Analysis of Glutathione Transferase P Gene Regulation with Liver Cells in Primary Culture

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Abstract
Glutathione transferase P (GST-P) gene is specifically and highly activated during rat chemical hepatocarcinogenesis. We have previously cloned the GST-P gene and have identified putative regulatory regions. To further explore regulatory mechanisms, deletion constructs of the GST-P gene fused to the chloramphenicol acetyltransferase (CAT) structural gene were introduced into primary cultured rat hepatocytes by electroporation, and their activity was determined. The expression of the GST-P-CAT fusion gene is quite low in these cells as compared to that in both a rat fibroblast cell line, 3Y1 cells, and a rat hepatoma cell line, dRLh84. The presence of the strong enhancer GPEI did not elicit any enhancing activity at its original position, or when it was located 3' of the CAT gene, although this element does enhance CAT activity significantly when located adjacent to the promoter. Cotransfection of either c-jun nor c-fos expression vector, nor both vectors, could enhance the CAT activity, even though GPEI consists of two phosphol ester response element-like sites. Furthermore, the expression of jun family gene was not correlated with GST-P gene expression either in primary cultured hepatocytes or in hepatoma cell lines.

Introduction
GST-P is an isozyme of a family of multifunctional enzymes which catalyze the conjugation of electrophilic metabolites of xenobiotics (1). GST-P is known to be expressed specifically from the early stages of chemical hepatocarcinogenesis of the rat (2–5). To elucidate the molecular mechanism of this regulatory change, we have cloned the GST-P gene and identified multiple regulatory elements in the 5'-flanking region (6, 7).

The promoter region of the GST-P gene (−60−−20) consists of a TATA box, a GC box, and a consensus AP-1 binding site (TRE), the former two of which are essential for the basal level of GST-P gene expression (7). An enhancer, designated GPEI, consisting of two TRE-like sequences arranged in a palindrome, is located 2.5 kb upstream from the transcription start site (8). A silencer region has also been identified between 100 and 400 bases upstream from the transcription start site. The function of these elements is not yet clear, but they may work as general regulators of basal gene expression. At least two proteins bind to these silencer elements, one of which has been cloned from rat liver and was found to be identical to the LAP/IL-6DBP (9–12).

The GPEI is an efficient enhancer showing an activity higher than that of SV40 enhancer in dRLh84 cells, a rat hepatoma cell line (7). Furthermore, the 5'-flanking region (−2.9 kb) of the GST-P gene fused to the coding sequence of the CAT gene also showed a high level of CAT activity in 3Y1 and L cells, which do not appreciably express GST-P (7). Transfection experiments with the above fibroblast cell lines thus did not seem appropriate for elucidating the mechanisms of the liver-repressive and hepatoma-specific expression of the GST-P gene. On the other hand, primary cultured hepatocytes, which have been shown to reflect the in vivo situation at least partially (13), may provide better information on the expression of the GST-P gene in normal and transformed liver cells. Therefore, we explored a system of primary cultured hepatocytes into which DNA could be introduced efficiently by electroporation (14, 15).

We have previously shown that the amount of c-jun mRNA increases during the early stages of rat liver carcinogenesis (16). Since the GST-P gene has a TRE near the core promoter region and the enhancer GPEI contains two TRE-like sequences, c-jun expression was suspected to be causing the high expression of the GST-P gene. To examine this possibility, we cotransfected a reporter construct containing GST-P 5'-regulatory region (GST-P-CAT) together with the jun family and fos expression vectors into primary cultured hepatocytes and examined the effect of their expression on GST-P gene transcription. To correlate the results of primary hepatocytes with the expression of these genes in vivo, we also examined the levels of jun family mRNA in normal liver, primary hepatocytes, and hepatoma cell lines in comparison with GST-P mRNA.

Results
Characterization of Regulatory Elements of GST-P Gene in Primary Rat Hepatocytes. After a number of trials, we found that electroporation was the method of choice for the highly efficient introduction of DNA into primary
cultured hepatocytes while maintaining high cellular viability (14). The conventional calcium phosphate procedure was ineffective because calcium phosphate at the required concentration was too toxic for these cells. We first examined whether the endogenous GST-P gene was expressed in primary cultured hepatocytes (Fig. 1). To determine the physiological function of liver cells, expression of the albumin gene was also monitored. GST-P mRNA was not appreciably detected in normal liver nor in primary cultured hepatocytes at up to 24 h, but it became slightly detectable at 48 h and increased thereafter. Albumin mRNA decreased dramatically after 48 h. These results indicated that the normal state of GST-P gene control could be maintained until 24 h after hepatocyte culture. Therefore, the electroporation was performed immediately after isolation of hepatocytes, and the CAT assay was carried out at 24 h.

We obtained CAT activity of pSV2CAT regulated by the SV40 enhancer in the range of 0.45 nmol/min/mg protein in these cells, a level of expression that is approximately 15–20-fold higher than that obtained by previous workers (0.03 nmol/min/mg protein) (14). We confirmed that the l-pyruvate kinase promoter (a liver-specific gene promoter) is also active in these cultured hepatocytes (data not shown). These data indicate that the conditions described here are better for primary cultured hepatocytes than that used by Tur-Kaspa et al. (14), likely as a result of a higher transfection efficiency.

A series of deletion constructs were introduced into the primary cultured hepatocytes, and the CAT activity was examined (Fig. 2). Truncation of the 5'-flanking sequence from −2.9 to −0.4 kb (E to 4CAT) decreased the CAT activity only slightly, not more than 1.6-fold. However, deletion from −0.4 to −0.14 kb (4 to 5CAT) caused a 2-fold increase in CAT activity. This phenomenon was reproducible and is consistent with the presence of silencer sequences in this region (7, 9). The CAT activity of ECAT was almost identical to Δ-56CAT, a construct containing only the minimal core promoter of the GST-P gene and which has at least 10-fold lower activity than that of pSV2CAT in primary cultured hepatocytes. This was in contrast with our previous data with a rat hepatoma cell line, dRLh84 cells, in which the activity of ECAT was more than 10-fold higher than that of Δ-56CAT and was even 2-fold higher than that of pSV2CAT (7). These results indicate that the 5'-flanking region of the rat GST-P gene containing the GPEI enhancer is inactive in primary cultured hepatocytes, as it is in normal liver, suggesting that a similar control mechanism is operating in the former as in the latter.

Further deletion analysis confirmed that the 56-bp sequence upstream of the start site is necessary and sufficient for basic promoter activity in primary hepatocytes just as it is in hepatoma and other cell lines. Other constructs, such as Δ-41CAT construct (−41 to +59), which lacks the SP1 binding site but has an intact TATA box and pSVOCAT, showed virtually no CAT activity in these cells (data not shown). GPEI is located −2.5 kb upstream between the EcoRI site (ECAT) and the Accl site (1CAT). It is a very active enhancer in dRLh84 and, to a lesser extent, in the rat fibroblast cell line 3Y1 (7). We then examined whether GPEI can also work as an enhancer in primary hepatocytes. Two plasmids were constructed in which the GPEI fragment was inserted...
Table 1  GPEI enhancer activity in dRLh84, 3Y1, and primary hepatocytes

<table>
<thead>
<tr>
<th>Construct used</th>
<th>Relative CAT activity</th>
<th>dRLh84</th>
<th>3Y1</th>
<th>Primary hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ-56CAT</td>
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<td>1.0</td>
<td>1.0</td>
<td></td>
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<tr>
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<td>4.9</td>
<td>4.5</td>
<td></td>
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<tr>
<td>Δ-56CAT GPEI</td>
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<td>2.6</td>
<td>0.7</td>
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</table>

Each number is the mean of at least 3-5 independent transfection experiments. GPEI enhancer in GPEI Δ-56CAT and Δ-56CAT GPEI is located 56 bp upstream and 1.65 kb downstream of the promoter, respectively.

either into the 5′ region of the GST-P promoter at -56 bp (GPEI Δ-56CAT) or downstream to the CAT gene (Δ-56CAT GPEI). In the former, GPEI is in close proximity with the TATA box, whereas in the latter, GPEI is located more than 1.65 kb away. dRLh84 and 3Y1 cells were also used for comparison. In the construct GPEI Δ-56CAT, GPEI enhanced promoter activity about 13.0-fold in dRLh84, 4.9-fold in 3Y1, and 4.5-fold in the primary hepatocytes. When Δ-56CAT GPEI was used, we again saw enhancement in dRLh84 (7.2-fold) and 3Y1 (2.6-fold) cells. However, in primary cultured hepatocytes, no enhancement by the GPEI located downstream was obtained (Table 1). These results suggest that the enhancing activity of GPEI does not work at a distance in hepatocytes and is likely to be repressed in normal liver, whereas it is activated in hepatomas.

Effect of jun Family Expression on Transfected GST-P Gene. GPEI enhancer is known to consist of two TRE-like sequences that are arranged in a palindrome (8). This raises a possibility that GPEI may be activated by jun family proteins. We therefore examined the response of GST-PCAT (ECAT) to cotransfected c-jun, junB, or junD expression vectors in primary hepatocytes as well as to c-fos. As shown in Fig. 3A, expression of these vectors could not trans-activate ECAT in the 24-h posttransfection assay used. We next tested the effect of c-fos expression. The cotransfection of ECAT with a c-fos expression vector also had no trans-activation capacity, nor did the cotransfection of c-jun and c-fos expression vectors together (Fig. 3B). When 1XTRE, 3X TRE, and 5XTRE, containing one to five copies of synthetic TRE derived from collagenase TRE sequence, were used as a reporter, no trans-activation was observed by cotransfection of jun and/or fos expression vectors, although 3XTRE and 5XTRE showed relatively high basal CAT activities (data not shown).

Expression of Endogenous jun Family and GST-P Genes in Primary Hepatocytes and Hepatoma Cell Lines. We examined the expression of the endogenous jun family genes in primary cultured hepatocytes. In normal liver, neither c-jun nor junB was appreciably expressed, whereas junD was expressed significantly (Fig. 4, Lane 1).
Expression of GST-P and Jun Genes in Rat Hepatocytes

Interestingly, all of the Jun family genes (c-jun, JunB, and JunD) became highly induced during the early stages of hepatocyte culture. The c-jun mRNA appeared as early as after just 24 h of culture and remained high until at least 96 h (Fig. 4, Lanes 2–5). The amount of c-jun mRNA at 24 h of primary culture was about 10-fold higher than that of the c-jun mRNA in dRLh84 (Fig. 4, Lanes 2–6).

The amount of JunB gradually increased until 72 h, whereas JunD mRNA increased and reached a maximum level at 48 h after culture (Fig. 4, Lane 3). The concentration of JunB mRNA in these cells was approximately 2–3-fold lower than that in dRLh84 cells, whereas that of JunD mRNA was 2–3-fold higher than that of dRLh84 cells. These data clearly suggest that c-jun, JunB, and JunD are differentially regulated in rat liver and behave differently than during primary culture. c-fos mRNA, which is not present in normal liver, was not significantly induced in primary hepatocytes during culture up to 96 h (Fig. 4), although it was superinduced by cycloheximide (Fig. 4, Lane 7).

We also examined the expression of Jun family, albumin, and GST-P genes in four different rat hepatoma cell
which are moderately differentiated, the level of c-jun mRNA is comparable to that in normal liver. When the level of GST-P gene expression was analyzed in these various cell lines, it was found to be highly expressed in all instances. These data indicate that there is no correlation between the level of c-jun expression and that of the GST-P mRNA. We also examined the level of junB and junD mRNA in these cells. In each case, both mRNAs were highly expressed, but the magnitude of change between these mRNAs and GST-P mRNA was not the same as that observed in primary cultured hepatocytes.

Discussion

To approach the mechanisms of GST-P gene regulation in normal liver and hepatoma cells, we used primary cultured hepatocytes into which DNA constructs were introduced by electroporation. In our previous transfection analyses, the 5'-flanking region (−2.9 kb) of the GST-P gene was connected to the coding sequence of the CAT gene (ECAT), and its activity was examined in a variety of cell lines such as a rat fibroblast cell line (3Y1), a human hepatoma cell line (HepG2), a mouse fibroblast cell line (L cells), and a mouse embryonal carcinoma cell line (F9), all of which did not express appreciable amounts of the GST of π class (7). In each case, ECAT activity was very high, even though only the hepatoma cells expressed endogenous GST-P. The present study has expanded these initial studies and shows that, in the case of primary cultured hepatocytes, the activity of ECAT is very low and comparable to that of the core GST-P promoter containing a GC box and a TATA box (Δ-56CAT) (Figs. 2 and 3). Deletion experiments have shown that the distally located enhancer GPEI does not have trans-activating capacity in primary hepatocytes. These results indicate that GPEI can act as a cell type-specific enhancer.

The effect of the distance from the promoter on the enhancing activity of GPEI is an interesting effect (Table 1). In both dRLh84 and 3Y1 cells, the construct containing the GPEI enhancer at about 1.65 kb from the promoter, downstream of the CAT gene, as well as the constructs containing the GPEI enhancer just upstream of GST-P promoter, showed considerable enhancer activity. In the primary cultured liver cells, however, GPEI showed enhancing activity only when it was placed adjacent to the promoter. Since GPEI is located at −2.5 kb from the promoter in the native GST-P gene, these results suggest that the GPEI element is not working as an enhancer in normal liver cells. Thus, primary cultured hepatocytes appear to mimic the mode of GST-P regulation by GPEI in normal rat liver. Perhaps the difference in GPEI enhancing activity between primary cultured hepatocytes and hepatoma cells may be the major cause of hepato-specific GST-P expression.

Because GPEI is composed of two palindromic TRE-like sequences and one consensus sequence of TRE exists at −61 to −55 bp of the GST-Pgene promoter (8), c-jun or another member of the jun family of proteins may regulate (activate) the GST-P gene. In addition, c-jun expression was found to be elevated during early stages

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Fig. 5. Northern blot analysis of albumin, GST-P, and jun family mRNA in rat hepatoma cell lines. Total RNA (10 μg) was prepared from normal rat liver (Lane 1), Reuber H4llE cells (Lane 2), dRLh84 cells (Lane 3), Kagura-1 cells (Lane 4), and Kagura-2 cells (Lane 5), transferred, and hybridized with the albumin, GST-P, and jun probes, as described in "Materials and Methods."

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*S. Morimura, A. Okuda, M. Sakai, M. Imagawa, and M. Muramatsu, unpublished data.*
of chemical hepatocarcinogenesis (16), suggesting the possibility that the activation of the GST-P gene through GPEI is caused by c-jun activation. In the present study, however, the expression of c-jun with or without the cotransfection of c-fos could not trans-activate GST-P-CAT gene in primary cultured hepatocytes; JunB or JunD was also without effect. Furthermore, neither 1xTRECAT, 3xTRECAT, nor 5xTRECAT showed any trans-activation by jun and/or fos expression vectors. It is unlikely that expression vectors used here may not be active in primary hepatocytes, for the following reasons. First, the expression vectors used here were always active in cultured cell lines and could trans-activate several reporter constructs, including GPEI Δ-56CAT and 1xTRECAT. Second, both RSV and 5RsA, derived from SV40 expression systems, are active in primary hepatocytes, since both RSVCAT and pSV2CAT showed high CAT activities, as shown in Fig. 2B. Thus, AP-1-related transcription factors themselves may not be active in primary hepatocytes and require some other factor(s), as discussed below. These data suggest that the rise of c-jun expression during the early stages of hepatocarcinogenesis is not sufficient for the activation of the GST-P gene.

Endorsement of the interpretation of the cotransfection data was obtained by examining the correlation between the endogenous levels of jun family and GST-P mRNA in primary hepatocytes and in several hepatoma cells. Whereas the c-jun mRNA level was elevated markedly as early as 24 h after primary culture, the GST-P mRNA increased only slightly after 48 h and then gradually increased by 96 h. JunB and JunD mRNA also increased during the early stages of culture. However, no correlation between the expression of any of the jun and GST-P could be established. GST-P was found to be inducible in primary cultured hepatocytes (17–21). GST-P induction in serum-free primary cultured hepatocytes by the addition of either epidermal growth factor or insulin was associated with enhancement of transcription of c-jun and c-fos (22). However, our findings indicate that the process of GST-P gene activation is not always accompanied by the elevation of jun family expression in primary cultured hepatocytes or hepatoma cell lines.

GPEI was shown to be active in F9 embryonal carcinoma cells that have virtually no AP-1 activity (23), whereas it is inactive in primary cultured hepatocytes that express the jun family of genes. The presence of other trans-acting factors may help to explain the apparent paradox. Indeed, we have recently identified a factor from F9 nuclear extracts that specifically binds to GPEI.7 IP-1 has been identified as a dominant transcriptional inhibitor of AP-1 activity (24). These factors may regulate the GPEI activity and the GST-P gene. Whether these factors are involved in the activation of GST-P gene during hepatocarcinogenesis remains to be determined. A resolution of this issue awaits isolation and biochemical characterization of these factors.

Materials and Methods

Cell Culture and Transfections. Hepatocytes from adult male Wistar rats (approximately 180–200 g) were isolated by cannulating the portal vein and perfusing the liver with collagenase (25). The isolated cells were then subjected to isodensity Percoll centrifugation (26). Hepatocytes were transfected with plasmid DNA by electroporation using a Gene Pulser (Bio-Rad). After resuspending at a density of 5 × 10⁶ cells/ml in phosphate-buffered saline, DNA (10 or 20 μg) was added to 800 μl of cell suspension containing 4 × 10⁶ cells. The mixture was transferred to a Gene Pulser cuvet and subjected to a single pulse of 960 μF at 300 V. After electroporation, the cells were incubated on ice for 10 min, transferred to collagen-coated dishes containing Williams E medium supplemented with 10% fetal calf serum, and then incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. Cells were harvested at specified times, and extracts were prepared for CAT assay.

3Y1, dRLb84, and H4IE cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum. Transfection of these cells was performed using the calcium phosphate precipitation method (27). All of the transfection experiments were performed three to four times by using two different preparations of DNA, and the mean values are shown in the “Results.” Kagura-1 and Kagura-2 cells were grown as described previously (28).

CAT Assay. CAT assays were carried out as described by Gorman et al. (29), except that 0.15 μCi of [14C]-chloramphenicol (54 mCi/mmol) was present in each reaction, and extracts were heated at 65°C for 10 min before assay.

Northern Blot Analysis. Total cellular RNA was isolated from rat tissues and from cell lines by the guanidinium thiocyanate/hot phenol method (30). Total RNA (10 μg) was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a nitrocellulose filter (31). Hybridization was carried out in 50% formamide, 0.65 M NaCl, 0.1 M sodium piperazine-N,N’-bis(2-ethanesulfonic acid) (pH 6.8), 10% dextran sulfate, 5X Denhardt’s solution, 0.1% sodium dodecyl sulfate, 5 mm EDTA, and 100 μg/ml salmon sperm DNA at 42°C for 18 h. The filters were washed four times in 2X standard saline citrate, 0.2% sodium pyrophosphate, and 0.1% sodium dodecyl sulfate for 30 min at 60°C.

Plasmid Constructions. A 2595-bp fragment between −2900 and +59 bp relative to the cap site of the GST-P gene was inserted into the HindIII site of the pSVOCAT, designated as ECAT, and 5’-deletion mutants (1–5CAT and Δ-56CAT) were constructed as previously described (7). GPEI Δ-56CAT and Δ-56CAT GPEI, containing a GPEI enhancer at a site upstream (−56 bp) and downstream (1.65 kb) to the GST-P promoter, respectively, have been described (8). As probes for hybridization studies, a 2.0-kb HindIII-BamHI fragment of rat c-jun cDNA (16), a 1.5-kb EcoRI cDNA fragment of mouse junB (32), a 1.7-kb EcoRI cDNA fragment of mouse junD (33), a 1.2-kb HindIII fragment of rat albumin cDNA (34), a 0.75-kb EcoRI-Sall fragment for rat GST-P cDNA (35), and a 1.0-kb PstI/PstI fragment of p-fos-I (36) were used. c-fos expression vector was constructed by ligating a human c-fos cDNA clone into the EcoRI site of pcDSRα (23). RSV c-jun, RSV-junB, and RSV-junD expression vectors were constructed as follows. NheI-ScaI 1.3-kb fragment (mouse c-jun), SmaI-EcoRI 1.3-kb fragment (mouse junB), or EcoRI 1.6-kb fragment (mouse junD) was fused by blunt-end ligation to HindIII-Hpal fragment of pRSVOCAT containing the RSV long terminal repeat poly-

7 M. B. Diccianni, M. Imagawa, and M. Muramatsu, unpublished data.
adenylated addition signal of SV40 early gene and vector plasmid sequence after filling in with Klenow enzyme.

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References