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Trans-activation of glutathione transferase P gene during chemical hepatocarcinogenesis of the rat

(tumor marker gene/transgenic rat/Solt–Farber procedure/precancerous lesion/chloramphenicol acetyltransferase)

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ABSTRACT **Glutathione transferase P (GST-P; glutathione transferase, EC 2.5.1.18) is known to be specifically expressed at high levels in precancerous lesions and in hepatocellular carcinomas from a very early phase of chemically induced hepatocarcinogenesis in the rat. The almost invariable occurrence of this phenotype in these lesions strongly suggests a mechanism by which GST-P gene is activated together with a crucial transforming gene of liver cells. To distinguish the two alternative possibilities—either the GST-P gene is coactivated with a closely located transforming gene by a cis mechanism or it is activated in trans by a common trans-acting factor—we carried out carcinogenesis experiments using transgenic rats harboring the bacterial chloramphenicol acetyltransferase reporter gene ligated to the upstream regulatory sequence of the GST-P gene. In each of three independent lines tested, liver foci and nodules produced by chemical carcinogens (Solt–Farber procedure) were found to express high levels of chloramphenicol acetyltransferase activity, indicating clearly that the GST-P gene is activated by a trans mechanism during hepatocarcinogenesis.**

Empirically, certain proteins are known to be highly expressed in specific tumor cells and are called "tumor markers" (1). Although they are often valuable for early diagnosis of various cancers and for postoperative management and chemotherapy, the molecular mechanisms underlying their expression in cancer cells are unknown. Why and how are these genes activated concomitantly with the malignant transformation of certain cell types? Elucidation of the mechanisms by which those genes are activated may shed some light on the mechanism of malignant transformation of these cells. Glutathione transferase P (GST-P; glutathione transferase, EC 2.5.1.18), a member of a family of enzymes that detoxify xenobiotics by glutathione conjugation, is present in small quantities in rat tissues such as lung, kidney, testis, spleen, and placenta, but is present only in trace amounts in normal liver (2–5). This protein is highly expressed (50–100 times), however, in putative precancerous foci and nodules as well as in hepatocellular carcinomas induced by hepatocarcinogens such as diethylnitrosamine (DEN), aflatoxin B_1, 2-acetylaminofluorene (AAF), and 3′-methyl-4-dimethylaminoazobenzene (2, 3), with the rare exception of peroxisome proliferators such as clofibrate. Because GST-P is not present in fetal liver and does not increase in regenerating liver, it is not a so-called oncofetal antigen nor is its expression related to cell growth alone (2).

To study the mechanism by which GST-P gene is regulated, we cloned the gene (6, 7) and analyzed the upstream control regions using various cells including the hepatoma cell line dRLh84 and an embryonal carcinoma cell line, F9. Two enhancers were found clustered at around −2.7 kb (8). The stronger one, termed GST-P enhancer I (GPE I), consists of two 4β-phorbol 12-tetradecanoate 13-acetate-responsive element (TRE)-like sequences that are palindromically oriented with 3 bp in between (9). Although GPE I is composed of two TRE-like sequences, it is active in F9 embryonal carcinoma cells that lack c-Jun protein, suggesting that it can function with some trans-activator other than AP-1 (c-Jun/c-Fos heterodimer) (10). Indeed, we have recently identified two factors that bind specifically to GPE I sequence (data not shown). In addition to the positively regulating regions, a negatively regulating region (silencer) was also found at around −300 bp. This region contains several sequences that resemble each other to a certain extent and specifically bind at least three factors, termed SF-A, SF-B, and SF-C (11). Recently, cDNA of SF-B has been cloned and found to be identical to that of liver-enriched transcriptional activator or inhibitory protein LAP/LIP/NF-IL6 (12). Thus, GST-P gene appears to be regulated by multiple DNA elements and protein factors in a complex manner that remains to be clarified in future studies.

Apart from the individual controlling elements, the mechanism of activation of the GST-P gene as a tumor marker is a central issue, because some gene(s) that leads the liver cell to a malignant state (here termed a "hepatooncogene" for simplicity) is thought to be activated together with the GST-P gene. Categorically, two different mechanisms may be considered for the simultaneous activation of two genes. (Fig. 1). One is cis activation, in which two closely located genes are coactivated by local activation of chromatin (Fig. 1*A*). Although the precise mechanism is not clear, a number of such examples are known, including the activation of c-*myc* gene by translocation of active immunoglobulin heavy chain gene in mouse plasmacytoma cells (13, 14) and the activation of c-*abl* oncogene by translocation to the *bcr* region of chromosome 22 in human leukemia cells (15). Another well-studied example is the locus-activating region (16, 17) or dominant control region (18, 19) that is present far upstream of the human β-globin gene and activates a large cluster of the gene family by changing local chromatin structure (20, 21). The other mechanism to be considered is trans activation, in which the putative hepatooncogene and the GST-P gene need not be located near each other. Fig. 1*B* depicts only the simplest model in which one common regulator transactivates both genes. There may be variations in this category—

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Abbreviations: GST-P, glutathione transferase P; CAT, chloramphenicol acetyltransferase; DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; GPE I, glutathione transferase P enhancer I.

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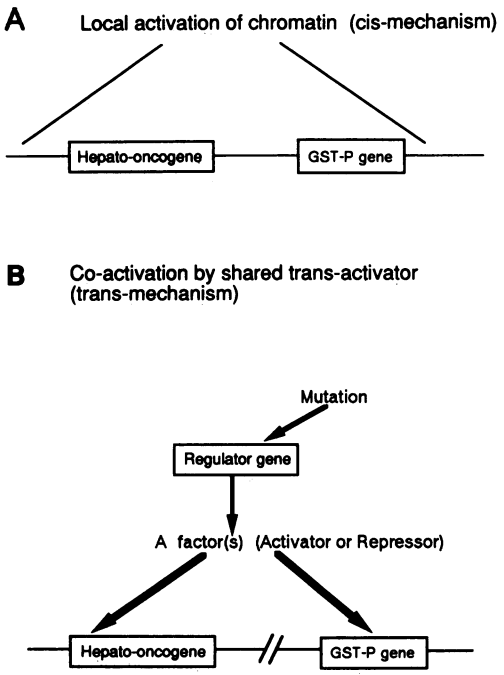

FIG. 1. Two alternative mechanisms of the simultaneous activation of a tumor marker (here GST-P) gene and an as yet unidentified gene presumed to play a crucial role in carcinogenesis in a specific tissue (here termed "hepatooncogene"). (*A*) A cis-mechanism, in which local activation of chromatin simultaneously activates the hepatooncogene and the GST-P gene. (*B*) A trans mechanism, in which the GST-P gene and the hepatooncogene are not linked but share a trans-activator (regulator) and are coactivated by some mutation affecting its function or expression. More complex models for trans-activation are possible; only the simplest example is shown (see text).

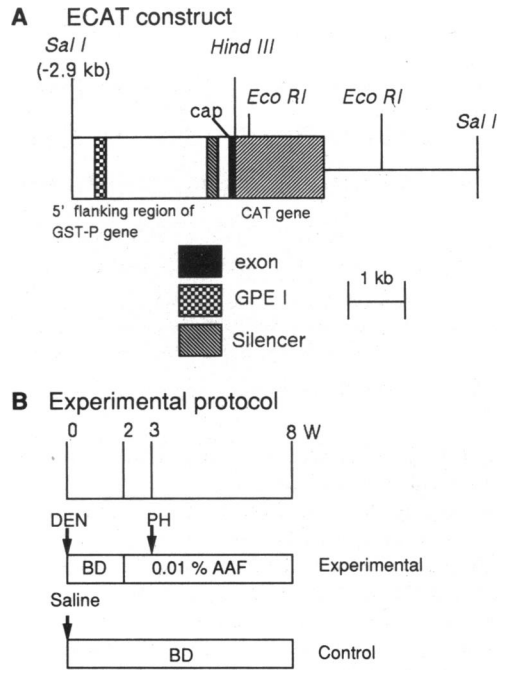

FIG. 2. (*A*) GST-P-CAT construct used to produce transgenic rats. Sequence elements (8–10) are shown as different boxes and the vector plasmid is shown as a horizontal bar. Major restriction sites are also shown. Cap represents the transcription start site. (*B*) Schematic representation of the Solt–Farber method for chemically induced hepatocarcinogenesis (23). PH, partial hepatectomy; BD, basal diet.

e.g., intervention of another gene between the regulator and effector or hierarchal control between hepatooncogene and GST-P gene, etc.

In this communication, we describe a decisive experiment with transgenic rats by which the GST-P gene is shown to be activated by a trans mechanism.

MATERIALS AND METHODS

Production of Transgenic Rats. A 2959-bp fragment of the GST-P gene from −2900 to +59 bp relative to the cap site of GST-P mRNA was inserted into the *Hin*dIII site of pSV0CAT and the resulting plasmid was designated ECAT (8). ECAT plasmid digested with *Sal* I was microinjected into the male pronuclei of fertilized eggs from Wistar rats. Injected eggs were transferred to oviducts of pseudopregnant rats (22). Seven independent founders, each with intact copies of the transgene, were identified by Southern blot analysis. Three lines, designated line 1, line 4, and line 5, were able to transmit the transgenes to their offspring and were bred out into permanent lines. The copy numbers of the integrated transgenes were 13, 30, and 25 for lines 1, 4, and 5, respectively.

Carcinogenic Experiments. The animals in each line were divided into two groups. Experimental and control groups, each consisting of three independently derived male transgenic rats, were subjected to the Solt–Farber protocol (23) as outlined in Fig. 2*B*. Experiments were initiated by injecting 200 mg of DEN per kg into 6-week-old Wistar male transgenic rats. After feeding the rats the basal diet for 2 weeks, the diet was changed to basal diet containing 0.01% AAF. Partial hepatectomy was performed at the beginning of the third week and most of the rats were sacrificed at the end of the eighth week. Controls were fed basal diet.

Protein Preparation and Chloramphenicol Acetyltransferase (CAT) Assay. Rat tissues were excised, 1 g of which was homogenized in 2.0 ml of cold 0.25 M Tris-HCl (pH 7.5) and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatants were stored at −20°C. The supernatants were heated for 10 min at 65°C to inactivate endogenous acetyltransferase activity, and samples containing 100 μg of protein were assayed for CAT activity as described (24) and quantified using the Fuji-BAS 2000 system (Fuji Film, Tokyo).

Western Blot Analysis. For Western blot analysis, proteins of liver extracts were separated by electrophoresis on a 12.5% sodium dodecyl sulfate/polyacrylamide gel, transferred to nitrocellulose membrane, and probed with the GST-P-specific polyclonal antibody (Bioprep, Dublin, Ireland). The reactive Immunoglobulins were detected with an affinity-purified goat anti-rabbit IgG alkaline phosphatase conjugate (Zymed Laboratories).

Histological Method. Tissue samples from experimental ECAT line 1 male rats were fixed in ice-cold acetone, and paraffin-embedded sections were used for immunohistochemical staining with either rabbit anti-CAT antibody or rabbit anti-GST-P antibody (a generous gift from Kiyomi Sato, Hirosaki University, Hirosaki, Japan). Serial sections were visualized by the avidin–biotin–peroxidase complex method (25) (Vector Laboratories).

RESULTS AND DISCUSSION

To determine whether the GST-P gene and the putative transforming gene for liver cells are activated by a cis or trans mechanism during hepatocarcinogenesis, we constructed transgenic rats harboring the upstream control region of the GST-P gene ligated to the CAT structural gene. The principal reason for using rats rather than mice is that the GST-P

counterpart is significantly expressed in normal mouse hepatocytes, especially in the male (26), and therefore does not serve as a specific marker of precancerous lesions. A number of transgenic founder rats were produced by injecting a GST-P-CAT construct, termed ECAT (Fig. 2*B*), into male pronuclei of fertilized eggs (22). The founder rats were back-crossed to normal rats to obtain F_1 animals. After confirming that ECAT gene was transmitted to F_1 (through germ line), three independent lines were chosen for hepatocarcinogenic experiments according to the Solt–Farber procedure (23) (Fig. 2*B*). Southern blot analysis showed that rats of lines 1, 4, and 5 integrated 13, 30, and 25 copies of ECAT, respectively, in tandem arrays (data not shown). At the end of 8 weeks, the rats were killed, the livers (which had a large number of foci and nodules) were excised, and CAT activity was measured.

The data shown in Fig. 3*A* clearly indicate that in each of the three transgenic lines tested, the livers of rats subjected to the Solt–Farber protocol exhibited high CAT activity. By contrast, liver tissue from untreated transgenic rats showed no CAT activity (Fig. 3*B*). Although we observed little variation among individual animals within a given line, the degree of the inducibility was dependent on the transgenic line. Interestingly, following exposure to DEN and AAF, line 4 rats tended to show significantly higher expression of ECAT transgene than rats from other lines, not only in liver tumor but also in other normal tissues. The livers of untreated line 4 rats, however, did not show any CAT activity. As mentioned above, normal kidney, lung, and spleen are known to express small amounts of GST-P in normal rats. The variability in these tissues was emphasized in this line for some unknown reasons. Since the ECAT transgene copy number in line 4 is not much higher than that in line 5, its high inducibility is presumably due to the chromosomal location of its integration. Although the precise nature of the variability is unknown, this observation is consistent with the assumption that the transgenes are integrated at different sites in each founder strain.

To corroborate the observation that CAT and the endogenous GST-P gene are coexpressed during carcinogenesis, a time course study was carried out (Fig. 4). In each line tested, CAT activity appeared in 3 weeks when the number of GST-P-positive cells became significant and increased gradually to a high activity at 6 weeks (Fig. 4*A*). Endogenous GST-P increased in a similar fashion (Fig. 4*B*), suggesting that the introduced CAT gene was regulated in the same way as endogenous GST-P gene. As another control, regenerating liver was examined. In each line tested, CAT activity did not increase 1 day after partial hepatectomy, indicating that the introduced GST-P genes are not activated in regenerating liver (data not shown). This is in line with the previous observation that GST-P does not increase in regenerating

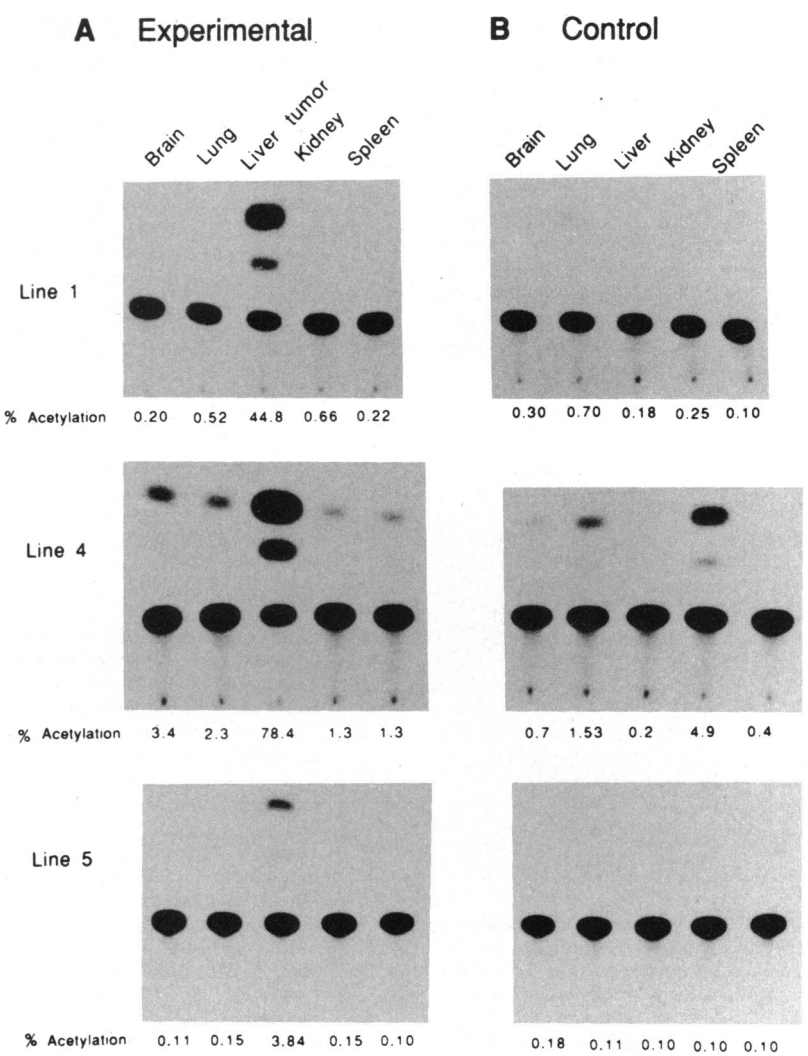

FIG. 3. Liver tumor-specific expression of GST-P-CAT (ECAT) in transgenic rats. (*A*) Experimental. Tissues were taken from a rat that was subjected to the Solt–Farber protocol for 8 weeks. (*B*) Control. Tissues were from an untreated rat (see Fig. 2). Lines 1, 4, and 5 are independent transgenic rat lines. Typical results from two or three experiments that showed similar data for each are shown.

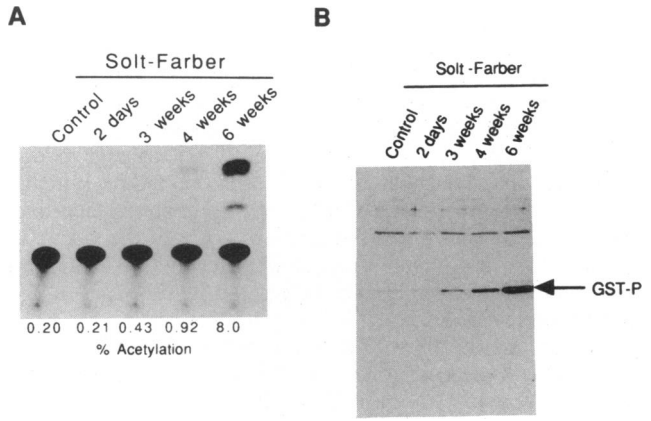

FIG. 4. (*A*) Expression of ECAT at early stages of chemical hepatocarcinogenesis. Liver extracts were prepared from a transgenic line (line 5) at specified times and CAT activity was assayed. The numbers of days or weeks at the top indicate times after DEN injection in the experimental protocol (Fig. 2). (*B*) Expression of GST-P at early stages of chemical hepatocarcinogenesis. Aliquots of the above samples were electrophoresed and examined with anti-GST-P antibody. The arrow marks the GST-P protein band.

liver (2). This result favors the idea that ECAT transgenes are more or less normally regulated in the liver.

Finally, an immunohistochemical study was made to characterize the alteration of gene expression at each cell level. As shown in Fig. 5, anti-CAT antibody specifically stained the same foci as did the anti-GST-P antibody, indicating that the increase in CAT activity detected by biochemical determination is indeed due to the altered cells by this carcinogenic protocol.

Given the near-random nature of transgene integration, the probability that the three ECAT genes in the independent transgenic rat founder lines are all integrated into chromosomal loci close to the putative hepatooncogene is virtually zero. Therefore, we interpret the above data to mean that the ECAT gene was activated during the course of carcinogen treatment by a trans mechanism. The above study has thus demonstrated that at least one tumor marker can be activated by a trans mechanism. Whether this mechanism can be generalized for other tumor markers remains to be established. This study also demonstrates that the sequence present in the GST-P gene between −2.9 kb and +59 has sufficient sequence information for GST-P gene activation during hepatocarcinogenesis. Experiments are necessary to determine the sequence essential for this activation in transgenic rats.

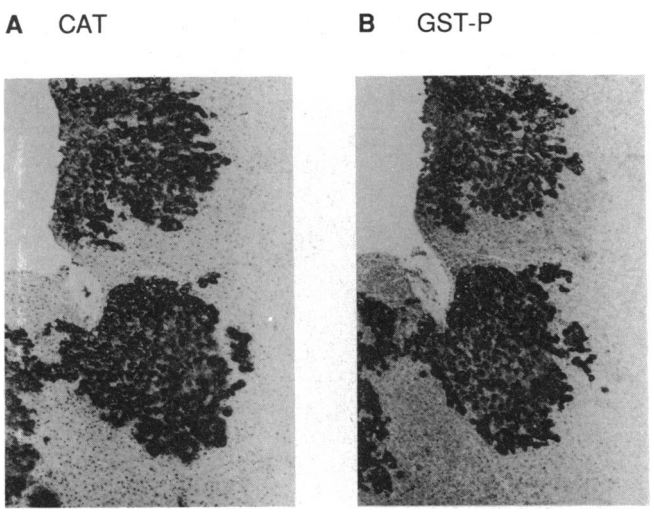

FIG. 5. Immunohistochemical demonstration of the coincidental expression of ECAT and GST-P in focal lesions of altered hepatocytes. Serial sections of liver from a transgenic rat (line 1) that was subjected to the Solt–Farber protocol for 8 weeks were immunostained with either rabbit anti-CAT antibody (*A*) or rabbit anti-GST-P antibody (*B*) with the immunoperoxidase staining method. (×30.)

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