Transcriptional and post-transcriptional mechanisms can regulate cell-specific expression of the human Pi-class glutathione S-transferase gene

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Previous studies from this laboratory have identified transcriptional mechanisms that are utilized to increase expression of the human glutathione S-transferase gene *GSTP1* in a multidrug-resistant derivative (VCREMS) of the human mammary carcinoma cell line MCF7 [Moffat, McLaren and Wolf (1994) J. Biol. Chem. 269, 16397–16402]. The data presented here provide strong evidence that post-transcriptional mechanisms can also play an important role in determining cell-specific expression of the *GSTP1* gene. *GSTP1* mRNA levels were shown to be elevated 3.1-fold in the human bladder carcinoma cell line EJ compared with VCREMS cells. Despite this observation, transient transfection assays revealed a decreased rate of *GSTP1* promoter activity in EJ cells. Indeed, *GSTP1* transcriptional repressor activity, mediated by a region located between nucleo-

tides –105 and –86 (as we have previously described in MCF7 cells), was observed in EJ cells. However, in contrast with our results in MCF7 cells, the EJ repressor activity did not displace the essential nuclear complex bound to the C1 promoter element (–73 to –54) *in vitro*. In addition, competition experiments indicated that an AP-1-like protein is an integral component of the C1-bound complex in EJ cells. Interestingly, experiments utilizing actinomycin D to inhibit transcription demonstrated significantly greater stability of *GSTP1* mRNA in EJ cells than in VCREMS cells. These findings suggest that cell-specific differences in the rates of *GSTP1* mRNA decay provide the predominant mechanism responsible for elevated expression of the *GSTP1* gene in EJ cells.

INTRODUCTION

Glutathione S-transferases (GSTs) are important phase II drug-metabolizing enzymes that catalyse a detoxification mechanism involving the nucleophilic attack of glutathione on a wide range of electrophilic xenobiotics. Mammalian GSTs comprise five gene families: Alpha, Pi, Mu, Theta and microsomal, with each form exhibiting a degree of substrate specificity [1].

Understanding the regulatory mechanisms controlling expression of the GST Pi-class enzymes has become a major research focus since the observation that the *GSTP* gene is expressed at high levels in preneoplastic foci of rat liver. Moreover, expression of the rat *GSTP* gene remains elevated as the tumours develop and, therefore, increased GSTP levels are recognized as important markers of rat hepatocarcinogenesis [2–7]. In addition, there are a number of reports linking increased human *GSTP1* gene expression with the development of resistance to antineoplastic drugs [8–11].

In this regard, we have described the transcriptional mechanisms involved in up-regulating *GSTP1* gene expression in a multidrug-resistant derivative (VCREMS) of the human mammary carcinoma cell line MCF7 [12–14]. In these previous studies, we identified an essential promoter element, C1 (nucleotides –73 to –54), which bound the transcription factor AP-1 in VCREMS but not in MCF7 cells. Furthermore, we have located a negative regulatory element (nucleotides –105 to –86) that acted to suppress *GSTP1* transcription in MCF7 cells.

In the course of our studies, we have examined *GSTP1* gene expression in a number of cell lines. Of all the cell types tested thus far, the human bladder carcinoma cell line EJ expressed the highest level of GSTP1. Indeed, our results show that *GSTP1* mRNA levels within EJ cells exceed those found in VCREMS

cells by more than 3-fold. To help increase our understanding of the molecular mechanisms responsible for *GSTP1* overexpression in EJ cells, we examined *GSTP1* promoter activity and the stability of *GSTP1* mRNA in both the EJ and VCREMS cell lines. These studies have clearly demonstrated a role for mRNA stabilization in mediating the basal level of *GSTP1* gene expression in these two cell lines. Moreover, these data provide further insight into the regulatory mechanisms that control *GSTP1* transcription, with particular reference to the silencer element located between nucleotides –105 and –86.

EXPERIMENTAL

Cell culture

The VCREMS cell line (generously provided by Dr. Bridget Hill, Imperial Cancer Research Fund, London, U.K.) was derived by selecting the human mammary carcinoma cell line MCF7 for resistance to vincristine [15]. VCREMS, MCF7, EJ, HeLa and HepG2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and supplemented with L-glutamine and penicillin/streptomycin mixture (Gibco-BRL, Paisley, Scotland, U.K.).

For the mRNA stability experiment, the cells were treated with actinomycin D (10 µg/ml) and then harvested for RNA isolation at defined time points.

Northern analysis

Total cellular RNA was isolated from EJ, VCREMS, MCF7, HeLa and HepG2 cells using the procedure described by Chomczynski and Saachi [16]. RNA (10 µg/lane) was electrophoretically fractionated on a denaturing formaldehyde gel and

Abbreviations used: GST, glutathione S-transferase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic-mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear factor-κB; OR, oestrogen receptor.

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transferred to Hybond-N nylon membrane (Amersham International, Amersham, U.K.). A full-length (800 bp; *Eco*RI-digested) *GSTP1* cDNA fragment [17] was labelled by the random priming method [18] and used to determine the relative level of *GSTP1* mRNA in the five different cell lines. Northern blots were washed to high stringency: $0.1 \times$ SSC containing 0.1% SDS at 65 °C for 30 min. Autoradiography was performed using X-Omat AR film (Eastman Kodak Co.) and two intensifying screens at -70 °C.

Promoter deletion constructs

The *GSTP1* promoter deletion constructs p291CAT, p105CAT, p85CAT and p65CAT were prepared as described previously [12] by ligating deletion fragments of the human *GSTP1* promoter (-291 to +36, -105 to +36, -85 to +36 and -65 to +36) into the pCAT.Basic vector (Promega, Southampton, U.K.). Following preparation, all constructs were sequenced by the dideoxy-chain-termination method [19].

Transient transfection assay

DNA transfections were performed by the calcium phosphate method [20], as described by Gorman [21], with the exception that glycerol was omitted. Cell extracts were assayed for protein content [22], and chloramphenicol acetyltransferase (CAT) activity was determined as described previously [23] and modified by Sambrook et al. [24]. In all experiments, results were normalized for the activity of pCAT.Control (Promega), which contained the simian virus 40 enhancer and promoter, while the promoterless pCAT.Basic vector was used as a negative control.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from EJ, VCREMS and MCF7 cells were prepared using the method described by Dignam et al. [25]. EMSAs were performed as described previously [12]. Briefly, 10 µg of EJ, VCREMS or MCF7 cell nuclear protein was incubated for 20 min at room temperature with 2 ng of [γ -³²P]ATP-end-labelled 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 on to a pre-run (200 V for 2 h at 4 °C) 4% polyacrylamide gel (30:1 cross-linking ratio) containing $0.3 \times$ TBE (6.54 g of Tris, 3.3 g of boric acid and 0.45 g of EDTA in 2 litres). 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 pre-incubated for 20 min at room temperature with a 100-fold molar excess of unlabelled DNA before the addition of radiolabelled probe. The following oligonucleotides and their complementary sequences were used as probes and competitors: *GSTP1* promoter fragment -105 to -54 (5'-AGTCCGCGGGACCCTCCAG-AAGAGCGGCCGCGCCGTGACTCAGCACTGGGG-3'), *GSTP1* promoter fragment -85 to -54 (5'-AGAGCGGC-CGGCGCCGTGACTCAGCACTGGGG-3'), *GSTP1* C1 promoter fragment -73 to -54 (5'-GCCGTGACTCAGCACTGGGG-3'), the AP-1 binding site (5'-AGCTTGATGAGTCAGCCG-3') from the human collagenase promoter [26] and the nuclear factor- κ B (NF- κ B) response element (5'-AGTTGAGGGACTTTCCAGGC-3') from the immunoglobulin κ light-chain gene [27]. For annealing, equal amounts of complementary oligonucleotides were heated to 95 °C for 2 min and allowed to cool gradually to room temperature.

RESULTS

Increased expression of *GSTP1* mRNA in EJ cells

Total RNA was isolated from EJ, VCREMS, MCF7, HeLa and HepG2 cells and analysed by Northern blot analysis to determine the relative level of *GSTP1* mRNA expression. Figure 1 shows that the *GSTP1* gene was expressed at 3.1-fold higher levels in EJ cells than in the VCREMS cell line. Furthermore, *GSTP1* mRNA was undetectable in MCF7, HeLa and HepG2 cells. Therefore the experiments described in this paper were designed to investigate the molecular mechanisms responsible for the observed differences in *GSTP1* gene expression between EJ and VCREMS cells.

Transcriptional activity of the *GSTP1* promoter

To determine *GSTP1* promoter activity in EJ and VCREMS cells, 5' deletion fragments of the human *GSTP1* promoter were prepared and ligated upstream of the CAT reporter gene, as described elsewhere [12]. These *GSTP1* promoter deletion constructs were then used to perform transient transfection assays in both cell lines. Table 1 shows high levels of CAT activity

Figure 1 Increased expression of *GSTP1* mRNA in EJ cells

The relative levels of *GSTP1* mRNA in MCF7, VCREMS, EJ, HeLa and HepG2 cells were determined by Northern analysis (10 µg/lane) using a full-length human *GSTP1* cDNA fragment as a probe. The ethidium bromide-stained gel is shown to confirm equal loading of RNA samples.

Table 1 Transcriptional activity of the *GSTP1* promoter

The *GSTP1* promoter fragments -291 to +36, -105 to +36, -85 to +36 and -65 to +36 were subcloned into the pCAT.Basic vector to generate p291CAT, p105CAT, p85CAT and p65CAT respectively. These constructs were transfected into VCREMS and EJ cells and their relative CAT activities were determined. pCAT.Basic (contains no enhancer/promoter sequences) and pCAT.Control (contains the simian virus 40 enhancer and promoter) were used as negative and positive controls respectively. Results were compared between cell lines by correcting for pCAT.Control activity levels.

Cell line	CAT activity (units)			
	p291CAT	p105CAT	p85CAT	p65CAT
VCREMS	22.1 ± 3.2	23.2 ± 3.9	26.1 ± 0.9	2.0 ± 1.6
EJ	5.2 ± 1.3	6.1 ± 2.9	25.7 ± 1.6	1.7 ± 1.1

Figure 2 Transcriptional repressor activity in EJ cells does not displace the essential C1 nuclear complex

EMSA demonstrating the nuclear complexes (10 μ g of nuclear extract per reaction) in EJ, VCREMS and MCF7 cells that bound to the *GSTP1* promoter fragments -73 to -54 and -105 to -54 .

produced by p291CAT, p105CAT and p85CAT in VCREMS cells. Furthermore, p65CAT was inactive in both cell lines, thus emphasizing the importance of the previously identified C1 promoter region located between nucleotides -73 and -54 [12].

However, in contrast to the measured level of *GSTP1* mRNA, the activities of p291CAT and p105CAT were significantly decreased (4.8- and 3.2-fold respectively) in EJ cells, although p85CAT was equally active in the two cell lines. This result implies the presence of a transcription silencer element located between nucleotides -105 and -86 that is functional in EJ but not VCREMS cells. Interestingly, we have shown previously that this same promoter region mediates transcriptional repression of the *GSTP1* gene in MCF7 cells [12]. Therefore these results provide another example of the functional importance of the negatively acting promoter element (-105 to -86) in the control of *GSTP1* transcription.

Moreover, these findings provide strong evidence that over-expression of *GSTP1* in EJ cells is not mediated by enhanced transcriptional activity of the *GSTP1* promoter.

Transcriptional repressor activity in EJ cells does not displace the essential C1 nuclear complex

The major difference between regulation of the *GSTP1* promoter in EJ and VCREMS cells was the presence of a negative regulatory element (-105 to -85) that was functional in EJ but not VCREMS cells. As stated above, we have shown previously that this element suppressed *GSTP1* transcription in the human breast carcinoma MCF7 cell line. Furthermore, recent evidence from this laboratory has shown that binding of the MCF7 transcriptional repressor between nucleotides -105 and -86 of the *GSTP1* promoter mediates displacement of the essential C1-bound complex [13].

To investigate the possibility that a similar mechanism may account for the suppression of *GSTP1* promoter activity in EJ cells, band-shift assays were performed using the C1 promoter region (-73 to -54) and the *GSTP1* promoter fragment, -105 to -54 , which contains the negative regulatory element. Figure 2 shows that single nuclear complexes of similar mobilities were specifically bound to the C1 probe in EJ, VCREMS and MCF7

Figure 3 Binding of an AP-1-like complex to the C1 promoter region in EJ and VCREMS cells

Upper panel: sequence of the *GSTP1* promoter C1 region (nucleotides -73 to -54). The AP-1 consensus binding site (-69 to -63) is boxed. Lower panel: EMSA demonstrating the nuclear complexes (10 μ g of nuclear extract per reaction) in EJ, VCREMS and MCF7 cells that bound to the *GSTP1* promoter fragment -73 to -54 and the AP-1 binding site from the human collagenase promoter. Competition analysis confirmed that the denoted C1 complex was the only specific complex bound to the element (results not shown).

cells. However, unlike the MCF7 C1 complex, binding of the EJ and VCREMS C1 nuclear complexes was retained by the -105 to -54 probe. Therefore these *in vitro* data suggest that inhibition of binding of the C1 complex to the *GSTP1* promoter is not required for the negative regulatory mechanism observed in EJ cells.

Binding of an AP-1-like complex to the C1 promoter region in EJ and VCREMS cells

Examination of the sequence contained within the C1 promoter region revealed the presence of a perfect consensus AP-1 binding site (TGACTCA; positions -69 to -63 ; Figure 3, upper panel). Indeed, the p65CAT construct was designed to disrupt this putative response element, and in both cell lines this deletion abrogated *GSTP1* transcriptional activity (see Table 1), suggesting that AP-1 comprised the major complex bound to this essential regulatory region of the *GSTP1* promoter.

Studies in this laboratory have demonstrated that the essential complex bound to the C1 promoter element (-73 to -54) is composed of a Jun/Fos heterodimer in VCREMS but not MCF7 cells [12]. Moreover, recent data have indicated that differences in the composition in the C1-bound nuclear complex may be important for repressor activity [13].

However, despite the observed repression of *GSTP1* transcription in EJ cells, the lack of C1 displacement activity in these cells (Figure 2) suggested mechanistic differences in the function of the repressor in EJ and MCF7 cells. Therefore it was important to characterize the nuclear proteins bound to this promoter region in EJ cells.

Figure 4 Efficient competition for the EJ and VCREMS C1-bound complexes by the AP-1 binding site from the human collagenase promoter

Oligonucleotides containing the known AP-1 binding site from the human collagenase promoter were used at 10-, 50-, 100- and 500-fold molar excess (relative to the radiolabelled *GSTP1* promoter fragment -73 to -54) to compete for the EJ and VCREMS nuclear complexes (10 μ g of nuclear extract per reaction) bound to the C1 region of the *GSTP1* promoter. Competition with a 100-fold molar excess of the known NF- κ B response element from the immunoglobulin κ light-chain gene was used as a negative control. Non-specific complexes are marked by an asterisk.

As shown above, single nuclear complexes of similar mobility were bound to the C1 promoter fragment (-73 to -54) in EJ, VCREMS and MCF7 cells (Figure 3, lower panel). Furthermore, a 20 bp probe containing the known AP-1-binding site from the human collagenase promoter bound a single nuclear complex of mobility identical to that of the C1 complex in both EJ and VCREMS cells. However, in agreement with previous results [12], no detectable binding of MCF7 nuclear extract to the AP-1 probe was observed.

To corroborate these data, the ability of the AP-1 binding site from the human collagenase promoter to compete for the C1 complexes from EJ and VCREMS cells was tested. Figure 4 clearly shows that efficient competition for both the EJ and VCREMS C1 nuclear complexes was observed using the AP-1 oligomer. Therefore the cumulative data from Figures 3 and 4 provide strong evidence that members of the Jun and Fos protein families are major components of the C1 nuclear complex in EJ cells.

***GSTP1* mRNA stabilization in EJ cells**

Our results have strongly indicated that *GSTP1* overexpression in EJ compared with VCREMS cells is not due to increased transcription of the *GSTP1* gene in the bladder carcinoma cell line. Indeed, our data show diminished *GSTP1* promoter activity in the EJ cell line. These findings suggested that other modes of regulation play the predominant role in mediating the observed cell-specific pattern of *GSTP1* expression.

In this regard, post-transcriptional mechanisms were investigated by treating EJ and VCREMS cells with actinomycin D to inhibit RNA synthesis. Figure 5 shows that *GSTP1* mRNA levels in VCREMS cells began to decrease 4–5 h after treatment with actinomycin D. Moreover, the *GSTP1* transcript was virtually undetectable after 6 h. In contrast, the *GSTP1* mRNA levels in EJ cells remained unchanged 6 h after treatment with actinomycin D. As a control, levels of glyceraldehyde-3-phos-

phate dehydrogenase (GAPDH) mRNA were demonstrated to be unaffected in both cell lines. Therefore these results suggested that the decay of *GSTP1* mRNA was significantly more rapid in VCREMS cells than in the EJ cell line.

Thus our data have shown that *GSTP1* transcriptional mechanisms differ significantly in EJ and VCREMS cells. However, these differences do not appear to be primarily responsible for the elevated levels of *GSTP1* in EJ cells. In contrast, the mRNA decay experiment shown in Figure 5 strongly indicated that enhanced post-transcriptional stabilization of *GSTP1* mRNA provides the most important mechanism governing *GSTP1* overexpression in EJ cells.

DISCUSSION

Previous studies from this laboratory [12] have outlined the transcriptional mechanisms responsible for *GSTP1* overexpression in a multidrug-resistant derivative of the human mammary carcinoma cell line MCF7. In contrast, the data presented here highlight the important role that the post-transcriptional regulation of *GSTP1* gene expression can play in elevating *GSTP1* levels in the human bladder carcinoma cell line EJ. Indeed, these data provide the first evidence to date that modulating the stabilization of *GSTP1* mRNA can act as the primary mechanism governing the cell-specific expression of this gene.

Changes in mRNA turnover rate are recognized as a major control point in the regulation of gene expression. The complexity of these mechanisms is exemplified by the observation that, within the same cell, some eukaryotic mRNAs are degraded with a half-life of 20 min while other mRNAs remain intact for up to 24 h (for a review, see [28]). Furthermore, the stabilities of specific mRNAs can be modulated in response to cellular conditions (e.g. transferrin receptor mRNA in response to iron levels [29]), while alterations in mRNA-specific destabilizing factors have been shown to occur in certain cancers (e.g. monocytic tumours [30]). In addition, the physiological importance of the role played by mRNA degradation in differential gene expression was demonstrated by the transformation phenotype associated with mRNA stabilization mutations in *c-myc* and *c-fos* mRNAs [31].

The data from our studies indicate that differences in *GSTP1* mRNA stability in human bladder carcinoma EJ cells compared with the multidrug-resistant breast cancer VCREMS cell line are likely to account for the relative overexpression of *GSTP1* in EJ cells.

Post-transcriptional regulatory mechanisms have been postulated previously to mediate *GSTP1* gene expression [32]. This hypothesis arose from studies which demonstrated the existence of an inverse correlation between *GSTP1* gene expression and oestrogen receptor (OR) levels within breast cancer cell lines [33] and breast tumour specimens [34]. No differences in *GSTP1* transcription rates were observed between OR-negative and OR-positive cell lines, which led the authors to conclude that changes in mRNA stabilization controlled the variable levels of *GSTP1* detectable in these cells.

However, this theory was difficult to prove, since *GSTP1* mRNA is undetectable in OR-positive cell lines. Interestingly, though, in the three OR-negative cell lines tested, the *GSTP1* mRNA half-life was estimated to be in the range 12–18 h [32]. In contrast, we have shown that, in VCREMS cells (OR-negative), *GSTP1* mRNA was undetectable 6 h after actinomycin D treatment. Therefore relating our results to work by other investigators suggests that an uncharacteristically rapid decay of *GSTP1* mRNA in VCREMS cells rather than increased *GSTP1*

Figure 5 *GSTP1* mRNA stabilization in EJ cells

Total cellular RNA was isolated from VCREMS and EJ cells at specific time points after treatment with actinomycin D and examined by Northern analysis (10 μ g/lane) to follow the decay rates of *GSTP1* mRNA. Levels of GAPDH mRNA were quantified as a control, and the ethidium bromide-stained gels are shown to confirm equal loading of RNA samples. C, control (cells not treated with actinomycin D).

mRNA stabilization in EJ cells provides the primary mechanism responsible for our observations.

Despite overexpression of the *GSTP1* gene in EJ cells, our results surprisingly indicated the presence of *GSTP1* transcriptional repressor activity in this cell line. This repressor activity was mediated by an element contained between nucleotides –105 and –86 in the *GSTP1* promoter. Interestingly, this identical promoter region controls negative regulation of *GSTP1* transcription in MCF7 cells [12]. However, unlike in MCF7 cells, EJ repressor activity failed to inhibit DNA binding of the essential C1 complex. This finding strongly indicates that C1 displacement activity is not required to repress *GSTP1* transcription. It seems more likely that the suppressive effects on *GSTP1* transcription in EJ cells are exerted by the inhibition of C1-complex-mediated transcriptional activation of the *GSTP1* promoter. One potential mechanism may be the formation of a transcriptionally inactive repressor–C1 complex.

Promoter-specific repression of transcription has been reported for a number of genes, including the γ -crystallin [35] and P-glycoprotein [36] promoters. Work in this laboratory on *GSTP1* transcriptional regulation in MCF7 cells has highlighted similar promoter-specific properties of the *GSTP1* transcriptional repressor [13]. Indeed, these studies have clearly shown differences in the essential C1 complex (bound between nucleotides –73 and –54) between MCF7 and VCREMS cells. It is likely that these differences in composition may allow interaction with the *GSTP1* transcriptional repressor (bound between nucleotides –105 and

–85) and thus mediate repression of *GSTP1* promoter activity. Subtle differences in the composition of the VCREMS and EJ C1 complexes may also be important for repressor activity in EJ cells, and this requires further investigation.

Clearly, evidence that both transcriptional and post-transcriptional mechanisms can contribute to regulating *GSTP1* gene expression provides a number of potential regulatory pathways for modulating the *GSTP1* concentration in response to changes in the intracellular environment.

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