Sp1-mediated Transcriptional Activation of the Human Pi Class Glutathione S-Transferase Promoter*

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Previous studies in this laboratory have identified an essential AP-1 recognition sequence (C1 region; −69 to −63) in the human Pi class glutathione S-transferase (GSTP1) promoter and a negatively acting regulatory element (−105 to −86) that acts to suppress GSTP1 transcription in the human mammary carcinoma cell line, MCF7 (1). The data presented here further delineate the functional characteristics of the GSTP1 promoter by examining the significance of two potential binding sites for the transcription factor, Sp1 (−57 to −49 and −47 to −39). The introduction of mutations within these Sp1-like elements and the use of Sp1 antisera in electrophoretic mobility shift assays demonstrated that Sp1 was bound to this region of the GSTP1 promoter in three different cell lines, MCF7, VCREMS, and EJ. Moreover, these in vitro studies indicated that only one of the two putative Sp1 response elements was utilized. Transient transfection assays using GSTP1 promoter constructs that incorporated mutations of the Sp1 elements clearly demonstrated that binding of Sp1 to the GSTP1 promoter was absolutely required for optimal levels of GSTP1 transcription. In particular, disruption of the distal Sp1 recognition motif (−57 to −49) markedly reduced GSTP1 promoter activity in each cell line, thus indicating preferential binding of Sp1 to the distal site. However, insertion of the repressor binding site (−105 to −86) into these constructs suggested that Sp1 was not involved in mediating the suppressive effects of the GSTP1 transcriptional repressor in MCF7 cells, because inhibition of Sp1 binding did not alleviate repressor activity. Therefore, these studies provide strong evidence that Sp1 plays a central role in regulating basal levels of GSTP1 transcription.

Glutathione S-transferases (GSTs) are an important family of enzymes primarily responsible for the detoxification of a large number of electrophilic xenobiotics by catalyzing the nucleophilic conjugation of these compounds with glutathione. There are five mammalian subclasses of GSTs, namely Alpha, Pi, Mu, Theta, and microsomal, with different classifications of substrate specificity (2, 3).

Overexpression of the Pi class of GST enzymes has been associated with tumor development and carcinogenesis (4–9) and in the acquisition of antineoplastic drug resistance (10–13). Therefore, understanding the transcriptional regulatory mechanisms of this particular gene family has stimulated much research activity.

In this regard, functional studies of the rat GSTP promoter have identified several regulatory elements that are important for basal and inducible expression (14–18). Moreover, preliminary analysis of the human GSTP1 promoter had identified a putative AP-1 response element (19–23).

Recent studies in this laboratory have established that the AP-1 recognition sequence is absolutely required for transcriptional activity of the GSTP1 promoter (1). The transcription factor AP-1 is comprised of members of the Jun (c-Jun, J unB, and J unD) and Fos (c-Fos, FosB, Fra1, and Fra2) protein families (24, 25). We have demonstrated that a Jun-Fos heterodimer is an integral component of the nuclear complex bound to this essential GSTP1 promoter element in a multidrug-resistant derivative (VCREMS) of the human mammary carcinoma cell line, MCF7. In addition, we have identified a negative regulatory element (−105 to −86) that acts to suppress GSTP1 transcription in MCF7 cells (1).

Analysis of the GSTP1 proximal promoter revealed the presence of two putative Sp1 binding sites (−57 to −49 and −47 to −39) located downstream of the AP-1 response element. The transcription factor Sp1 was first identified as a regulatory protein that bound multiple GC-rich boxes in the 21-bp repeat elements of the SV40 early promoter (26–30). Since this initial observation, Sp1 has been shown to regulate the activity of a large number of viral and cellular promoters. The DNA binding domain of Sp1 consists of three zinc finger motifs (31) and recognizes the 9-bp sequence GGGCGGGG, in which each triplet is thought to be contacted by one of the three zinc fingers (32–35).

These present studies examine the ability of these potential Sp1 response elements in the GSTP1 promoter to bind Sp1 in vitro. In addition, the functional significance of these DNA-protein interactions in regulating GSTP1 promoter activity is addressed. Finally, we investigate the involvement of Sp1 in mediating the suppressive effects of the GSTP1 transcriptional repressor in MCF7 cells.

EXPERIMENTAL PROCEDURES

Cell Culture—The VCREMS cell line (a generous gift from Dr. Bridget Hill) was derived by selecting the human mammary carcinoma cell line, MCF7, for resistance to vincristine (36). VCREMS, MCF7, the human bladder carcinoma cell line, EJ, HepG2, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and supplemented with l-glutamine and penicillin/streptomycin mixture (Life Technologies, Inc., Paisley, Scotland).

Northern Analysis—Total cellular RNA was isolated from MCF7, VCREMS, EJ, HepG2, and HeLa cells using the procedure described by Chomczynski and Sacchi (37). RNA (10 μg/lane) was electrophoretically fractionated and transferred to Hybond-N nylon membrane (Amersham International, Amersham, UK). A full-length (800 bp, EcoRI-digested) GSTP1 cDNA fragment (38) was labeled by the random priming method.
(39) and used to determine the relative levels of GSTP1 mRNA in each cell line. Northern blots were washed to high stringency: 0.1 × SSC containing 0.1% SDS at 65 °C for 30 min. Autoradiography was performed using X-Omat AR film (Eastman Kodak) and two intensifying screens at −70 °C.

Electrophoretic Mobility Shift Assay—Nuclear extracts from MCF7, VCREMS, and EJ cells were prepared using the method described by Dignam et al. (40). Electrophoretic mobility shift assays were performed as described previously (1). Briefly, 10 μg of MCF7, VCREMS, or EJ nuclear extract was incubated for 20 min at room temperature with 2 ng of [γ-32P]ATP end-labeled DNA fragment in a 20-μl reaction mixture containing 12 mM HEPES, pH 7.9, 12% glycerol, 60 mM KCl, 0.12 mM EDTA, 1 mM dithiothreitol, and 2 μg of poly(dI-dC). Each reaction mixture was then loaded onto a prerun (200 V for 2 h at 4 °C) 4% polyacrylamide gel (301 cross-linking ratio) containing 0.3 × TBE. Electrophoresis was performed at 200 V for 2 h at 4 °C, and the gel was then dried and autoradiographed.

In competition experiments, the reaction mixture was preincubated for 20 min at room temperature with 100-fold molar excess of unlabeled DNA for 3 μl of polyclonal anti-human Sp1 antisera; a generous gift from Dr. Stephen Jackson) before the addition of radiolabeled probe.

The following oligonucleotides and their complementary sequences were used as probes and competitors: the GSTP1 promoter fragment −61 to −32, 5′-CACTGGGAGCGGCGGGGACACCCT-3′; 5′-CACGGCCAGGCGGAGCGGGACCACCCT-3′ (M1); 5′-CACTGGAGCGGCGGGGACACCCT-3′ (M2); 5′-CACGGCGGGAGCGGGACCACCCT-3′ (M3); 5′-CACTGGAGCGGCGGGGACACCCT-3′ from the SV40 early promoter (32) and the AP-1 binding site from the human collagen promoter, 5′-AGCTTGTAGTACCGGAC-3′ (41).

Promoter Deletion Constructs—The GSTP1 promoter deletion consensus p105CAT, p73CAT, and p65CAT were prepared as described previously (1) by ligating deletion fragments of the GSTP1 promoter (−105 to +36, −73 to +36, and −65 to +36 with HindII and SalI linkers) into the HindII/SalI-digested pCAT.Basic vector (Promega, Southamptom, UK).

Using p73CAT as a template, mutated GSTP1 promoter fragments were generated by PCR using the following upstream primers (with HindII linkers): 5′-CGGCGGAGCGGCGGGGACACCCT-3′ (p73Sp1M1CAT), 5′-CCTGGAGCGGCGGGGACACCCT-3′ (p73Sp1M2CAT), and 5′-CACTGGAGCGGCGGGGACACCCT-3′ (p73Sp1M3CAT). The ability of these potential recognition sequences in the promoter region, the three p73CAT and three p105CAT mutant constructs was revealed three potentially important features that are high-lighted in Fig. 2, namely one putative AP-1 binding site (−69 to −63) and two sequences homologous to Sp1 recognition elements (−57 to −49 and −47 to −39). Previous studies in this laboratory have demonstrated a functional role for the AP-1 response element, which binds a Jun-Fos heterodimer in VCREMS cells (1). However, the importance of the two putative Sp1 binding sites had not been investigated.

The ability of these potential recognition sequences in the GSTP1 promoter to bind Sp1 was assessed by electrophoretic mobility shift assays. For these studies, four double stranded DNA probes spanning the GSTP1 promoter region, −61 to −32, were prepared (Fig. 3A). The wild-type sequence (−61 to −32) contained both of the Sp1-like elements. M1 contained a 4-bp mutation in the distal site (−57 to −49), whereas a 4-bp mutation was introduced into the proximal site (−47 to −39) to generate M2. In addition, M3 contained 4-bp mutations in both elements. The ability of each of these four GSTP1 promoter fragments to bind proteins in nuclear extracts prepared from MCF7, VCREMS, and EJ cells was then tested.

Fig. 3B shows that the wild-type sequence bound three major complexes. Interestingly, both the mobility pattern and the relative intensity of these bands were similar in each cell line. Despite the mutations described above, M1 and M2 both gave the same result as the wild-type sequence. This finding suggests that perhaps sequences flanking the Sp1 motifs were responsible for the observed band shift pattern or alternatively that only one of the two Sp1 elements was occupied at one time. The latter explanation seemed more likely because M3, which contained specific mutations in both of the Sp1-like elements, failed to bind any nuclear proteins.
A

**Spl binding site**

\[
\text{CATGCGCCGGGAGCGGCGGCCGCCACCT} \quad \text{Spl binding site}
\]

**SplM1**

\[
\text{CATGCGCCGGGAGCGGCGGCCGCCACCT}
\]

**SplM2**

\[
\text{CATGCGCCGGGAGCGGCGGCCGCCACCT}
\]

**SplM3**

\[
\text{CATGCGCCGGGAGCGGCGGCCGCCACCT}
\]

B

| MCF7 | VCREMS | EJ |
|------|--------|----|
| M    | V      | E  |
| M    | V      | E  |
| M    | V      | E  |

**GSTP1 promoter fragment, -53 to -32**

**Sp1 consensus sequence**

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Fig. 3. *In vitro* binding of nuclear proteins to the GSTP1 promoter fragment -61 to -32. A, mutational analysis of the two potential Sp1 binding sites located within the GSTP1 promoter. B, electrophoretic mobility shift assays demonstrating the nuclear complexes (10 μg of nuclear extract per reaction) in MCF7, VCREMS, and EJ cells that bound the GSTP1 promoter fragment -61 to -32 and the mutated fragments M1, M2, and M3. C, electrophoretic mobility shift assays demonstrating the nuclear complexes (10 μg of nuclear extract per reaction) in MCF7, VCREMS, and EJ cells bound to a single Sp1 binding site. The GSTP1 promoter fragment -53 to -32 and the Sp1 consensus sequence from the SV40 early promoter were used as probes. WT, wild type.

To further emphasize this possibility, a GSTP1 promoter fragment spanning nucleotides -53 to -32 and thus containing only the proximal Spl motif and a probe containing a single Spl consensus sequence from the SV40 early promoter, produced the same results as the wild-type sequence, which contained two potential Spl binding elements (Fig. 3C).

DNA Binding Specificity of the Nuclear Complexes Bound to the GSTP1 Promoter Fragment -61 to -32—To determine the binding specificity of these nuclear proteins, the ability of M1, M2, and M3 to compete for the three complexes bound to the wild-type sequence was assessed (Fig. 4). Consistent with results shown in Fig. 3B, a 100-fold molar excess of unlabeled M1 or M2 was able to efficiently compete for the nuclear proteins bound to the wild-type sequence, whereas no competition was observed with excess unlabeled M3.

Furthermore, efficient competition for the three nuclear complexes was observed with the single Spl consensus sequence from the SV40 early promoter but not with the nonspecific AP-1 binding site from the human collagenase promoter. As shown for the experiments presented in Fig. 3 (B and C), identical results were observed in all three cell lines.

Sp1 Polyclonal Antisera Inhibited Binding of Two of the Nuclear Complexes Bound to the GSTP1 Promoter Element -61 to -32—Results presented above have demonstrated that three major complexes were specifically bound to the GSTP1 promoter region -61 to -32. However, despite the presence of two sequence motifs exhibiting strong homology with an Spl binding site, this data did not prove that Spl was represented by any of these nuclear complexes.

To clarify this matter, nuclear extracts were incubated with a polyclonal Spl antibody for 20 min before the -61 to -32 probe was added to the reaction. Fig. 5 clearly shows that the Spl antibody disrupted binding of the complexes I and II in all three cell lines, although the high mobility complex III was unaffected. Importantly, preimmune sera did not interfere with the DNA binding activity of any of the three complexes. These results conclusively demonstrated that Spl was an integral component of complexes I and II in MCF7, VCREMS, and EJ cells. However, the protein composition of complex III remains unknown.

In the Absence of the AP-1 Element, the Two Spl Binding Motifs Were Insufficient to Support GSTP1 Transcription—To assess the functional importance of these Spl binding sites for GSTP1 transcription, transient transfection assays were performed with the GSTP1 promoter deletion constructs, p73CAT and p65CAT (containing the GSTP1 promoter fragments, -73 to +36 and -65 to +36, respectively). p73CAT contained an intact AP-1 response element (-69 to -63) as well as the two Spl motifs, but the AP-1 site had been disrupted in p65CAT. Fig. 6 clearly shows that p73CAT was strongly active in MCF7, VCREMS, and EJ cells. However, in all three cell lines, loss of the AP-1 element abolished transcriptional activity of the GSTP1 promoter. This result clearly indicated that the two Spl motifs, in the absence of the AP-1 element, were not sufficient for GSTP1 transcription.

However, the role played by the two Spl binding sites in contributing to the high level of CAT activity observed for p73CAT was not clear, i.e. could the essential AP-1 element still confer optimal GSTP1 promoter activity? To clarify this matter, nuclear extracts were incubated with the Spl antibody, and results of these experiments showed that in all three cell lines, mutation of the distal Spl site (Fig. 7). The results of these experiments showed that in all three cell lines, mutation of the distal Spl site (-57 to -49; p73Sp1M1CAT) reduced GSTP1 promoter activity by 45, 57, and 78% in MCF7, VCREMS, and EJ cells, respectively. In contrast, mutation of the proximal binding motif (-47 to -39; p73Sp1M2CAT) had only a relatively marginal effect on GSTP1 transcription. Consistent with these results, mutation
of both Sp1-like elements (p73Sp1M3CAT) produced effects on GSTP1 promoter activity similar to those of mutation of the distal site alone.

Taken together with the in vitro binding data, these results demonstrated that in MCF7, VCREMS, and EJ cells, Sp1 was preferentially bound to the distal Sp1 recognition sequence and that this interaction was required for optimal activity of the GSTP1 promoter.

Loss of Sp1 Binding to the GSTP1 Promoter Did Not Inhibit GSTP1 Transcriptional Repressor Activity—Previous studies in this laboratory have identified a negative regulatory element (−105 to −86) in the GSTP1 promoter, which acts to suppress transcription of the GSTP1 gene in MCF7 cells (1). From the data described above, we have shown that Sp1 interaction with the distal Sp1 recognition sequence and that this interaction was required for optimal activity of the GSTP1 promoter. However, the ability of Sp1 to mediate upstream transcriptional events on GSTP1 promoter activity remained unclear. In this regard, we investigated the effect of the loss of Sp1 binding on repressor activity.

These experiments were performed by introducing the mutations M1, M2, and M3 (see Fig. 3A) into the GSTP1 promoter fragment −105 to +36 and subcloning the resultant three mutants into pCAT.Basic. Transient transfection assays were performed in MCF7 cells and the promoter activities of the mutant constructs, p105Sp1M1CAT, p105Sp1M2CAT, and p105Sp1M2CAT were compared with that of p105CAT (Fig. 8).

Similar to results presented in Fig. 7, the CAT activities of p105Sp1M1CAT and p105Sp1M3CAT were significantly reduced, whereas the activity of p105Sp1M2CAT was relatively unaffected compared with the wild-type construct. These results show that loss of Sp1 binding did not inhibit GSTP1 transcriptional repressor activity. Therefore, it appeared unlikely that a protein-protein interaction between Sp1 and the repressor was required to confer the suppressive effects on GSTP1 transcription.

DISCUSSION

These studies have clearly demonstrated that interaction of Sp1 with the GSTP1 promoter is required for basal transcription of the GSTP1 gene. Indeed, in the absence of Sp1 binding, the GSTP1 promoter exhibited only 17–40% optimal activity in three different cell lines. Moreover, by mutational analysis, we have shown that the distal Sp1 binding motif (−57 to −49) was functionally more important than the proximal element in terms of regulating basal levels of GSTP1 transcription. However, despite the requirement for Sp1, disruption of the adjacent C1 element deemed the GSTP1 promoter inactive. Therefore, it appeared unlikely that a protein-protein interaction between Sp1 and the repressor was required to confer the suppressive effects on GSTP1 transcription.
fore, the presence of Sp1 binding sites alone were insufficient to support GSTP1 transcription.

In general, sequence-specific transcription factors can be classified into two main categories: proximal promoter factors, which only function close to the transcriptional start site, and enhancer-binding proteins, which can exert their effects over much larger distances (48). Due to the fact that functional Sp1 binding sites are generally found within a few hundred nucleotides of the transcriptional start site, Sp1 has been placed in the former category (28). Moreover, cloning of Sp1 sites far upstream results in a marked decrease in promoter activity (49). These findings have led investigators to conclude that the primary function of Sp1 is to regulate basal levels of transcription. In this regard, recent advances have established that Sp1-mediated transcription has been shown to involve an interaction of the glutamine-rich activation domain of Sp1 with the TAF\textsubscript{i110} component of the basal TFIIID complex (50–52).

However, it has also been proposed that proximally bound transcription factors mediate the regulatory effects of enhancer-binding proteins, i.e. the two classes of control factors activate transcription synergistically. This idea was originally formulated from observations that Sp1 interactions with the SV40 early promoter mediated activity of the SV40 enhancer (53, 54). Interestingly, Sp1 recognition motifs are often found located near binding sites for other transcription factors such as CTF/NF-1 (55), AP-1 (56), NF\textsubscript{k}B (57), and the sterol regulatory element-binding protein, SREBP (58). Indeed, synergistic activation of the HIV promoter by NF\textsubscript{k}B and Sp1 (57) and of the low density lipoprotein receptor gene by SREBP and Sp1 (58) have recently been demonstrated.

Our results have indicated that a similar mechanism may account for Sp1-mediated activation of the GSTP1 promoter. In the absence of Sp1 binding, the essential C1 element was only able to confer 17–40% optimal activity on the GSTP1 promoter. Conversely, in the presence of the Sp1 binding motifs, disruption of the C1 element rendered the GSTP1 promoter inactive. These data strongly indicate that basal transcriptional activity of the GSTP1 promoter is mediated by a synergistic mechanism requiring both Sp1 and the C1 nuclear complex. Given the critical importance of these two elements, the above conclusion suggests that cooperativity between these two complexes would mediate changes in transcriptional rates of the GSTP1 promoter.

In this regard, it was important to examine the role of Sp1 in controlling GSTP1 transcription in three cell lines, MCF7, VCREMS, and EJ, which exhibited widely different levels of GSTP1 gene expression. However, in the presence of the Sp1 binding motifs, disruption of the C1 element rendered the GSTP1 promoter inactive. These data strongly indicate that basal transcriptional activity of the GSTP1 promoter is mediated by a synergistic mechanism requiring both Sp1 and the C1 nuclear complex. Given the critical importance of these two elements, the above conclusion suggests that cooperativity between these two complexes would mediate changes in transcriptional rates of the GSTP1 promoter.
Regulation of GSTP1 Gene Expression

Many studies have indicated that Sp1 is not involved in determining cell-specific differences in the constitutive level of GSTP1 gene expression, our data do not exclude the possibility that changes in Sp1 binding to the GSTP1 promoter may be important in mediating transient alterations in GSTP1 transcription. In this regard, our studies have indicated that only one of the two potential Sp1 binding sites was preferentially utilized in three different cell lines. However, occupation of multiple GC boxes has been shown to synergistically activate transcription (62). As stated above, the major function of GSTP1 inside the cell is to provide a detoxification mechanism in its role as an important drug metabolizing enzyme. Therefore, it is intriguing to perceive an involvement for the proximal Sp1 element in the transient induction of GSTP1 transcription in response to xenobiotics.

In summary, the data presented in this report have highlighted an absolute requirement for Sp1 in maintaining activity of the GSTP1 promoter. Moreover, our results have provided strong evidence that Sp1 is principally involved in regulating basal transcription of the GSTP1 gene but does not appear to mediate directly the different steady state levels of GSTP1 mRNA in VCREMS, and EJ cells.

However, it is interesting to note that recent developments have characterized several other transcription factors that can bind to the GC-rich Sp1 binding motif. Evidence suggests that these regulatory proteins can compete for this promoter element to either potentiate (eg. EGR-1 (63)) or repress (eg. Sp3 (64)) Sp1-mediated transcriptional activation. Therefore, further work is required to determine whether similar control mechanisms can modulate GSTP1 gene expression in a coordinated stress response to dramatic but transient changes in the intracellular environment.

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