Glutathione transferase P (GST-P) is specifically induced in rat liver and kidney by lead cation. The increase of GST-P mRNA after lead administration is blocked by actinomycin D, suggesting that GST-P production by lead is regulated at the transcriptional level. To further determine which part of the flanking region of the GST-P gene has the lead-responsive cis-element in vivo, we utilized transgenic rats with five different constructs having GST-P and/or chloramphenicol acetyltransferase coding sequence. We studied the effect of lead on these transgenic rats and on transfected NRK (normal rat kidney) cells and found that GST-P induction by lead is indeed regulated at the transcriptional level and that the GST-P enhancer I (GPEI) enhancer is an essential cis-element required for the activation of the GST-P gene by lead. GPEI consists of two AP-1 (c-Jun/c-Fos heterodimer) site-like sequences that are palindrome arranged and can bind AP-1. c-Jun mRNA in the liver increased after lead administration and GST-P, and c-Jun had patchy expression in the same hepatocytes 24 h after lead exposure. These results suggest that activation of the GST-P gene by lead is mediated in major part by enhancer GPEI and that AP-1 may be involved at least partially. GPEI has been shown to have essential sequence information for the trans-activation of the GST-P gene during chemical hepatocarcinogenesis of the rat (Morimura, S., Suzuki, T., Hochi, S., Yuki, A., Nomura, K., Kitagawa, T., Nagatsu, I., Imagawa, M., and Muramatsu, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2065–2068; Suzuki, T., Imagawa, M., Hirabayashi, M., Yuki, A., Hisatake, K., Nomura, K., Kitagawa, T., and Muramatsu, M. (1995) Cancer Res. 55, 2651–2655). The present study establishes that the same enhancer element does operate in the activation of the GST-P gene by lead regardless of the trans-activators involved.

Glutathione transferase P (GST-P) is an enzyme that catalyzes the glutathione conjugation of electrophilic xenobiotics (1). This enzyme is known to be dramatically increased during chemical hepatocarcinogenesis of the rat (2, 3). This is apparently an induction at the transcriptional level (3, 4), but is somewhat different from usual induction in that it is not reversed by withdrawal of the carcinogen but becomes constitutive in the precancerous liver cells. It has also been reported that GST-P is induced by lead nitrate (5–8) and lead acetate (9), although other metals can hardly affect its production (10). Glutathione is supposed to serve as a first line of defense against heavy metal cytotoxicity prior to induction of metallothionin (11). Rats given lead acetate induce metallothionein-like bound protein and zinc-metallothionein. The binding of lead to metallothionein-like bound protein seems not so tight but metallothionein-like bound-bound protein bound lead accounts for about 60% of the lead in the rat liver cytosol at maximum (12, 13). Zinc-metallothionein is supposed to sequester lead and donate zinc to other zinc-dependent enzyme (11). Therefore GST-P may play an important role in cooperation with metallothionein-like lead-bound protein and zinc-metallothioneins in the detoxification of lead.

To understand the mechanisms of tumor-specific expression of this gene during chemical hepatocarcinogenesis, we have cloned the GST-P gene (4, 14) and identified at ~2.5 kb of the 5′-flanking region a strong enhancer, termed GPEI, whose core consisting of two AP-1 site-like sequences (1-base mismatch for each) is arranged in a palindrome (14–17). By using transgenic rats we have recently demonstrated that the GST-P gene is activated by some transactivator(s) during chemical hepatocarcinogenesis and that the activation requires 5′-flanking region of GST-P gene containing GPEI (18). We have also shown that GPEI itself is the necessary cis-element for GST-P gene expression during this process (19).

Question arises as to whether GST-P expression at lead exposure is regulated at the transcriptional level and whether a common regulatory mechanism of GST-P gene expression is operative between the precancerous liver cells and lead-treated liver cells. To answer these questions, we have utilized transgenic rats having various transgene constructs. We have also transfected NRK fibroblast cells with a series of ECAT deletion mutant genes and determined CAT activity in order to narrow down the lead-responsive DNA region of the GST-P gene. The results indicate that GST-P induction by lead is regulated at...
the transcriptional level and that the essential cis-element for GST-P gene activation by lead is also the enhancer GPEI. Furthermore, to clarify the role of c-jun in the activation of GST-P gene by lead, we studied the expression of c-jun and GST-P in the liver cells at acute lead exposure using immunohistochemistry.

The data show that GPEI is an essential element for the activation of the GST-P gene by lead and that trans-acting factor AP-1 is likely to be involved at least in part in the transcriptional activation of GST-P gene by lead through GPEI sequence.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions—** A 3.0-kb fragment between −2.9 kb (EcoRI) and +59 bp (Acl) of the GST-P gene was inserted into the HindIII site of pSVOCAT and designated as ECAT. A series of 5′ deletion mutants, including 3CAT, were constructed from the ECAT by using appropriate restriction enzymes (35). Δ−56CAT is one of the deletion mutants, containing minimum GST-P promoter (−56 bp to +59 bp) fused with CAT coding region. Although the GST-P gene has one authentic AP-1 site near the promoter at −56 bp, it is disrupted in the Δ−56CAT construct (15). Genomic 122-bp sequence containing GPEI (15, 17) was subcloned into downstream BamHI site of CAT gene in Δ−56CAT and designated as GPEI-Δ−56CAT. Synthetic oligonucleotides having 17-bp GPEI core sequence was inserted into the BamHI site of Δ−56CAT and named as nCAT. We made six lines of nCAT transgenic rats. nCAT gene is activated in the liver of only one line (line 4) during chemical hepatocarcinogenesis (19). Therefore we used nCAT line 4 transgenic rats in the following experiments. The above constructs are depicted in each figure.

**Transgenic Rats, Detection of Transgene, and Animal Experiments—** Transgenic rats having DNA of ECAT, 1CAT, Δ−56CAT, Δ−56CAT GPEI, or nCAT were established as described previously (19). Southern blot analysis of the high molecular weight DNA extracted from the transgenic animals. Each rat transmitted 5–33 transgenes integrated into a surgically removed tails of newborn rats was used to select the transgene insertion. The above constructs are depicted in each figure.

**Fig. 1. Western blot analysis that shows the increase of GST-P in the liver of control rats by lead nitrate.** Lead salt was injected intraperitoneally three times at 24h intervals and livers removed 24 h after the last administration. 100 μmol/kg injection of lead nitrate treatment increased the amount of GST-P most effectively. 10 μg of protein were subjected to Western blots with anti-GST-P antibody. Lane1, control; lane 2, 100 μmol/kg injection of lead nitrate; lane 3, 200 μmol/kg injection of lead nitrate.

**RESULTS**

**Lead Cation Increases GST-P Protein in Liver and Kidney of Rats—** To confirm the effect of lead nitrate on the induction of GST-P in rats and to set the experimental conditions, we first investigated the GST-P protein expression in liver of control rats. Lead nitrate caused accumulation of GST-P protein in liver of control rats as seen by immunoblot analysis (Fig. 1). The amount of basal GST-P was quite low in the rat liver, although the image being intensified on the Fig. 1. GST-P protein in the liver increased 1.8-fold 24 h after intraperitoneal injection of 100 μmol/kg of lead nitrate once a day for 3 days. GST-P protein in the kidney increased in a similar fashion by lead (data not shown). Higher doses, such as 200 μmol/kg, caused lower increases, probably due to the toxic effect to the animal. The results were confirmed by more than two experiments. To confirm that GST-P production was stimulated by lead cation but not by nitrate ion, we tested the effect of lead acetate on the rat liver and kidney. Lead acetate stimulated the production of GST-P, even more effectively than lead nitrate did in the liver and kidney of the rats (data not shown).
GST-P Induction by Lead Requires GPEI

Fig. 2. A, Northern blot analysis that shows the changes of the GST-P, c-jun, junB, junD, and c-fos mRNA contents in the liver of control rats by single intraperitoneal lead acetate administration at a dose of 100 \( \mu \)mol/kg. Time after lead administration is indicated as 'T' (min) and h. Ten \( \mu \)g of total RNA was applied for each lane. Blotted filter was hybridized with GST-P, c-jun, junB, junD, c-fos, and GAPDH cDNA, successively. GAPDH mRNA contents are almost constant throughout the course of the study, which also indicates that almost equal amounts of undegraded RNA are loaded. B, Northern blot analyses of GST-P and c-jun mRNA expression after lead administration in another independent experiment different from that is shown in A. Note that the expression patterns shown here are essentially the same.

Confirming that lead cation was indeed responsible for the induction of GST-P, the result is compatible with the previous report by others (9).

Lead Increases GST-P mRNA at the Transcriptional Level—We next investigated the effect of lead acetate on the content of GST-P mRNA in the liver of rats. As seen in Fig. 2, lead acetate increased GST-P mRNA content significantly at about 12 h after lead administration and decreased by 48 h. After lead injection, c-jun mRNA increased by 15–30 min, once decreased, and then increased at 3 h and decreased and again increased at about 12 h and finally decreased by 48 h. Thus, the amount of c-jun mRNA apparently had three peaks of expression, which was confirmed by repeated experiments (Fig. 2, A and B). We infer that some oscillation of transcription regulators may occur after lead treatment; the first increase of c-jun mRNA may be the result of self-defense immediate early response to acute invasion of xenobiotics such as lead salt, the second peak may have occurred due to the up-regulation of the c-jun transcription by c-jun oncoprotein itself produced by the immediate early response, since c-jun is known to have an AP-1 site at its promoter and to be up-regulated by c-jun (31), and the third peak may be the result of complex interactions of the transcription factors, including c-jun involved in the detoxification of the xenobiotics. Fig. 2 also shows that the third peak of c-jun expression correlates well with the marked increase of GST-P mRNA, suggesting that the third peak may play a role in the expression of GST-P gene. We also studied the mRNA expression of c-fos, junB, and junD, which attenuate the transformation and transcription activities of c-jun by heterodimer formation (c-jun/junB and c-jun/junD) (32, 33), and also of c-fos, which forms more stable Jun/Fos heterodimer (AP-1 complex) (34, 35). Fig. 2A shows that first peak of junB and junD mRNA accumulation occurred at 3 h in a similar fashion as c-jun mRNA. The amount of c-fos mRNA increased at 30 min only once and thereafter kept the low expression level until 48 h. The amount of GAPDH mRNA did not change significantly during the time course studied, which indicates that almost equal amounts of undegraded RNA were loaded. The mechanisms of these oscillations of the Jun family mRNAs are interesting but remain to be elucidated.

To see whether lead-induced GST-P mRNA accumulation was regulated at the translational level, we administered actinomycin D to stop mRNA synthesis (21) before lead injection. Fig. 3 shows the results obtained from control animals. The GST-P mRNA of rat liver increased 2.5-fold 12 h after lead nitrate treatment (Fig. 3, lane 1 versus lane 2), whereas actinomycin D completely blocked the increase (lane 3). In the kidney, GST-P mRNA increased 1.8-fold by lead nitrate (lane 4 versus lane 5), which was also blocked completely by actinomycin D (lane 6). The filter was dehybridized and rehybridized with GAPDH cDNA to confirm that RNA was not degraded by lead nitrate treatment. The result is compatible with the previous report (9) that the expression patterns shown here are essentially the same.

Identification of the Lead-responsive Region of GST-P Gene by Cultured Cell System—To define preliminarily the lead-responsive cis-element of the 5'-flanking region of GST-P gene, we utilized a series of ECAT deletion mutants (Fig. 4A). We first tested the effect of lead nitrate on ECAT gene transfected into the primary culture of rat liver cells. Various concentrations of lead nitrate were tested to primary culture of liver cells without any effect (data not shown), suggesting that the ECAT transgene was suppressed in these cells as the endogenous GST-P gene was in vivo. We then tested the effect of lead nitrate on the ECAT gene transfected into the dRLH 84 rat hepatoma cells. Basal expression of the ECAT gene was rather high in these cells, but no stimulation was observed with lead nitrate (data not shown). Hep G2 human hepatoma cell line gave similar negative results (data not shown). Thus, we found it difficult to study the in vitro effect of lead on ECAT gene using cultured hepatocytes.

Because the expression of the GST-P mRNA was higher in the kidney than in the liver (see Fig. 3), we next examined the effect of lead on the ECAT gene transfected into NRK fibroblast cells (Fig. 4B). The results were shown by the mean values of three independent experiments. Basal ECAT activity was low, but lead could enhance the activity of the ECAT gene 2.7-fold of the control in these cells. Lead nitrate could not activate 1CAT, 2CAT, 3CAT, 4CAT, nor 5CAT deletion mutant genes, from which the GPEI had been deleted. Lead nitrate slightly, but reproducibly, reduced the activity of pSV2CAT transfected into...
NRK fibroblast cells (0.9-fold), and this might be due to the general toxicity of lead nitrate to the cells.

The above results show that the lead-responsive cis-element of the GST-P gene is located between 2.9 and 2.2 kb upstream from the transcription start site of GST-P gene. In this restricted region, there is a strong enhancer GPEI that we have reported previously (14–17).

Demonstration of the Transcriptional Regulation of the GST-P Induction by Lead and the Determination of the Lead-responsive Sequence by Means of Transgenic Rats—To investigate whether GST-P induction by lead is regulated at the transcriptional level and whether the lead-responsive cis-element of the GST-P gene is also mapped in the region 2.9 and 2.2 kb in vivo, we tested the effect of lead acetate on the CAT expression in the transgenic rats having ECAT and 1CAT DNA (Fig. 5). We used three lines each of ECAT and 1CAT transgenic rats. Thirteen, 30, 25, 33, 11, and 26 copies of the transgene were integrated in tandem arrays into the chromosomes of lines 1, 4, and 5 of ECAT and lines 3, 4, and 7 of 1CAT rats, respectively (36). CAT expression was enhanced by lead in the livers and kidneys of all lines of ECAT rats, although the enhancement was rather low in the liver of ECAT line 1 transgenic rat. The observation that CAT expression was enhanced by lead only in the liver or kidney of the ECAT rats, but not in the 1CAT rats, clearly indicates that the cis-element of GST-P gene present in ECAT, but missing in 1CAT, is responsible for the activation of GST-P gene by lead. The results were confirmed by more than two experiments. We have also shown that lead salt induces the acute accumulation of CAT mRNA in the liver of ECAT transgenic rat.2 The results clearly show that GST-P induction by lead is regulated at the transcriptional level. Because CAT mRNA is relatively unstable in animal cells (37), the increase of CAT mRNA by lead should reflect the in vivo enhancement of transcription of the GST-P gene by lead. The result is compatible with the run-on analysis by others (8).

To pinpoint, in vivo, the enhancer region that is essential for GST-P gene expression by lead, we used two more types of transgenic rats. Fig. 6A illustrates the constructs. The construct Δ-56CAT has the minimum GST-P promoter connected

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to CAT coding region. The construct Δ-56CAT GPEI has the genomic 122-bp GPEI (Fig. 6B) subcloned into the Δ-56CAT. We intentionally placed the GPEI element at the 3' end of CAT coding sequence, i.e. 2.0 kb downstream from the transcription initiation site, because we previously noted with transfected primary hepatocytes that the GPEI enhancer had a constitutive enhancing activity when it was located adjacent to the promoter (38). We were afraid that CAT might be constitutively expressed in the liver of transgenic rats, if the GPEI is located too close to the promoter. We used each one line of Δ-56CAT and Δ-56CAT GPEI rat. Southern blot showed that 5 and 21 copies of the transgene were integrated in tandem arrays into the chromosome of Δ-56CAT and Δ-56CAT GPEI rats, respectively (36). CAT activity did not change significantly by lead in the liver of these transgenic rats (data not shown). However, CAT activity of the kidney samples from Δ-56CAT GPEI rats was enhanced 2-fold with lead acetate (Fig. 6C). By contrast, CAT activity of the kidney samples from Δ-56CAT rat was not stimulated at all. The results show that lead stimulates the GST-P expression through the 122-bp GPEI sequence. Our previous data had suggested that 17-bp GPEI core consisting of two AP-1 site-like sequences (see Fig. 7B) alone could confer a similar enhancing activity to 122 bp of genomic GPEI in a cultured cell transfection system (16, 17). To determine whether lead responsiveness of the GST-P gene depends upon the GPEI core sequence or not, we have further made nCAT transgenic rats having only the 17-bp GPEI core sequence inserted into Δ-56CAT at the 3' end (Fig. 7A) and tested the effect of lead acetate on the transgene. Six copies of the transgene were introduced into the chromosome of nCAT rat (line 4) in a tandem manner (36). The CAT activity in the kidney was 2-fold enhanced by lead acetate administration (Fig. 7C), but the CAT activity in the liver did not change significantly (data not shown). The results clearly indicate that GPEI core sequence is sufficient for the GST-P gene expression in the kidney by lead under favorable conditions. Although we could test the effect of lead only on each one line of the transgenic rat having Δ-56CATGPEI or nCAT, we do not think that this activation of transgene by lead was fortuitous due to mere positional effect, for both of the transgenes were activated only...
after lead administration.

Trans-activator AP-1 May Be Involved at Least Partially in the Activation of GST-P by Lead—The next crucial question is how the GST-P gene is activated through the GPEI core sequence by lead. Because GST-P gene activation requires GPEI that has two AP-1 binding site-like sequences, one of the candidate trans-activators is c-jun. In addition, our data show that c-jun mRNA increases prior to the increase in GST-P mRNA (Fig. 2). Therefore, we analyzed the c-jun and GST-P protein distribution in rat liver by immunohistochemistry. In normal liver, both GST-P and c-jun were weakly expressed in the bile duct cells, but not in the hepatocytes under lead-free conditions (Fig. 8, A and B). Forty-eight hours after lead treatment, GST-P had “patchy” distribution in the lobules, and the staining was in the cytoplasm of hepatocytes. We found c-jun increased exclusively in the nuclei of the same hepatocytes expressing GST-P (Fig. 8, C and D). Thus we could demonstrate the expression of both the c-jun and GST-P in lead-treated liver cells at the cellular level. To further clarify the role of AP-1 in the activation of GST-P gene by lead, we studied the effect of antisense oligonucleotides against c-jun mRNA on the GST-P induction by lead using primary culture of the rat liver cells. Our preliminary results indicated that c-jun antisense oligomers attenuated the lead-responsive GST-P production when c-jun expression was repressed (data not shown).

Taken together, we conclude that GPEI has an essential sequence information for the activation of the GST-P gene by lead and that AP-1 may be involved at least partially in the induction of GST-P by lead.

DISCUSSION

We have demonstrated by means of transgenic rats that induction of the GST-P gene, a well known tumor marker for chemical hepatocarcinogenesis, by lead is regulated at the transcriptional level by means of the enhancer GPEI.

Although GST-P has been known to be induced by lead nitrate (5–8), notwithstanding the relative insensitivity to other substances, including heavy metals (10), and most of the previous studies were performed with this compound, we confirmed that lead acetate could induce GST-P in the liver and kidney as good as or even better than lead nitrate did as reported by others (9). This was probably because acetate ion was less toxic to cells covering the peritoneal cavity than nitrate ion, and this was confirmed by the morphological observation.

Two categorically different mechanisms may be considered for the activation of GST-P gene by lead. First, the GST-P gene may be activated, because the chromatin structure of a certain chromosomal locus is specifically altered and activated by lead cation. Second, some trans-acting factor(s) that is induced or activated by lead cation may bind to cis-elements of GST-P gene and thus activate GST-P gene. The present study showing that GST-P gene introduced into transgenic rats is activated in a locus-independent manner supports the latter hypothesis. The GST-P gene is thus activated by some trans-activator(s) at lead exposure just as during chemical hepatocarcinogenesis (18).

Actinomycin D blocked the lead-responsive increase of GST-P mRNA in rat liver and kidney, suggesting that the activation occurred at the transcriptional level. This idea is clearly confirmed by the fact that CAT transgenes, ECAT, Δ-56CATGPEI, and nCAT, but not the Δ-56CAT, were activated by lead in vivo. Others reported that transcription rate of the GST-P gene is enhanced by lead using run-on assay (8). Thus, our transgenic system was found to be useful for investigating in vivo transcription rate of the gene.

The next crucial question is what kind of trans-acting factor(s) will bind to the GPEI and activate GST-P gene after lead exposure. One of the candidates of the trans-activator would be AP-1, since c-jun mRNA elevation does precede GST-P mRNA accumulation, GPEI that is essential for the GST-P gene activation has two AP-1 binding site-like sequences, and GST-P protein is expressed in the hepatocytes having c-jun 48 h after lead treatment. The data suggest that AP-1 may play an important role in the transient expression of GST-P after lead exposure.

The question as to whether activation of the GST-P gene by lead has any similarity to the activation during hepatocarcinogenesis is of special interest in view of the apparent difference in the mode of induction; the former is a transient induction,
and the latter is a semi-constitutive change of gene expression during cell transformation. We note that the expression pattern of c-jun mRNA after lead exposure is complex. It has three peaks of expression after intraperitoneal injection of lead. N-Nitrosodiethylamine, which is given as an initiator for chemical hepatocarcinogenesis of Solt-Farber procedure (39), also causes c-jun mRNA increase (40). The pattern of c-jun mRNA expression after N-nitrosodiethylamine injection, however, was quite different from that after lead treatment. The amount of c-jun mRNA was kept unchanged at a high level from 2 h to 24 h after N-nitrosodiethylamine administration and GST-P mRNA content had its peak at 12 h (40). The difference in the expression pattern of c-jun mRNA might have some relationship to the difference between semiconsitutive and transient GST-P activation during carcinogenesis and lead exposure. The expression pattern of junB and junD mRNA resembled but was slightly different from that of c-jun, indicating that these Jun family proteins might partially contribute to the regulation of GST-P expression by attenuating the activity of AP-1. Involvement of other trans-activators such as Maf family proteins (41-44) that bind to the AP-1 site-like sequence cannot be ruled out and is now under study.

Activation of CAT enzyme activity by GPEI alone in Δ56CAT GPEI rat or GPEI core alone in nCAT rat was significantly lower than that seen in ECAT rat, an observation different from the transient transfection into cell cultures (16). This is probably due to the flanking sequences that might affect the expression of the integrated gene GPEI in the chromosomal context. The larger ECAT may be free from various effects of the integration site, but the smaller GPEI or its core may be subject to the effects of the adjacent sequences that are different from one integration site to another. It is known that a larger flanking region is often required for physiological regulatory phenomenon when analyzed with transgenic animals in a laboratory phenomenon when analyzed with transgenic animals in vitro (45). Although introduced GST-P genes were activated by lead in a somewhat locus-independent manner in GST-P transgenic rats, the degree of activation was different between liver and kidney. It is reported that both c-jun un and c-Fos are rich in kidney, while they are trace-positive or absent in liver (43, 46). Thus, the difference of GST-P activation between organs would be caused by the difference in the distribution of trans-activators.

Identification and cloning of the transcription factor(s) other than c-jun that bind to GPEI and activate or repress the GST-P gene expression are required for further understanding of the regulation of GST-P gene by lead.

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