations.

case was very similar to the background obtained with pICCAT. This indicates that the consensus AP1 binding site is essential for the basal level promoter activity of GST π in all three cell lines.

Identification of the Downstream cis-acting Element of the GST π Gene

The fragments used previously for basal promoter activity by Dixon et al. and Morrow et al. were nucleotides -99 to +72 or -80 to +314 respectively (12,13). In order to determine whether the downstream region (including nucleotides +8 to +72) is involved in modulating the transcription, three GST π-CAT fusion genes (Fig. 2) were tested by transfection into cultured cells and compared with the activities of pSV2CAT and pICCAT. Hela, HepG2, EJ and MCF7 cell lines were used. Transfection of pCR0.15CAT (nucleotides -99 to +8, 'minimal promoter')



B

	Hela	HepG2	MCF7	EJ
pICCAT	0.04±0.01	0.08±0.06	0.08±0.03	0.10±0.02
pSS0.1CAT	0.05±0.01	0.08±0.05	0.08±0.01	0.10±0.04
pCR0.15CAT	1.00±0.20	1.00±0.13	1.00±0.10	1.00±0.10
pSS0.2CAT	4.00±1.00	5.40±2.70	2.90±0.31	3.90±0.08
pSV2CAT	26.0±4.80	6.90±2.40	4.08±0.70	7.20±0.48

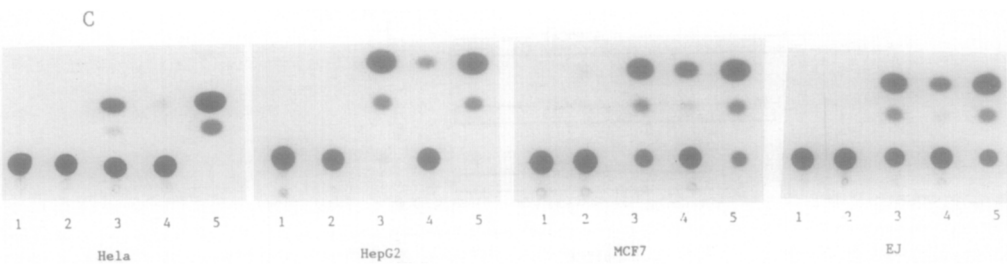


Fig. 2. A). GST π-CAT Chimeric Constructs Sites for Sst I (SI), Stu I (S), and Sst II (SII) are shown. Exon 1 is shown as box E1. The fragments shown were either isolated from restriction enzyme digestion or generated by PCR and were cloned directionally into the multicloning sites of pICCAT. **B). Relative CAT Activities Generated by GST π Chimeric Gene Constructs** Values were calculated as percentage of chloramphenicol acetylation, corrected for protein concentration and are expressed relative to the activity obtained with pCR0.15CAT containing the GST π promoter. Transfections were carried out in triplicate and the results are means of three independent experiments. **C).** CAT assay with HepG2, Hela, MCF7 and EJ cells transfected with GST π-CAT chimeric constructs. Transient assays were performed in all four cells using pSV2CAT (lane 5), pCR0.15CAT (lane 4), pSS0.2CAT (lane 3), pSS0.1CAT (lane 2) and pICCAT (lane 1).

resulted in CAT activities significantly above those obtained with pICCAT in all four cell lines tested. However, CAT transcription from pSS0.2CAT (nucleotides -99 to +72) gave results which were 2.9-5.4 fold higher than those from pCR0.15CAT. CAT activities expressed from pSS0.1CAT (nucleotides -8 to +72) were the same levels as the background generated from pICCAT (Fig. 2). When pSS0.2CAT was cotransfected with pSS0.1CAT into HepG2, EJ and MCF7 cell lines, the CAT activities observed were 2.8-4.8 fold lower than those cotransfected with pICCAT (Fig. 3). These results indicate that there is a cis-acting regulatory element located in the sequence +8 and +72 downstream of the transcription start site, which is functional in all four cell lines examined. The observation that the cotransfection of pCR0.15CAT and pSS0.1CAT, which has little overlapping sequence, gave CAT activities 4.6-7.0 fold lower than those of pCR0.15CT and pICCAT (Fig. 3), indicates that the pSS0.1CAT competes with one or more factors essential for pCR0.15CAT activity. This suggests that the trans-acting factor, which binds to the downstream cis-acting element, is related to a transcription factor active in the minimal promoter.

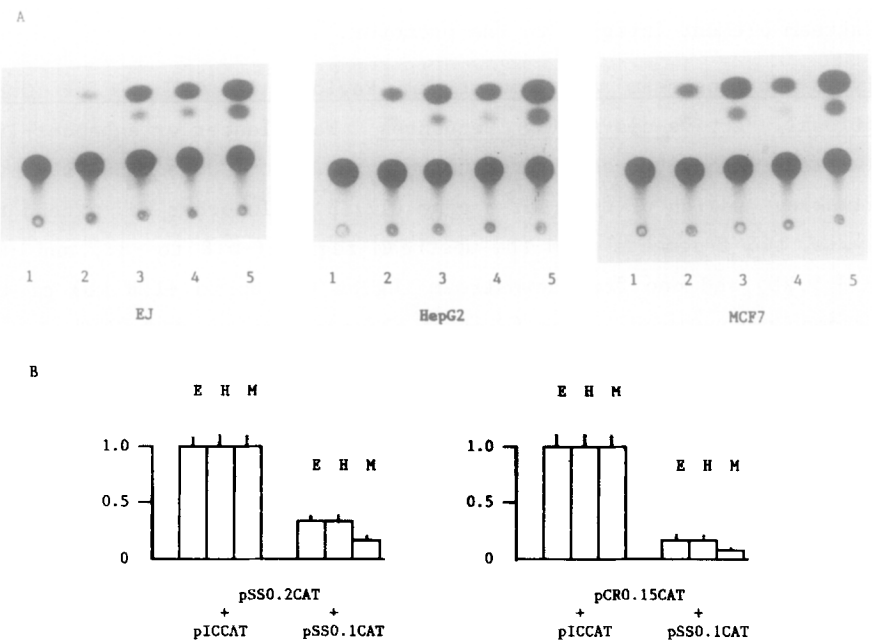


Fig. 3. The Effects of Cotransfection with pSS0.1CAT on pSS0.2CAT and pCR0.15CAT. A) pSS0.2CAT and pCR0.15CAT were cotransfected with pICCAT (lanes 5,3) and pSS0.1CAT (lanes 4,2) into EJ, HepG2 and MCF7 cells. pSS0.1CAT was cotransfected with pICCAT as a negative control. **B)** The CAT gene expression of pSS0.2CAT and pCR0.15CAT are shown as activity relative to the pICCAT cotransfection control. The results were obtained in triplicates from two independent experiments.

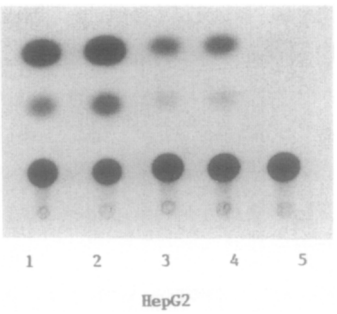


Fig. 4. CAT Assay with HepG2 Cells Transfected with GST π -CAT Chimeric Constructs. Transient assays were performed using pSV2CAT (lane 1), pSS0.2CAT (lane 2), pCR0.25CAT (lane 3), pCR0.15CAT (lane 4) and pICCAT (lane 5).

In order to determine whether the downstream cis-acting element is either an enhancer or part of the promoter, a construct (pCR0.25CAT) was made by inserting nucleotides -8 to +72 in front of the minimal promoter and was tested by transfection into HepG2 cell (Fig. 4) along with pCR0.15CAT and pSS0.2CAT. The level of CAT transcription from pCR0.25CAT was much lower than that from pSS0.2CAT and was about the same level from pCR0.15CAT. This indicates that the activity of the element located between -8 and +72 is position-dependent and thus is a downstream element integral to the promoter.

Analysis of Upstream and Downstream Regions of GST π for Other Functionally Active cis-Acting Sequences No silencer or enhancer-like activities have yet been located between -80 and -6000 basepairs in the 5' flanking region of the GST π gene (12,13). In order to extend the analysis, two fragments from the upstream region (-4.8 to -8.3 and -8.3 to -13.5 kb) and one from downstream region (+8 bp to +1.8 kb) of the GST π gene were placed in the front of the promoter of pSS0.2CAT plasmid and the levels of CAT expression were determined in EJ, HepG2 and Hela cells. The CAT expression from these constructs was not significantly different from that of the control (pSS0.2CAT) (data not shown), showing that no regulatory element was detectable in these fragments.

DISCUSSION

Many genes are responsive to the transcriptional activation of TPA (16,17) via the recognition of a consensus AP1 binding site by a number of related trans-acting factors such as Jun and Fos. However, in the case of the consensus AP1 binding site present in the GST π promoter region, both Dixon et al and Morrow et al (12,13) have shown that it is unresponsive to the transcriptional activating effects of TPA and

insensitive to the above regulatory factors. However, by means of transient expression of 5' deletion mutants of the GST π promoter from -71 to -43, we have shown that the integrity of the AP1 binding site is essential for the basal activity of the GST π promoter. These paradoxical results may be due to the fact that some consensus AP1 binding sites can also be bound by trans-acting factors from the CREB/ATF families (16) in addition to the Fos/Jun family. A further complexity is that the deletions in the sequence -99 to -71 result in some modest changes of downstream CAT expression (Fig. 1). There is therefore the possibility that weak enhancing and silencing elements are present adjacent to the consensus TRE sequence. However, another possibility of aberrant results arising from contextual differences particular to each construct is now under investigation.

Studies have shown that in each cell type, the transient expression obtained with pCR0.15CAT (-99 to +8 nucleotides) was much lower than that obtained with pSS0.2CAT (-99 to +72 nucleotides) implying that a transcriptionally active sequence is present at nucleotides between +8 to +72. The finding that cotransfection of pSS0.2CAT with pSS0.1CAT (-8 to +72 nucleotides) resulted in lower CAT expression than that with pICCAT supports this supposition. Transfection of pCR0.25CAT (contains nucleotides -99 to +8 and -8 to +72 in reverse), which yielded CAT activity of the same level as that of pCR0.15CAT, suggests that the sequence present at nucleotides -8 to -72 is part of the promoter.

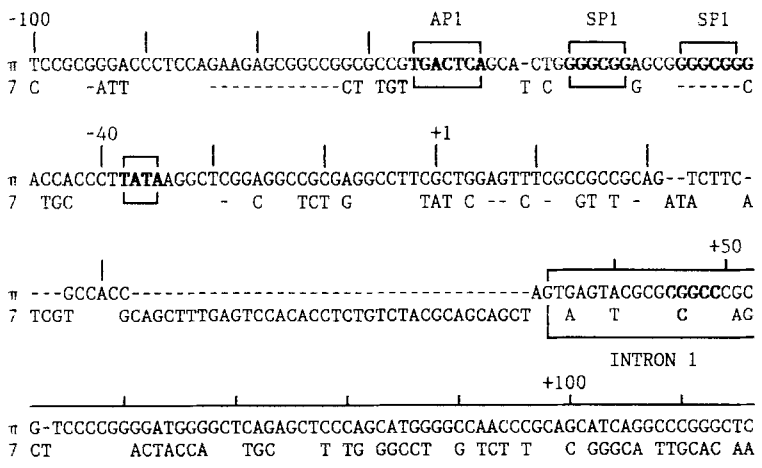


Fig. 5. Comparison of the Promoter Region, Exon 1 and Intron 1 of the Human and Rat GST pi Genes. Top sequence, human GST π gene; bottom sequence, rat subunit 7 gene. Only bases which differ from those of human gene are shown for the rat gene. Gaps "-" are introduced for maximum alignment. Numbering refers to the nucleotide position relative to the transcription initiation site (No. 1) of the GST π gene. The TATA box, G/C box core motifs, consensus AP1 binding site and intron 1 are boxed.

Sequence comparison reveals that the rat subunit 7 and human GST π gene are 73% conserved in the first 26 basepairs of intron 1, but there is no homology in exon 1 (9,10,18, Fig 5). Interestingly, there is a G/C box (consensus SP1 binding site) positioned in rat subunit 7 intron 1 between +83 and +88, while in GST π intron 1, there is a G/C box-like sequence (CGGCCC) located at the similar position (+46 and +51).

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