Persistent infection by hepatitis B virus (HBV) and exposure to chemical carcinogens correlates with the prevalence of hepatocellular carcinoma in endemic areas. The precise nature of the interaction between these factors is not known. Glutathione S-transferases (GST) are responsible for the cellular metabolism and detoxification of a variety of cytotoxic and carcinogenic compounds by catalysis of their conjugation with glutathione. Diminished GST activity could enhance cellular sensitivity to chemical carcinogens. We have investigated GST isozyme expression in hepatocellular HepG2 cells and in an HBV-transfected subline. Total GST activity and selenium-independent glutathione peroxidase activity are significantly decreased in HBV transfected cells. On immunoblotting, HBV transfected cells demonstrate a significant decrease in the level of GST Alpha class. Cytotoxicity assays reveal that the HBV transfected cells are more sensitive to a wide range of compounds known to be detoxified by GST Alpha conjugation. Although no significant difference in protein half-life between the two cell lines was found, semi-quantitative reverse transcription-polymerase chain reaction shows a reduced amount of GST Alpha mRNA in the transfected cells. Because the HBV x protein (HBx) seems to play a role in HBV transfection, we also demonstrated that expression of the HBx gene into HepG2 cells decreased the amount of GST Alpha protein. Transient transfection experiments using both rat and human GST Alpha (rGSTA5 and hGSTA1) promoters in HepG2 cells show a decreased CAT activity upon HBx expression, supporting a transcriptional regulation of both genes by HBx. This effect is independent of HBx interaction with Sp1. Treatment with oltipraz, an inducer of GST Alpha, partially overcomes the effect of HBx on both promoters. Promoter deletion studies indicate that oltipraz works through responsive elements distinct from AP1 or NF-xB transcription factors. Thus, HBV infection alters phase II metabolizing enzymes via different mechanisms than those modulated by treatment with oltipraz.

An estimated 350 million people worldwide contract some form of hepatitis per year. Epidemiological and experimental data have demonstrated that individuals chronically infected with HBV have a high incidence of developing HCC (1–4), and HBV DNA sequences have been shown to be integrated into cellular DNA of human HCC (5). Strikingly, the incidence of HCC is even greater in areas where there is also exposure to liver carcinogens such as aflatoxins (6–8).

Different mechanisms have been suggested as significant in the development of HCC following HBV infection. An activated host immune response and increased production of reactive oxygen species have been shown to be important in triggering abnormal liver cell growth (9). In addition, HBV encodes a small protein x (HBx) that seems to play a critical role in hepatocarcinogenesis both in humans and in animal models (10, 11). HBx has been found to be expressed in chronic hepatitis, cirrhotic liver, and HCC from individuals infected with HBV (11–15). The HBx protein demonstrates trans-activating ability for viral and cellular genes through protein-protein interaction with several components of the transcription machinery and signaling cascades (16–20). HBx has been shown to bind and inactivate the p53 tumor suppressor protein, alter the cell cycle, and interfere with apoptosis and DNA repair mechanisms (21–26). The processes that associate development of HCC with viral infection, HBx biological properties, and the role and action of concomitant exposure to liver carcinogens are still not clear.

The glutathione S-transferases are members of a family of detoxification enzymes that metabolize a variety of carcinogens by conjugating lipophilic electrophiles to glutathione. They also bind nonsubstrate ligands including bile acids and bilirubin (27, 28). The mammalian cytosolic GST isozymes are dimeric and have been divided into seven classes: Alpha, Kappa, Mu, Pi, Sigma, Theta, and Zeta. Alpha GSTs in all species are designated as GSTA1–GSTA5. The human GSTA1 shows a high degree of homology with rat GSTA5. In human and rodent liver, GST Alpha is the predominant form expressed, and GST Pi protein is not found in normal adult hepatocytes. GST Mu is absent in approximately 50% of the general population as a result of a frequent mutation (29). The fact that GSTs represent as much as 5% of the cytosolic protein in the liver suggests that they may play an important role in maintaining cellular homeostasis. The various GST isozymes have different cata-

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1 The abbreviations used are: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; GST, glutathione S-transferase; CAT, chloramphenicol acetyl transferase; nt, nucleotide; RT, reverse transcription; PCR, polymerase chain reaction; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CMV, cytomegalovirus; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AFB1, aflatoxin B1.
lytic activities and patterns of tissue distribution, suggesting that they may be a contributing factor in tissue-specific susceptibility to the carcinogenic process. Some highly reactive chemicals are able to conjugate glutathione directly; however, in most cases the biotransformation of these compounds via phase I activation results in a more electrophilic molecule. For instance, AF81-8,9-epoxide, the hepatocarcinogenic derivative of AF81, is detoxified by the formation of a glutathionyl-AFB1 conjugate. Studies with purified rat isozymes demonstrate that this reaction is catalyzed by enzymes containing Alpha class subunits of GST (30, 31).

The GST Alpha isozyme can be induced by agents such as phenobarbital and oltipraz (31, 32). Oltipraz (4-methyl-5-pyrazinyl-H-1,2-dithiole-3-thione) has been found to have cancer chemopreventive properties (33, 34). Inhibition of phase I enzymes, induction of phase II xenobiotic metabolizing enzymes, regulation of oxygen reactive metabolites, and enhancement of DNA repair processes are known properties of oltipraz (35–41). Because GST Alpha from human liver is inducible by oltipraz, Alpha GSTs may also reduce the susceptibility to hepatocarcinogenesis by enhancing carcinogen detoxification and elimination (36, 38).

Although much is known about the structure and function of GSTs, little work has focused on the changes that occur in the different GSTs following liver disease. In malignant hepatocytes from clinical specimens, Alpha subunit expression is almost always dramatically decreased. The reduction in expression appears to parallel the cellular transformation process, because adenomas demonstrate intermediary levels of GST Alpha (42–46). This loss of GST protection could increase the susceptibility of preneoplastic populations of hepatocytes to further contribute to genotoxic injury by chemicals during malignant progression.

In these studies, we reasoned that HBV transfection could diminish normal cellular detoxification potential, and thus, concomitant exposure to chemical carcinogens would be more likely to contribute to HCC development. We have identified changes that occur specifically in GST Alpha following the expression of HBV gene products in hepatocytes. Furthermore, we have determined the potential of oltipraz to overcome this effect.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The human hepatoblastoma cell line, HepG2, and its HBV transfected counterpart, HepG2/HBV (kindly provided by Dr. Wands, Massachusetts General Hospital, Boston, MA; Ref. 47), were grown in α-minimum essential medium with nonessential amino acids, sodium pyruvate, Earle’s balanced salt solution, and 90% and 10% fetal bovine serum (FBS). The rate of growth of both cell lines was shown to be similar.2 CCL13 cells are human liver epithelial cells. These cells were maintained in Dulbecco’s modified Eagle’s medium, 10% FBS, and 90% and 10% fetal bovine serum (FBS) and kept at room temperature.

**Northern Blot Analysis**—RNA extractions and Northern blot analyses were performed according to standard protocols (49). 15 μg of each RNA sample was electrophoresed in 2% agarose/formaldehyde gel at 100 V for 3 h, followed by transfer onto Zeta-Probe membrane (Bio-Rad). An EcoRI fragment of pGem-adw2 containing the entire HBV viral genome was radiolabeled with [α-32P]dCTP by random primer extension (Oligo Labeling Kit; Amersham Pharmacia Biotech) and used as probe. Hybridization was carried out at 42 °C using 50% formamide, 4X SSC, 4X Denhardt’s reagent, 1.2% SDS, and 0.2 mg/ml salmon sperm DNA. Final washes of blots were done with 2X SSC and 0.1% SDS at 65 °C. The same membrane was probed with β-actin cDNA to control for sample loading and transfer efficiency.

**Protein Turnover Studies**—The turnover of GST Alpha was measured in exponentially growing HepG2 and HepG2/HBV cultures. Cells were incubated in complete medium supplemented with 150 μg/ml cycloheximide (ICN Biomedicals Inc., Mississauga, Canada) for 2, 4, and 10 h. Following these time periods the cells were washed twice with PBS and lysed by resuspending them in 1 ml of lysis buffer (10 mX Tris-HCl, pH 8, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml apro- tinin/4 × 10^5 cells).

**Pharmacological Assays**—Cytosolic extracts from the various cell lines were prepared by cell lysis and centrifugation of debris at 12,000 × g for 1 h. Protein concentrations were determined according to the method of Lowry, using bovine serum albumin as the standard. Total GST activity was assayed using 2-chloro-1,3-dinitrobenzene as the substrate (43). Selenium-independent glutathione peroxidase activity was assayed using cumene hydroperoxide (44). Student’s t test was used for statistical analysis.

**Cytotoxicity Assays**—Cells were plated in 100 μl of medium at a concentration of 2–10 × 10^3 cells/flat bottomed well in 96-well microtiter plates that were incubated for 24 h at 37 °C in an atmosphere of 5% CO₂. 100 μl of medium containing drug dissolved in appropriate solvent were added to triplicate wells and incubated for a further 72 h. Medium (100 μl) was then added to each well and replaced by 150 μl of medium containing 10 μg 1,4-piperizinediethanol succinyl hydrazide, acid pH, 7.4, and 50 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) at 2 mg/ml in PBS. Plates were then wrapped in aluminum foil and incubated for 4 h at 37 °C. The formazan crystals were dissolved in 180 μl of dimethyl sulfoxide (Fisher) and 25 μl glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) by mixing on a shaker for 5 min. The formazan product formed by viable cells was then quantitated by measuring the absorbance at a wavelength of 570 nm on a microtiter plate reader. IC₅₀ was determined by plotting the percentage of surviving cells versus the log of drug concentration. Statistical analysis was performed using Student’s t test for paired samples.

**Plasmid Constructs**—The expression vectors for the HBx protein used in this study were: pCMV-HBx, consisting of the HBx gene from the HBV ayw subtype (nucleotides 1241–1991) ligated into the HindIII site of the pRc-CMV vector (Invitrogen, La Jolla, CA). The HBx gene was kindly provided by Dr. J. Cromlish (21). The pRc-CMV plasmid was used as a negative control and referred to as pRc-CMV. pAP2 is a retroviral expression vector encoding for a bicistronic nonsplicing retrovector that incorporates a multiple cloning sites, allowing insertion of sequences flanked by an IRES to the enhanced green fluorescent protein (GFP) reporter. The HBx gene from the HBV subtype ayw (nucleotides 1241–1991) (21) was subcloned into the XhoI-BamHI site of the pAP2 vector and referred to as pAP2-HBx. The pAP2 vector was used as a negative control. Retroviral producer cell lines were generated for both pAP2 and pAP2-HBx by stably transfecting the 293GPG packaging cell line (48). VSVG pseudotyped retroparticles were constructed as described (46). These were utilized to transduce target cells. The rGSTA5-CAT reporter plasmid was a generous gift from Dr. Board (50). The 5′ deletion constructs of the rGusta5 promoter (accession number S82821; Ref. 51) were created by PCR amplification followed by subcloning into the pCAT-Basic reporter plasmid. The primers were RP1 (190 bp): 5′-ggccagcagcctgatagtcgcttcg-3′; FP1 (190 bp): 5′-ggtgagtagttacagctgctgcttcg-3′; FP2 (939 bp): 5′-gtaaacgagagctgtcagagag-3′; FP3 (761): 5′-gagggaggcaagttcaggttctggg-3′; FP4 (476 bp): 5′-gagggaggcaagttcaggttctggg-3′; and FP5 (353): 5′-ccctgagctctgggtcctcgcttcg-3′. The position of each deletion site is indicated in Fig. 4A. The deletion clones produced by PCR were sequenced using T7 Sequenase v2.0 (Amersham Pharmacia Biotech). The chloramphenicol reporter construct pERE3-CAT containing API-responsive elements (52) and the reporter construct 5′b containing NF-κB sites were kindly provided by Dr. Mader (Université de Montréal, Montreal, Canada) and Dr. J. Hiscott (McGill University, Montreal, Canada).

**Retroviral Production**—293GPG cells were plated at a concentration of 4 × 10^5/ml in the day before transfection in the appropriate medium. 5 μg of linear DNA containing either pAP2 or pAP2-HBx were co-transfected with the Zeocin resistance plasmid pJ6122bIce (48) using LipofectAMINE according to the manufacturer’s recommendations (Life Technologies, Inc.). Selection for cells stably transduced with pAP2 or pAP2-HBx was performed in 3 weeks in 293 cell medium containing 100 μg/ml of Zeocin (Invitrogen). Fluorescence-activated cell sorter analysis was performed to determine the percentage of producer cells that expressed the GFP reporter protein. Cells expressing GFP were selected using a fluorescence-activated cell

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1 I. Jaitovitch-Groisman, N. Fotouhi-Ardakani, R. L. Schecter, A. Woo, M. A. Alauii-Jamali, and G. Batist, unpublished data.
softer STAR PLUS TURBO (530–30; FL-1) cell sorter and immediately sorted for hGSTA1 expression. The predominant GST subtype in HepG2 cells is class A. The cytosolic protein from HepG2 liver, the predominant GST subtype in HepG2 cells is class A. The quantification of the reaction products in the CAT assay was performed using a Hoefer scanning densitometer, model GS300 (Hoefer Scientific, San Francisco, CA).

**RESULTS**

**Expression of HBV in HepG2 Cells**—The integrity of the hepatitis B virus DNA sequence in HepG2 cells was verified by Northern blot analysis. Fig. 1 shows the results where total RNA from HepG2 and HepG2/HBV cells were hybridized with an HBV cDNA. Only infected cells carry the message for this gene. Our result showing more than one HBV transcripts is in agreement with the earlier reports indicating that different viral transcripts are produced prior to viral replication (5). The same membrane was reprobed with \( \beta \)-actin cDNA to verify for sample loading and transfer efficiency.

**Biochemical Characterization**—The activities of GST and selenium-independent glutathione peroxidase in HepG2 and HepG2/HBV are shown in Table I. There was, however, a significant difference in total GST activity toward 2-chloro-1,3-dinitrobenzene between the two cell lines. GST activity in HepG2 was 3.2-fold higher than in HBV transfected HepG2 cells. Using cumene hydroperoxide to measure GST Alpha (glutathione peroxidase) activity specifically, transfected cells demonstrated an 86% decrease in GSHPx activity relative to control cells.

**Cytotoxicity Assays**—The drug sensitivity of HBV transfected cells was examined in cytotoxicity assays. The results of these experiments are presented in Table II. The chemicals studied are known substrates for GST Alpha detoxification. HBV transfected cells were hypersensitive to the antiproliferative effects of melphalan, cisplatin, and BCNU. The increase in sensitivity ranged from 1.8- to 3-fold relative to uninfected cells.

**GST Protein Expression**—The cytosolic protein from HepG2 and HepG2/HBV cells was analyzed by immunoblotting to examine the pattern of GST isozyme expression. Significant differences in protein expression were seen (Fig. 2A). As in normal liver, the predominant GST subtype in HepG2 cells is class A. GST Alpha protein expression is strongly decreased in HBV transfected cells. GST Mu isozyme protein is slightly decreased in HepG2/HBV cells, and the absence of GST Pi was a consistent feature in both the parental and HBV transformed cells (data not shown). The turnover time of GST Alpha in HBV transfected cells was compared with that in untransfected cells. Both cell lines were exposed to 150 \( \mu \)g/\( \mu \)l of cycloheximide for up to 10 h. After consecutive time points, extracts were analyzed by radioautography. The membrane was reprobed with \( \beta \)-actin cDNA for verification of sample loading and transfer efficiency.

**Semi-quantitation of GST Alpha mRNA by Polymerase Chain Reaction**—Total RNA was isolated from HepG2 and HepG2/HBV treated and untreated with 45 \( \mu \)M oltipraz for 36 h, using the RNeasy Total RNA Kit (Qiagen, Inc., Chatsworth, CA). The RNA was treated with RNase-free Dnase I and further cleaned (RNeasy Total RNA kit). Each RNA sample (1 \( \mu \)g) was reverse transcribed using murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and oligo(dT) primer for 1 h at 37 °C. PCR amplification of hGSTA1 and GAPDH cDNAs were performed using 50 pmol of each primer, 5 \( \mu \)Ci of \( [\text{\textsuperscript{32}P}]\)dCTP, and 2.5 units of Taq polymerase (Amerham Pharmacien Biotech) for 30 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The appropriate cDNA volumes were \( \frac{1}{100} \) for hGSTA1 and \( \frac{1}{5000} \) for GAPDH. These dilutions were determined to be in a linear range of each standard curve. Samples were run on a 10% acrylamide gel dried and exposed. The quantification was performed using the Bio-Rad Molecular Imager (Bio-Rad) software program.

**Expression and Analysis of HBV mRNA**—20 \( \mu \)g of total RNA from HepG2 cells (lane 1) and HBV transfected HepG2 cells (lane 2) were hybridized with an HBV cDNA, which recognizes the viral genome. Transcripts of expected sizes were visualized by radioautography. The membrane was reprobed with \( \beta \)-actin cDNA for verification of sample loading and transfer efficiency.
prepared and subjected to immunoblotting using anti hGSTA1 antibody (Fig. 2B). The stability of the protein did not appear to be any different between the two lines even up to 12 h in the presence of cycloheximide (not shown). The effect of cycloheximide on protein synthesis was confirmed by reacting the same samples with antibody to detect expression of NF-kB and demonstration of the anticipated protein half-life (data not shown). Although the levels of the hGSTA1 protein are decreased in HepG2/HBV cells, treatment with oltipraz increased the protein level, although not to the same level observed in the HepG2/HBV cells, treatment with oltipraz increased the protein half-life (data not shown).

### Table I

| Chemical | IC_{50} (means ± S.D.) |
|----------|------------------------|
| Melphalan | 7.91 ± 0.28            |
| CDDP     | 1.46 ± 0.36            |
| BCNU     | 271.0 ± 27.50          |

*p < 0.01.

### Table II

Cytotoxic effect of chemicals on HepG2 and HepG2/HBV cells

Drug sensitivity assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay. Results represent the means of three independent assays, each cell line being tested in quadruplicate in every individual assay. IC_{50} is the concentration that inhibits 50% of cell growth. CDDP, cis-diaminedichloroplatinum.

- **HepG2**
- **HepG2/HBV**
- **Melphalan**
- **CDDP**
- **BCNU**

*p < 0.01.

**Fig. 2. GST Alpha Protein and mRNA expression in HepG2 and HepG2/HBV cells.** A, 25 μg of protein from whole cell extract from HepG2 and HepG2/HBV cells were separated by polyacrylamide gel electrophoresis and transferred into nitrocellulose. The membrane was probed with a monoclonal antibody for hGSTA, stripped, and reprobed with a monoclonal antibody for GAPDH as described under "Experimental Procedures." B, GST Alpha turnover was measured after incubation with cycloheximide at different time points. Cells were lysed in protease inhibitors, and cell extracts were subjected to SDS-polyacrylamide gel electrophoresis. C, mRNA from HepG2 and HepG2 HBV cells treated with oltipraz and left untreated was reverse transcribed, PCR amplified with specific primers that amplify a fragment of 500 base pairs from hGSTA1 cDNA, and semiquantitated as described under "Experimental Procedures."
by oltipraz is disputable and, therefore, not yet defined. Oltipraz clearly transactivates these genes through an effect on their promoters; however, some data suggest that this effect depends on an AP1-like element sequence, whereas others support a rate-limiting role for the NF-κB motif (54). To examine their involvement in GST Alpha induction by oltipraz, we prepared a series of 5′-deletion constructs for the rGSTA5 promoter (Fig. 4A). Elimination of both NF-κB and AP1-like element consensus elements do not affect the induction of the rGSTA5 promoter (Fig. 4B), suggesting that oltipraz does not require the presence of either NF-κB or AP1 transcription factors.

Effect of HBx on the Endogenous Expression of GST Alpha Proteins—Because of a low transfection efficiency using lipid complexes and the abundance of GST Alpha in liver cells, a decrease in the endogenous levels of GST Alpha was difficult to prove in the whole cell population. To overcome this limitation, we subcloned the HBx gene into a bicistronic retroviral vector expressing the GFP, which serves as a marker for retroviral expression. Following HepG2 cell transduction, 81 and 77% of the cells were positive for GFP in cells transduced with AP2 and AP2-HBx retroviral particles, respectively. As a positive control, to demonstrate that the HBx protein produced following retroviral expression was functional, we evaluated its transactivation capacity on regulatory elements known to be transactivated by HBx such as AP1 or NF-κB. For this purpose, HepG2 cells transduced with AP2 or AP2-HBx retroviral particles were transfected with two different reporter plasmids, containing either Ap1 or NF-κB regulatory elements. CAT activity was fourfold higher in cells transduced with AP2-HBx than in cells transduced with the negative control. These results confirmed that the HBx protein produced was functional.

FIG. 3. Effect of HBx on hGSTA1-CAT and rGSTA5-CAT activities. HepG2 cells were transfected with increasing amounts of pCMV-HBx (A) or with 3 μg/well of pCMV-HBx (B) or the negative control (C). Cell extracts were used to determine CAT activity as described under "Experimental Procedures." The results, percentages of acetylated chloramphenicol to nonacetylated metabolites, are expressed as percentages of CAT Activity for each HBx transfected sample compared with the control. Each bar corresponds to the average ± S.D. for at least three independent experiments. For A hGSTA1 driven CAT expression was used, whereas in B we show the activity of both promoters upon HBx expression. C show and D CAT activity with and without oltipraz treatment following HBx expression or transfection with the negative control.

**Fig. 4.** Effect of HBx and oltipraz on the rGSTA5 promoter. A, schematic representation of the rGSTA5 promoter constructs. Known responsive elements are shown. B, promoter activity of the rGSTA5 deletion constructs. HepG2 cells were transfected with 3 μg of each rGSTA5 promoter construct (control) or co-transfected with 3 μg of pCMV-HBx expression vector (HBx). 24 h later, cells were treated with 45 μM of oltipraz (Olz and HBx + Olz, respectively). The transfection efficiency was normalized by cotransfection with the plasmid containing β-galactosidase gene. Each bar corresponds to the average ± S.D. for at least three independent experiments.
To determine the effect of HBx on endogenous GST Alpha protein, we examined its expression on HepG2 following cell transduction with AP2 or AP2-HBx retroviral particles. Compared with control cells, expression of HBx down-regulates the endogenous expression of hGSTA1 (Fig. 5A), whereas no significant differences were observed between HepG2-AP2 transduced and HepG2 nontransduced cells (data not shown). To confirm that this effect is not cell restricted, CCL13 (Chang) cells were transduced following the same protocol. The levels of hGSTA1 were also reduced in CCL13 cells (Fig. 5B).

The transcriptional regulation of endogenous GST Alpha protein by HBx was confirmed by semiquantitative RT-PCR on HepG2 transduced cells. Cells expressing HBx show lower level of hGSTA1 mRNA compared with cells transduced with the negative control (Fig. 5C). When HepG2 cells were treated with oltipraz, the hGSTA1 mRNA levels increased even upon expression of HBx (Fig. 5C).

**DISCUSSION**

An emerging theme among DNA tumor viruses is that viral encoded oncoproteins interact specifically with critical cellular regulatory proteins and that the oncogenic effects of these viruses are at least in part, a consequence of these specific interactions. Published data indicate that HBV itself is not a transforming virus, and thus it must act in some way to enhance the transforming capacity of other factors such as environmental toxins. Indeed, HCC in particular, occurs more frequently in livers that have been damaged by both chemical toxins and HBV (6–8), but the mechanism of this synergism is not known. The recent observation that very high levels of DNA damage accumulate in hepatocytes of transgenic mice with HBV suggests that antioxidant and DNA repair mechanisms are suboptimal (9). In this regard, the organic peroxidase activity of GST Alpha in liver is highly significant and critical. In

**FIG. 5.** GST Alpha expression in cells expressing HBx. A and B are Western blots for hGSTA1 expression of whole cell extracts from HepG2 and CCL13 cells, respectively, that were transduced with AP2-HBx or AP2 retroviral particles as described under “Experimental Procedures.” C shows the results of a semiquantitative RT-PCR for hGSTA1 mRNA with and without oltipraz treatment.

To determine the effect of HBx on endogenous GST Alpha protein, we examined its expression on HepG2 following cell transduction with AP2 or AP2-HBx retroviral particles. Compared with control cells, expression of HBx down-regulates the endogenous expression of hGSTA1 (Fig. 5A), whereas no significant differences were observed between HepG2-AP2 transduced and HepG2 nontransduced cells (data not shown). To confirm that this effect is not cell restricted, CCL13 (Chang) cells were transduced following the same protocol. The levels of hGSTA1 were also reduced in CCL13 cells (Fig. 5B).

The transcriptional regulation of endogenous GST Alpha protein by HBx was confirmed by semiquantitative RT-PCR on HepG2 transduced cells. Cells expressing HBx show lower level of hGSTA1 mRNA compared with cells transduced with the negative control (Fig. 5C). When HepG2 cells were treated with oltipraz, the hGSTA1 mRNA levels increased even upon expression of HBx (Fig. 5C).

**Involvement of Sp1 Transcription Factor and p53 on the Regulation of GST Alpha by HBx**—Although no previous report exists on the involvement of Sp1 transcription factor and the tumor suppressor protein p53 on the regulation of the hGSTA1 and rGSTA5 promoter activity, HBx has been shown to interact with both proteins. Conversely, because both promoters contain Sp1-responsive elements (Fig. 6A), we further investigated whether the observed transcriptional down-regulation was linked to HBx interaction with Sp1.

Transient transfection experiments on HepG2 cells using the rGSTA5 deletion constructs suggest that Sp1 is not involved in the down-regulation of GST Alpha by HBx protein (Fig. 4B). To confirm our findings, we used the Drosophila SL2 cells in the next transfection experiments. The Drosophila SL2 cells provide an Sp1-deficient background to assess the activity of both promoters in the absence of Sp1. Both hGSTA1 and rGSTA5 promoters are active in this cell line. Furthermore, HBx decreased CAT activity of both hGSTA1 and rGSTA5 promoters in these cells, suggesting that the previously described HBx-Sp1 interaction is not the mechanism involved in GST Alpha transcriptional down-regulation (Fig. 6B). Because these cells do not express human wild type p53, we can also infer that hGSTA1 and rGSTA5 promoter down-regulation by HBx does not depend on p53 status in the cells. The absence of p53 consensus sequence on both promoters provide further support for p53-independent regulation of the GST Alpha by HBx.

**FIG. 6.** GST promoter activity in SL2 cells. A, several regulatory elements important for GST expression in both human and rat promoters are depicted. B, two reporter plasmids containing either hGSTA1- or rGSTA5-driven CAT expression were used. The expression vector for HBx (pCMV-HBx) or the empty vector (pRC-CMV) was cotransfected with either hGSTA1-CAT or rGSTA5-CAT. CAT activity for either promoter is relative to pRC-CMV. 100% CAT activity represents the percentage of acetylated to nonacetylated metabolite obtained after transfection with pRC-CMV. Each bar corresponds to the average ± S.D. for at least three independent experiments.
most animal models of hepatocarcinogenesis there is the early appearance of Pi class GST by neoplastic hepatocytes not present in normal adult liver cells. In humans, however, GST Pi expression has not been found during hepatic neoplasia. On the other hand, much more variability exists in the expression of both Alpha and Mu class isozymes. The concentration of Alpha class GST, the predominant isozyme found in normal liver, decreases dramatically in malignant hepatocytes (44, 45) and in HCC from HBV-infected individuals (55). A reduction in Alpha and Mu class GST is seen in tumor as compared with normal tissues from kidney and breast (56, 57).

HepG2 cells are moderately differentiated and produce a spectrum of normal hepatocyte proteins, and the pattern of expression of GSTs is similar to normal adult liver (58). Although these cells express α-feto protein, they are not tumorigenic in nude mice. We recognize that the oncogenic potential of HBV cannot be determined in HepG2 cells that are derived from hepatoblastoma. However, the HBV transfected cells can be utilized to test the effects of various xenobiotics given the observation that many are detoxified by GST Alpha enzymes (59, 60). We have shown that HBV transfected HepG2 cells have decreased GST Alpha subunit levels concomitant with depressed GST and glutathione peroxidase activity and are hypersensitive to the effects of DNA alkylating agents such as melphalan, BCNU, and cisplatin. We have found an increased sensitivity to AFB1 and B(a)P cytotoxicity using these assays, although not strong,2 probably because of the fact that these carcinogens require metabolic activation to a cytotoxic species by cytochrome P-450 enzymes. Furthermore, the effects of decreased GST Alpha may be more subtle regarding carcinogenicity than is obvious using a less sensitive cytotoxicity assay. Indeed, one study has provided evidence that expression of GST Alpha can protect differentially against genotoxic and potentially mutagenic effects without necessarily affecting the cytotoxicity of electrophiles. The susceptibility of HBV-infected cells to environmental toxins may be considerable, because it has been demonstrated that slight increases in Alpha class isozymes are able to decrease DNA adduct formation by 90% in AFB1-treated cells (61). Administration of AFB1 to woodchucks with viral hepatitis was shown to result in a significantly earlier appearance of hepatocellular neoplasm and a higher incidence of HCC compared with viral carriers not treated with AFB1 (62). In this model, the similarity of preneoplastic foci after both viral and chemical (AFB1) exposure suggest common underlying molecular mechanisms for carcinogenic development where GST Alpha may be implicated. Finally, expression of HBx in the liver cell line CCL13 sensitizes cells to carcinogens normally detoxified by GST enzymes (63). In addition, hepatocytes from adjacent areas of fibrosis and inflammation in sections of liver infected with HBV have shown a marked increase of cytochrome P-450 (64), thus increasing the susceptibility to AFB1 genotoxicity.

The regulation of GST genes have been examined in several different models. There is evidence of altered GST Pi half-life and its mRNA in chemically treated tumor cells (65). Increased rates of transcription have also been demonstrated as a mechanism to regulate the GST Alpha gene product (61). Hypomethylation of the GST Pi promoter was shown as a mechanism to down-regulate Pi in human prostate cancer (65). We have provided evidence that GST expression in HBV transfected HepG2 cells is regulated transcriptionally.

The decrease in GST activity correlates with diminished levels of Alpha class and to a lesser extent Mu class and suggests that propagation of HBV may be more favorable under these conditions. Interestingly, after SV40 infection of human fibroblasts, GST activity was found to decrease initially and then return to normal levels after passaging (66). We have shown here that the presence of an HBV genome may functionally compromise liver cells by significantly reducing the detoxification potential offered by GST Alpha. This change might favor the likelihood of increased susceptibility to carcinogenic development not only by exogenous chemicals but also by endogenous toxins normally metabolized by GST. Our data suggest that the interaction of HBV gene products with detoxifying enzymes may contribute to the synergistic effect of HBV and chemical carcinogens in the development of liver carcinogenesis.

Although we have not compared GST Alpha protein structure from transfected and untransfected cells, amino acid sequencing of GST Alpha from HepG2 and normal liver demonstrated that they were in fact identical (67). Cycloheximide inhibition of de novo GST Alpha synthesis, however, failed to show any difference between the rates of degradation in the two cell lines studied over the course of 12 h. Exposure for greater than 12 h was not performed because it has been previously demonstrated that cycloheximide degradation occurs after this time (68). If in fact a difference in turnover time exists between the protein in transfected versus untransfected cells, detection of this difference should have been apparent within 12 h. Semiquantitative RT-PCR clearly shows that the reduced protein levels are due to a decreased transcription of the hGSTA1 gene. Moreover, treatment with oltipraz induces transcription of hGSTA1 in both HepG2 and HepG2/HBV cells, partially overcoming the viral effect on mRNA levels.

Three highly related hepadnaviruses, the human hepatitis B virus, the woodchuck hepatitis virus, and the ground squirrel hepatitis virus, cause liver cancer in their hosts (5). Although there is still a poor understanding of the mechanisms that associate viral infection to carcinogenesis, it is striking that the three mammalian hepadnaviruses share the regulatory gene x, whereas no counterpart of this gene is found in the non oncogenic duck hepatitis B virus. Many different biological properties have been ascribed to this ~17-kDa protein. It has been reported that HBx affects transcription, signal transduction, DNA repair, cell cycle control, and apoptosis. One of the best documented activities of HBx is the transcriptional transactivation of a wide range of cellular promoters cellular genes such as interleukin 6, tumor necrosis factor α, and transforming growth factor β1 (69). Whereas directly or indirectly, HBx associates with several transcription factors resulting in promoter transactivation, this activity may also interfere with the regular expression of other genes.

We report in this study that following HBx expression, a down-regulation of hGSTA1 and rGSTA5 promoter activities was observed. Both the human and rat homologue promoters are equally affected by this viral protein, strongly suggesting common conserved regulatory elements in their gene structure. Reduced levels of GST Alpha protein were confirmed in HepG2 and CCL13 liver cell lines transduced with the AP2-HBx retroviral particles. The effect of HBx on hGSTA1 transcription was confirmed utilizing semiquantitative RT-PCR on HepG2 cells and upon HBx expression. Interestingly, when HepG2 cells expressing HBx were treated with oltipraz, the levels of hGSTA1 mRNA increased, overcoming in great proportion the effect of HBx. Several mechanisms may explain the protective effects attributable to oltipraz with respect to viral infection and chemical carcinogenesis. Inhibition of phase I enzymes, induction of phase II xenobiotic metabolizing enzymes, regulation of oxygen reactive metabolites, and enhancement of DNA repair processes are known properties of oltipraz (33–41). Oltipraz was shown to have potent inhibitory activity against the reverse transcriptase of HIV and to inhibit HBV transcription...
through elevation of p53 protein (70). In this study we report that oltipraz overcomes the HBx transcriptional down-regulation of hGSTA1, thus adding strength to the case for testing oltipraz treatment in HBV infection. Adjacent areas of fibrosis and inflammation in sections of liver infected with HBV have shown a marked increase of cytotoxicity P-450 (64). Interestingly, oltipraz decreases P450 expression (33). However, these experiments were performed independently.

The regulation of both hGSTA1 and rGSTA5 promoter activities has had limited study. Because decreased levels of the corresponding metabolizing enzymes have been shown to be decreased in neoplastic processes (65), we further investigated the involvement of two possible regulatory factors in down-regulation of GST Alpha promoters by HBx. Promoter deletion studies as well as transfection in Drosophila SL2 cells clearly shows that this effect is independent of HBx indirect association with Sp1 (71). Although neither hGSTA1 nor rGSTA5 promoters contain any known p53-responsive element, because of the known interaction between HBx and p53 the contribution of this association on GST Alpha transcriptional down-regulation by HBx could be also considered. The participation of the HBx/p53 association cannot be involved as mediating HBx transcriptional down-regulation of these enzymes, because the Drosophila SL2 cells do not express human wild type p53. We did find that expression of Sp1 in these cells strongly stimulates CAT activity from both promoters, whereas expression of human wild type p53 protein does not modify it.2 Neither Sp1 nor p53 expression modifies the effect of HBx on GST Alpha. Interestingly, the GSTP1Xenzymaticcatalystselenozymes

dependent enzymatic reactions is strongly transactivated by the p53 tumor suppressor protein (72).

Because expression of the GST family of proteins is affected in HBV and HBx transfected cells, two different mechanisms seem to regulate the activity of phase II metabolizing enzymes during this process. One of them is probably related to the oxidative damage induced by the surface/envelope viral protein, whereas the other is directly related to transcriptional modulation by HBx. We have shown that the use of oltipraz overcomes the effect of both HBV mechanisms in the regulation of GST isoforms expression. The mechanism of action of oltipraz at the molecular level is controversial and still unidentified. In this study, we presented evidence that the transcriptional induction of the GST Alpha genes by oltipraz is mediated through responsive elements distinct from NF-kB or AP1 transcription factors. The fact that a variety of cell defense mechanisms are modified during HBV expression and that oltipraz can at least partly overcome the effect on GST Alpha strengthens the case for studying the use of oltipraz as a chemopreventive agent in hepatocellular carcinoma.

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