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Chemical Inducers of Rodent Glutathione *S*-Transferases Down-Regulate Human *GSTA1* Transcription through a Mechanism Involving Variant Hepatic Nuclear Factor 1-C

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ABSTRACT

The regulation of human *GSTA1* by chemical inducers of rodent glutathione *S*-transferases (GSTs) and the regulatory role of hepatic nuclear factor (HNF) 1 was investigated in Caco-2 cells. Treatment of preconfluent and confluent cells with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), 3-methylcholanthrene (3-MC), 2-*tert*-butyl-4-hydroxy-anisol (BHA), and phenobarbital (PB) reduced GSTA1 mRNA levels in preconfluent and confluent cells. Constitutive levels of GSTA1 and HNF1α mRNA were elevated 6.25- and 50-fold, respectively, in postconfluent cells compared with preconfluent cells. Overexpression of HNF1α in cells transfected with a GSTA1 promoter-luciferase construct (pGSTA1-1591-luc) resulted in dose-related increases in reporter activity not observed when an HNF1 response element (HRE) in the proximal promoter was mutated (pGSTA1-ΔHNF1-luc). TPA, 3-MC, BHA, and PB reduced HNF1α mRNA levels in preconfluent and confluent cells and caused marked reductions in luciferase activity in pGSTA1-1591-luc transfectants. Transcriptional repression was abrogated with pGSTA1-ΔHNF1-luc and with truncated constructs that eliminated a functional HRE. Moreover, cotransfection of pHNF1α with pGSTA1-1591-luc partially prevented the reduction in luciferase activity by rodent GST inducers. Immunoblot analysis of DNA binding studies indicate that variant (v)HNF1-C binding to HRE is increased in preconfluent cells treated with 3-MC, BHA, and PB. In addition, overexpression of vHNF1-C repressed GSTA1 transcriptional activity in luciferase reporter assays. Finally, treatment with 3-MC, BHA, and PB increased vHNF1-C mRNA levels in preconfluent cells. These data demonstrate that repression of human *GSTA1* transcription by chemical inducers of rodent GSTs occurs, in part, through a mechanism involving the repressive action of vHNF1-C.

Glutathione *S*-transferases (GSTs) are a family of multifunctional proteins involved in the detoxification of a broad range of xenobiotics and therapeutic compounds, playing a critical role in protecting cells from reactive electrophiles. Rodent GSTs are inducible by drugs, carcinogens, antioxidants, and other dietary components. Inducers such as 2-*tert*-butyl-4-hydroxyanisole (BHA), phenobarbital (PB), 3-methylcholanthrene (3-MC), dithiolethiones, and ethoxyquin differentially control the regulation of mouse and rat hepatic GST isoenzymes and have led to the discovery of new inducible α- and μ-class subunits (Ding and Pickett, 1985; Hayes et al., 1991). Transcriptional activation of rodent α class GSTs occurs via the antioxidant-responsive element (ARE), required for induction by dithiolethiones and antioxidants, as well as the xenobiotic-responsive element, which was first noted as a mediator in the induction of cytochrome P450 1A1 by polyaromatic hydrocarbons. (Rushmore et al., 1990; Hayes and Pulford, 1995; Eaton and Bammler, 1999).

Only a few studies have addressed the regulation of human *GST* genes. Although the intron and exon structure of the two major human *GSTA* genes (*A1* and *A2*) is highly homologous with those of the rat and mouse, there are significant differences in the regulation of transcription between the rodent and the human *GSTA* genes (Morel et al., 1994; Suzuki et al., 1994). For example, the human *GSTA* 5′-flanking region lacks the ARE and the xenobiotic-responsive element through which the rodent α genes are regulated (Klone et al., 1992). Potential regulatory elements have been identified in the human *GSTA1* promoter, including AP-1 and AP-2 consensus sequence and a glucocorticoid response element

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ABBREVIATIONS: GST, glutathione *S*-transferase; BHA, 2-*tert*-butyl-4-hydroxy-anisol; PB, phenobarbital; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; 3-MC, 3-methylcholanthrene; ARE, antioxidant-responsive element; AP, activator protein; HNF, hepatic nuclear factor; v, variant; luc, luciferase; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); h, human; HRE, hepatic response element; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; ERE, electrophile responsive element.

(Whalen and Boyer, 1998). In addition, a hepatic nuclear factor (HNF) 1 site localized to −182 and −170 of the GSTA1 promoter region functions as an enhancer (Klone et al., 1992; Clairmont et al., 1994). Moreover, promoter activity was not elevated in HepG2 cells transfected with CAT reporter plasmids driven by the 5′-flanking sequence of the human *GSTA1* gene and subsequently treated with three archetypal rodent *GSTA* inducers: β-naphthoflavone, 3-MC, or *tert*-butylhydroquinone (Suzuki et al., 1994). Likewise, treatment of HepG2 cells with the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) caused a decrease of GSTA mRNA levels to below 5% of controls (Eickelmann et al., 1995). Thus, despite strong similarities in protein primary structure, tissue distribution, and cDNA sequence, lack of sequence homology between the promoter regions of the human and rodent *GSTA* genes suggests that the transcriptional regulation may be dramatically different.

The Caco-2 intestinal epithelial cell line is derived from a human colonic adenocarcinoma. Caco-2 cells are widely used as an in vitro model of the intestinal epithelium because they spontaneously differentiate, in the postconfluent state, into mature enterocyte-like cells that form polarized monolayers sealed by tight junctions and display a well developed apical brush-border membrane (Anderberg et al., 1993). Upon differentiation, there is an increase in specialized enzymatic and structural features detectable as differentiation markers, including expression of dipeptidyl peptidase IV, sucrase isomaltase, intestinal alkaline phosphatase, and villin (Vecchini et al., 1997). Moreover, GSTA1 activity and protein expression is significantly increased as Caco-2 cells differentiate (Peters and Roelofs, 1989), making this cell line particularly appropriate for studying *GSTA1* regulation and expression. Previous studies in our laboratory demonstrated that treatment with interleukin-1β down-regulates the expression of GSTA in Caco2 cells by a transcriptional mechanism involving an HRE (Romero et al., 2002). Therefore, we hypothesized that archetypical inducers of rodent GSTs reduced *GSTA1* transcriptional activity through a mechanism involving HNF1. We postulated that increases in HNF1α expression were responsible for the elevated levels of GSTA1 in differentiated Caco-2 cells and that higher levels of HNF1α might compensate for the repressive effects of rodent GST inducers. We found that GSTA1 mRNA was markedly reduced in Caco2 cells incubated for 24 h with TPA, 3-MC, BHA, and PB. Therefore, reporter activity also was reduced in similarly treated Caco2 cells transfected with GSTA1 luciferase reporter plasmids. Using truncated constructs, this down-regulatory effect was mapped to a region between −355 and −165 bp upstream to the coding region and mutation of an HNF1 site within this region abrogated the effect. We demonstrate that treatment with xenobiotics reduced expression of the transcriptional activator HNF1α and decreased its binding to HRE in preconfluent Caco-2 cells. Moreover, 3-MC, BHA, and PB increased mRNA levels of the dominant repressive isoform vHNF1-C and increased vHNF1-C-HRE complex formation in preconfluent cells. These results demonstrate clear differences in the transcriptional regulation of *GSTA1* by chemicals that normally induce GSTs in rodents and establish pivotal roles for HNF1α and vHNF1-C in the down-regulation of this gene.

Materials and Methods

Chemicals and Antibodies. 12-*O*-Tetradecanoyl phorbol-13-acetate, 3-methylcholanthrene, 2-*tert*-butyl-4-hydroxy-anisol, and dimethyl sulfoxide as well as monoclonal α-tubulin antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Sigma (Oakville, ON, Canada). Phenobarbital was obtained from BDH Inc. (Toronto, ON, Canada). Human recombinant GSTA1-1 antibody was obtained from Biotrin International (Dublin, Ireland), and HNF1-specific antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [γ-^{32}P]ATP (3000 mCi/mmol) was purchased from GE Healthcare (Piscataway, NJ).

Cell Culture and Treatments. Caco-2 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin under 5% CO_2 at 37°C. The cells were seeded in 60-mm dishes for mRNA isolation and cell extract preparation and in 75-cm^2 flasks for nuclear protein extraction. Cells were treated with 50 nM TPA, 5 μM 3-MC, 100 μM BHA, and 5 mM PB for 24 h at approximately 80% confluence. Test compounds were dissolved in dimethyl sulfoxide such that all cell cultures contained a final concentration of 0.1% dimethyl sulfoxide in the media.

Isolation of RNA and Real-Time RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions, and concentrations were determined by absorbance at 260 nm. Isolated RNA (1 μg/20-μl reaction volume) was used for first-strand cDNA synthesis using 0.1 μg of random primers, 20 units of RNase inhibitor, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time PCR was performed on a Roche light cycler using a DNA Master SYBR Green I kit (Roche Diagnostics, Mississauga, ON, Canada). The PCR reaction was done in a volume of 10 μl, containing 1 μl of SYBR Green I, 5 μM each primer, and 2 mM Mg^+. The PCR parameters were 1 cycle of 95°C for 1 min and 35 cycles of 95°C for 15 s, 70°C for 5 s, and 92°C for 15 s. The mRNA levels were normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA. Primer sequences used for real-time RT-PCR are shown in Table 1.

A standard curve was generated for the absolute quantification of vHNF1A/B and vHNF1-C mRNA levels by real-time RT-PCR. Complementary DNA for vHNF1A/B and vHNF1-C was produced by PCR amplification from total cDNA using primers specific for vHNF1A/B and vHNF1-C. Ten-fold dilutions of the PCR products were then used to construct the standard curve.

Plasmid Construction and Reporter Assays. The proximal −1591-bp promoter of the *hGSTA1* gene was cloned into the pGL3 basic vector (Promega, Madison, WI) upstream of the firefly luciferase gene, by using the XhoI and KpnI sites. In brief, the −1591-bp fragment was amplified with specific oligonucleotide primers (designed from the 5′-flanking region of the *hGSTA1* gene; accession no.

TABLE 1
Primer sequences for real-time RT-PCR

GSTA1	
Sense	5′-GACTTCACGCCACCCATCCT-3′
Antisense	5′-TGGGACAGGTGGGACTGGTT-3′
HNF1α	
Sense	5′-GACTTCACGCCACCCATCCT-3′
Antisense	5′-TGGGACAGGTGGGACTGGTT-3′
vHNF1-A/B	
Forward	ACGGCCTGGGCTCCAACTTGGTCACT
Reverse	AACCCTTAAACCAGATAAGATCCGT
vHNF1-C	
Forward	ACGGCCTGGGCTCCAACTTGGTCACT
Reverse	TTCAACCTCCTCCTGAGACTGAGATCAT
Glyceraldehyde-3-phosphate dehydrogenase	
Sense	5′-ACAGTCCATGCCATCACTGCC-3′
Antisense	5′-GCCTGCTTCACCACCTTCTTG-3′

X67663) using human genomic DNA as template. The amplified fragment was cloned into the pCR-TOPO cloning vector (Invitrogen) following product instructions. The *GSTA1* promoter was released by incubating with KpnI and XhoI and inserted into the luciferase vector to create pGSTA1-1591-Luc. A series of reporter constructs with promoter truncations were also designed including pGSTA1-850-Luc, pGSTA1-355-Luc, pGSTA1-165-Luc, and pGSTA1-84-Luc. An HNF1 site at −182 to −170 bp was mutated by site-directed mutagenesis from the pGSTA1-1591 plasmid, using a QuikChange site-directed mutagenesis kit (QIAGEN, Mississauga, ON, Canada) and designated as pGSTA1-ΔHNF1-Luc. Promoter sequences and the presence of the mutation were confirmed by direct sequencing. Primer sequences for the luciferase reporter gene constructs are shown in Table 2.

Promoter activity of the pGSTA1-Luc constructs was determined by the dual luciferase reporter assay system (Promega). Caco2 cells (1.2×10^5 cells/well in 24-well plates) were transfected for 18 h in suspension in fetal bovine serum-DMEM that included each of the various hGSTA1 promoter-luciferase constructs (1 μg/well) as well as the *Renilla reniformis* luciferase plasmid pRL-TK (10 ng/well) in the presence of LipofectAMINE reagent (3 μl/well) (Invitrogen). Transfected cells were then incubated in supplemented DMEM containing 50 nM TPA, 5 μM 3-MC, 100 μM BHA, or 5 mM PB for 24 h at 37°C. Cells were harvested and dual luciferase assays were performed according to the manufacturer's instructions. The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of *R. reniformis* luciferase. Similar control experiments were performed with a basic promoterless pGL3-luciferase plasmid. The expression plasmids pHNF1α and pvHNF1-C were kind gifts from Dr. Marie-Josée Vilarem (Institut National de la Santé et de la Recherche Médicale, Montpellier, France) and Dr. Moshe Yaniv (Institut Pasteur, Paris, France), respectively.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay. Nuclear extracts were isolated from confluent Caco2 cells grown on 10-cm dishes after treatment with chemicals for 24 h, by a modification of the Dignam method (Dignam et al., 1983). In brief, cells were washed in ice-cold phosphate-buffered saline, harvested by scraping, and resuspended in 100 μl of ice-cold buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9, 0.5 M DTT, and 0.5 mM phenylmethylsulfonyl fluoride, containing 2 μg/ml pepstatin, 10 μg/ml aprotinin, and 2 μg/ml leupeptin) in a prechilled 1.5-ml microfuge tube. The cells were lysed by passage through a 25-gauge needle several times. Nuclei were pelleted by centrifugation at 14,000*g* at 4°C, extracted in 60 μl of buffer C (20 mM HEPES, 25% glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, and 2 mM EDTA, pH 7.9, supplemented with protease inhibitors as described in buffer A), and incubated for 15 min on ice. Thereafter, 60 μl of buffer C (20 mM HEPES, 25% glycerol, and 0.2 mM EDTA, pH 7.9, with added protease inhibitors) were added. Cellular debris was then precipitated by centrifugation, and the supernatant was stored as nuclear extracts at −80°C. Protein concentrations of nuclear extracts were determined by the Bradford dye-binding technique using the Bio-Rad protein assay kit (Bio-Rad, Mississauga, ON, Canada). A double-stranded oligonucleotide of the HNF1 response element in the *hGSTA1* was end-labeled with [γ-^{32}P]ATP and T_4 polynucleotide kinase (GE Healthcare) according to manufacturer's instructions, except that reactions were allowed to continue for 1 h at 37°C. The sequence of the HNF1-containing oligonucleotide was 5′-GAAAAGGAACACATTAACCAGTTT-3′, and the mutant was 5′-GAAAAGGAACACATcgcCCAGTTT-3′. DNA binding reactions were performed in a 20-μl volume containing 0.5 ng of radiolabeled probe, 10 μg of nuclear extract, and 1× gel shift binding buffer using a Gel Shift Assay kit (Promega) according to manufacturer's protocol. Binding reactions were incubated at room temperature for 20 min. For competition assays, a 100-fold molar excess of unlabeled oligonucleotide was incubated with the samples. In supershift experiments, the nuclear extracts were preincubated with 2 μg of HNF1 antibody for 1 h. Protein-bound and free probes were separated by electrophoresis in 6% acrylamide gels in 0.5× Tris borate-EDTA buffer at 200 V for 15 min. The gels were dried and visualized by filmless autoradiographic analysis using the Typhoon 9410 gel and blot imager (GE Healthcare).

To identify the specific nuclear proteins bound to the HNF1α response element (HRE), biotinylated probes and streptavidin-agarose were used in an affinity purification procedure as described previously (Scotto et al., 1999). Binding reactions included 50 μg of nuclear extract and 5 μg of biotinylated HRE oligonucleotides identical in sequence to those used in EMSA. Streptavidin-agarose 50% (Novagen. Madison, WI) was treated with 1 mg/ml bovine serum albumin and 0.2 mg/ml salmon sperm DNA to block nonspecific binding and equilibrated for 1 h in binding buffer. Pretreated streptavidin-agarose was combined with DNA-protein complexes and incubated for 1 h at room temperature. Streptavidin-agarose was washed three times with phosphate-buffered saline, and proteins bound to biotinylated target DNA were eluted after a 1-h incubation in 1% SDS, 10 mM DTT.

SDS-Polyacrylamide Gel Electrophoresis, Western Blot Analysis, and Two-Dimensional Gel Electrophoresis. HNF1 protein was identified by Western blot analysis as described previously (Romero et al., 2002). In brief, equal volumes of affinity-purified nuclear protein were separated by SDS-PAGE on a 12% polyacrylamide gel and either stained with SYPRO stain or transferred to nitrocellulose and incubated for 1 h with either rabbit anti-human HNF1α or HNF1β (vHNF1) polyclonal antibody (Santa Cruz Biotechnology, Inc.) diluted to 1:250. After incubation with goat anti-rabbit peroxidase secondary antibody (Vector Laboratories, Burlington, ON, Canada), bands were detected by chemiluminescence (ECL Plus; GE Healthcare) and visualized using a Typhoon 9410 scanner (GE Healthcare).

Statistical Analysis. One-way analysis of variance was used to assess statistically significant differences among treatment groups. For each statistically significant effect, Fisher's least significant test was used for comparison between multiple group means. Significance was established at $P < 0.05$.

Results

Effect of Rodent GST Inducers on Human GSTA1 and HNF1α mRNA Levels. There is limited sequence homology between the 5′-flanking regions of human, mouse, and rat α class GSTs, suggesting that transcription may be regulated differently between these species. Indeed, previous studies have shown that GSTA1/2 mRNA levels in HepG2

TABLE 2

Primer sequences for the luciferase reporter gene constructs

The antisense primer sequence for all PCR reactions was TGAATTCCAGGTCCTAATGTATTT. KpnI restriction sites used in the primer design are underlined. Bases in lowercase letters are the mutated positions for the HNF1α response element (HRE).

pGSTA1-1591-Luc	5′-CAGATTTCCAAACTCCCCATA-3′
pGSTA1-850-Luc	5′-GGGGTACCGATTTGGGGCCTATCCAGA-3′
pGSTA1-355-Luc	5′-GGGGTACCTATTTTATTTGGCAACCCATGA-3′
pGSTA1-165-Luc	5′-GGGGTACCCTTCTGATAAGCAGATCACTTGC-3′
pGSTA1-84-Luc	5′-GGGGTACCTTCCCTAACTTGACCCTTCTTT-3′
pGSTA1-ΔHNF1-Luc	5′-CAACCTTGAAAAGGAACACATcgcCCAGTTTCTTCTGATAAGCAG-3′

and Caco-2 cells decrease when treated with TPA. To determine whether inducers of rodent GSTs induce mRNA levels of human GSTA1, we treated preconfluent, confluent, or postconfluent Caco-2 cells with TPA, 3-MC, BHA, or PB. GSTA1 mRNA levels in preconfluent cells were significantly reduced to 7% by TPA ($P < 0.001$), 39% by 3-MC ($P < 0.01$), 51% by BHA ($P < 0.01$), and 30% by PB ($P < 0.001$) relative to control levels (Fig. 1A). GSTA1 mRNA levels were also markedly reduced in confluent cells with reductions to 5% for TPA ($P < 0.05$), 12% for 3-MC ($P < 0.05$), 87% for BHA, and 46% for PB ($P < 0.05$) compared with control cells (Fig. 1B). There were no statistically significant reductions in GSTA1 mRNA levels in postconfluent cells; however, there was a 1.4-fold increase ($P < 0.05$) in GSTA1 mRNA levels in PB-treated cells (Fig. 1C). Compared with preconfluent control cells, there was a 6.25-fold ($P < 0.001$) increase in constitutive GSTA1 mRNA levels in postconfluent cells.

We have previously shown that reductions of GSTA1/2 mRNA by interleukin-1β were mediated by HNF1, so we examined HNF1α expression in Caco-2 cells treated with rodent GST inducers. Similar to GSTA1 repression observed in preconfluent cells, HNF1α mRNA levels were reduced to 8% by TPA ($P < 0.001$), 43% by 3-MC ($P < 0.05$), 50% by BHA ($P < 0.05$), and 19% by PB ($P < 0.001$) relative to control levels (Fig. 2A). In confluent cells, there were less marked

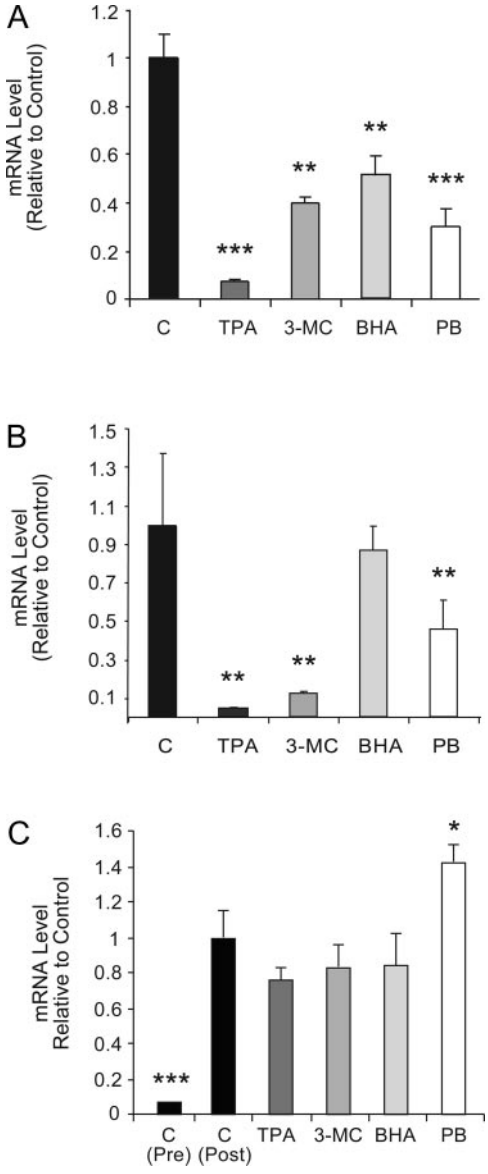

Fig. 1. Rodent GST inducers reduce human GSTA1 mRNA levels in preconfluent and confluent Caco-2 cells. Caco-2 cells at different stages of confluence: preconfluent (A), confluent (B), and postconfluent (C) were exposed for 24 h to TPA, 3-MC, BHA, and PB. Total RNA was isolated and GSTA1 mRNA levels were quantified by real-time PCR as described under *Materials and Methods*. Results are the mean of three independent experiments. Significant differences from control are designated as *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

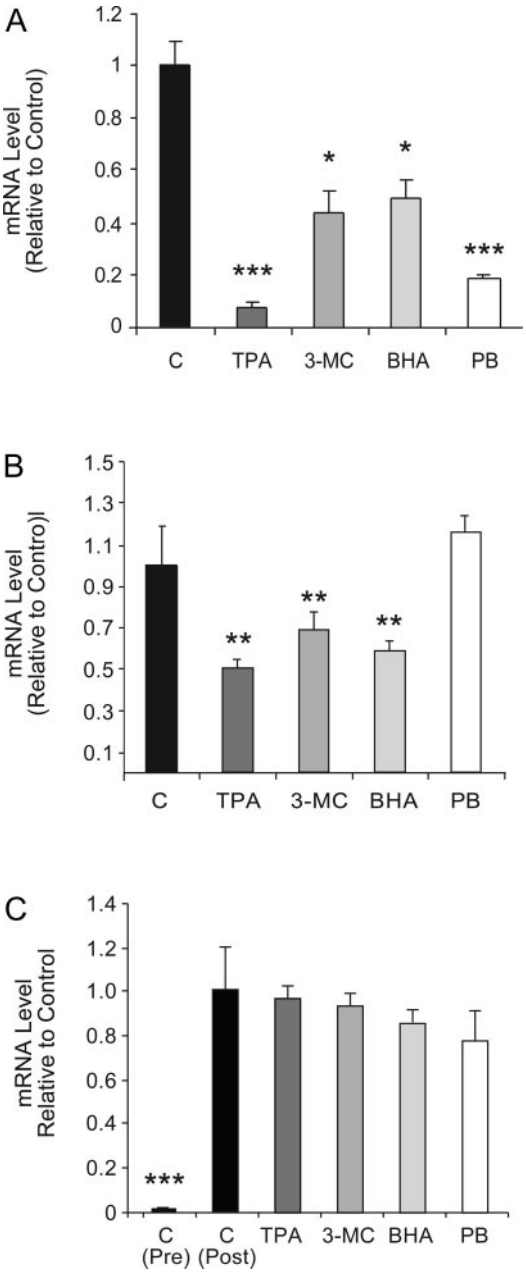

Fig. 2. HNF1α mRNA levels are reduced in preconfluent and confluent Caco-2 cells exposed to inducers of rodent GSTs. Caco-2 cells at different stages of confluence: preconfluent (A), confluent (B), and postconfluent (C) were exposed for 24 h to TPA, 3-MC, BHA, and PB. Total RNA was isolated and HNF1α mRNA levels were quantified by real-time PCR as described under *Materials and Methods*. Results are the mean of three independent experiments. Significant differences from control values are designated as *, $P < 0.05$; **, $P < 0.01$; or ***, $P < 0.001$.

reductions to 50% ($P < 0.05$) for TPA, 68% for 3-MC ($P < 0.05$), and 58% for BHA relative to controls (Fig. 2B). PB did not alter HNF1α mRNA levels. There were no statistically significant differences in treated and control postconfluent cells; however, there was a 50-fold increase in HNF1α levels in control cells compared with preconfluent control cells (Fig. 2C).

Role of HNF1α in GSTA1 Transcriptional Activity. To determine the role of HNF1α in transcriptional regulation of *GSTA1* and the involvement of HNF1α in down-regulation of *GSTA1* by rodent GST inducers reporter assays were preformed using various luciferase reporter plasmid constructs containing truncations of the 5′-flanking region of *GSTA1* (Fig. 3). Three of these constructs (pGSTA1-1591-Luc, pGSTA1-850-Luc, and pGSTA1-355-Luc) contained an HRE located between −182 and −170 bp upstream from the transcription start site. The HRE was excluded in the smallest truncation construct (pGSTA1-165-Luc) and was mutated in the largest construct (pGSTA1-ΔHRE-Luc).

Cotransfection of Caco-2 cells with pGSTA1-1591-Luc and increasing amounts of pHNF1α resulted in dose-related increases in luciferase activity of 2.8- and 3.7-fold ($P < 0.001$) in cells transfected with 0.5 and 1.2 μg of pHNF1α, respectively (Fig. 4). Reporter activity in cells cotransfected with pGSTA1-ΔHRE-Luc was reduced to approximately 65% of those cotransfected with pGSTA1-1591-Luc ($P < 0.05$). Overexpression of HNF1α did not result in any increase in luciferase activity in cells cotransfected with pGSTA1-ΔHRE-Luc.

Effect of Rat GST Inducers on Transcriptional Regulation of GSTA1. To locate the regulatory element within the GSTA1 promoter that is responsible for the decrease in GSTA1 mRNA levels by rodent GST inducers, luciferase activity was assessed in cells transfected with pGSTA1-1591-luc, pGSTA1-850-luc, pGSTA1-355-luc, and pGSTA1-ΔHNF1-luc and subsequently treated with TPA, 3-MC, BHA, and PB. In cells transfected with pGSTA1-1591-Luc, TPA, 3-MC, BHA, and PB reduced luciferase activity to 58, 67, 47, and 42% of control levels, respectively ($P < 0.001$) (Fig. 5). Statistically significant reductions in luciferase activities were also observed in cells transfected with pGSTA1-850-luc and pGSTA1-355-luc after treatment with TPA, 3-MC, BHA, and PB (Fig. 6, A–D). However, luciferase activity was either unchanged or mildly increased in cells transfected with the shortest construct pGSTA1-165-luc or pGSTA1-ΔHNF1-luc, neither of which contained a functional HRE. To confirm the involvement of HNF1 in the reduction of *GSTA1* transcriptional activity by rodent GST inducers and to determine whether overexpression of HNF1α reverses the transcriptional repression, cells were cotransfected with pGSTA1-1591-luc and either pHNF1α or the empty expression plasmid (pcDNA3.1) followed by treatment with TPA, 3-MC, BHA, and PB. Transfection with pHNF1α attenuated the transcriptional repression observed with TPA, 3-MC, BHA, and PB ($P < 0.05$) in BHA-treated cells (Fig. 7A). A similar trend was also observed at the mRNA level (Fig. 7B) although significant differences were only observed in BHA-treated cells.

Influence of Rodent GST Inducers on HNF1 Binding to GSTA1 HRE. To determine whether treatment of Caco-2 cells with TPA, 3-MC, BHA, and PB reduced binding of HNF1α to the HRE, gel shift assays were performed by incubating nuclear extracts from cells treated with the various rodent GST inducers and radiolabeled HRE probes (Fig. 8). The electrophoretic mobility shift observed with nuclear extracts incubated with the wild-type HRE probe and nuclear extract from control Caco-2 cells was abolished in the presence of 100-fold excess of nonradioactive HRE probe as well as in incubates in which the ΔHRE probe was used. Inclusion of anti-HNF1α antibodies in the binding reaction resulted in a supershift of the HRE-protein complex (data not shown). Densitometry did not reveal any statistically significant differences in the intensity of the shifted bands from nuclear extracts derived from cells treated with TPA, 3-MC, BHA, and PB compared with controls.

Expression of Repressive vHNF1-C in Caco-2 Cells. Although EMSA results did not reveal differences in the level of nuclear proteins bound to the HRE, the results of reporter assays clearly implicated an HNF-dependent mechanism in *GSTA1* repression by rodent GST inducers. HNF1-HRE complexes occur in the form of either homodimers or heterodimers of HNF1α and vHNF1; however, the composition of the dimers bound to DNA cannot be readily distinguished by conventional EMSA. We suspected that formation of complexes between the transdominant repressor isoform vHNF1-C and HRE may have profound implications in xenobiotic-mediated repression of *GSTA1*. To determine the capacity of vHNF1-C to repress *GSTA1* transcriptional activity, luciferase reporter assays were performed in Caco-2 cells cotransfected with pGSTA1–1591-Luc and the expression plasmid pRSV-vHNF1-C. GSTA1 promoter activity was reduced to 18% of control activity ($P < 0.05$) in cells in which vHNF1-C was overexpressed (Fig. 9A). To determine whether the ratio of vHNF1-C to vHNF1-B/C expression might explain variable constitutive *GSTA1* expression in Caco-2 cells at different stages of confluence, vHNF1-C mRNA levels were compared with vHNF1A/B levels in preconfluent and postconfluent cells by real-time RT-PCR (Fig. 9B). In preconfluent cells, vHNF1-C mRNA levels were 236-

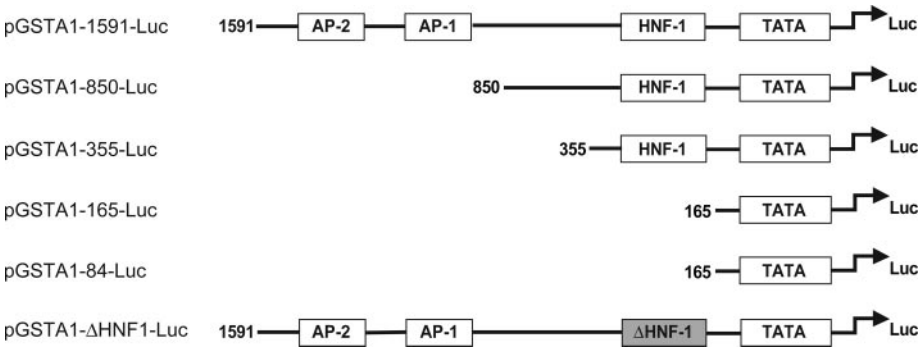

Fig. 3. Diagrammatic representation of GSTA1-LUC constructs.

fold higher than vHNF1A/B levels; however, levels were significantly reduced (96-fold higher than vHNF1A/B levels) in postconfluent cells ($P < 0.05$). More importantly, 3-MC, BHA, and PB increased vHNF1-C mRNA levels in preconfluent cells by 1.6-, 2.0-, and 1.5-fold, respectively ($P < 0.01$); however, vHNF1-C mRNA levels in TPA-treated cells were reduced to 50% of control levels ($P < 0.01$).

Immunodetection of HNF1α and vHNF1-C Protein Bound to HRE. To determine whether HNF1 proteins were responsible for DNA-protein complexes observed by EMSA, a DNA binding assay based on the interaction of HNF with biotinylated HRE probes, identical to those used in EMSA, was performed. HNF1 proteins in complexes were identified by purifying biotinylated probes on streptavidin-agarose beads followed by immunoblot analysis using polyclonal antibodies to either human HNF1α or vHNF1. Band intensity was assessed by densitometry. This assay clearly demonstrated that both HNF1α and vHNF1 proteins bind to the HRE (Fig. 10). HNF1α-HRE complex formation (detected at a molecular mass of approximately 92 kDa) was 1.4-fold

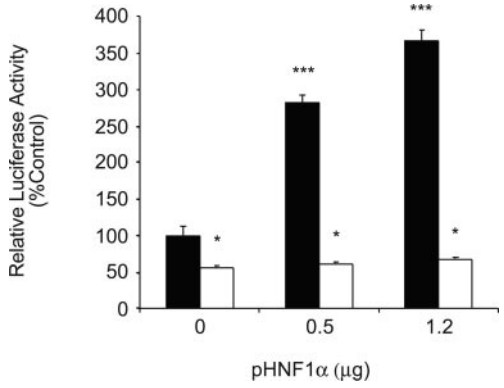

Fig. 4. Overexpression of HNF1α increases GSTA1 transcriptional activity. Caco-2 cells were transiently transfected with pHNF1α and cotransfected with either pGSTA1-1591-luc (black columns) or pGSTA1-ΔHRE-luc (white columns). Cells were harvested and luciferase activities were measured and corrected for differences in transfection efficiency based on *R. reniformis* activity. Luciferase activities (mean ± S.E.) are expressed relative to 100% control values from cell transfected with pGSTA1-1591-luc only. Results are derived from at least three separate experiments. Significant differences from control values are designated as *, $P < 0.05$ or ***, $P < 0.001$.

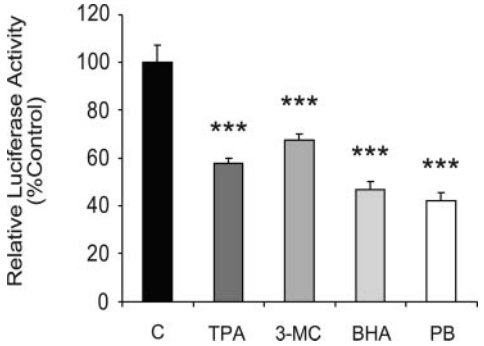

Fig. 5. Inducers of rodent GSTs reduce GSTA1 transcriptional activity. Caco-2 cells were transiently transfected with pGSTA1-1591-luc and then cultured for 24 h in the presence of TPA, 3-MC, BHA, and PB. Cells were harvested, and luciferase activities were measured and corrected for differences in transfection efficiency based on *R. reniformis* activity. Luciferase activities (mean ± S.E.) are expressed as a percentage relative to the 100% control values. Results are derived from at least three separate experiments. Statistically significant differences in luciferase activity are indicated as ***, $P < 0.001$.

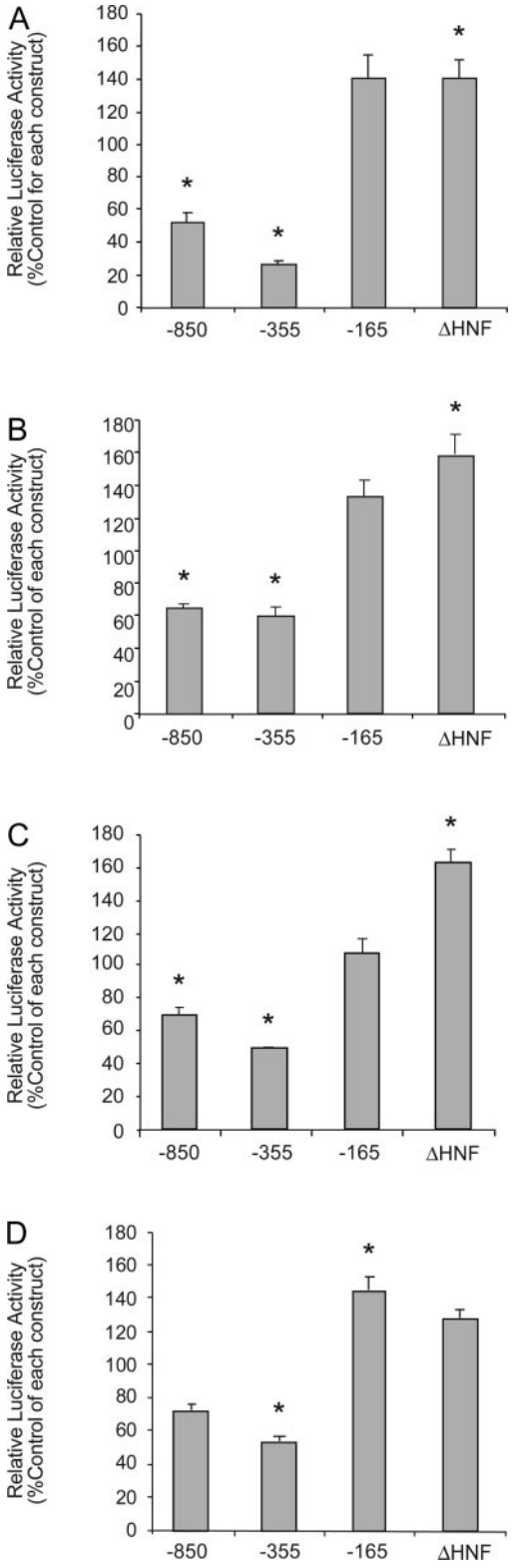

Fig. 6. Reduced transcriptional activity of GSTA1 by rodent GST inducers is mediated by HRE. Caco-2 cells were transiently cotransfected with either pGSTA1-850-luc, pGSTA1-355-luc, pGSTA1-165-luc, or pGSTA1-ΔHRE-luc and then cultured for 24 h in the presence of TPA (A), 3-MC (B), BHA (C), and PB (D). Cells were harvested, and luciferase activities were measured and corrected for differences in transfection efficiency based on *R. reniformis* activity. Luciferase activities (mean ± S.E.) are expressed as a percentage relative to the 100% control values. Results are derived from at least three separate experiments. Statistically significant differences in luciferase activity are indicated as * $P < 0.05$.

greater in untreated postconfluent cells than in preconfluent cells and vHNF1-C-HRE complexes (detected at approximately 72 kDa) were 1.3-fold greater in untreated preconfluent cells than in postconfluent cells. In preconfluent cells, treatment with all rodent GST inducers reduced HNF1α complex formation to less than 50% of control levels. In postconfluent cells, TPA and 3-MC had minimal effects on HNF1α binding to HRE, but treatment with BHA and PB reduced complex formation to approximately 40% of control levels. In preconfluent cells, treatment with 3-MC, BHA, and PB increased vHNF1 binding to HRE by 1.6-, 1.9-, and 2-fold, respectively; however, TPA reduced complex formation to approximately 30% of control levels.

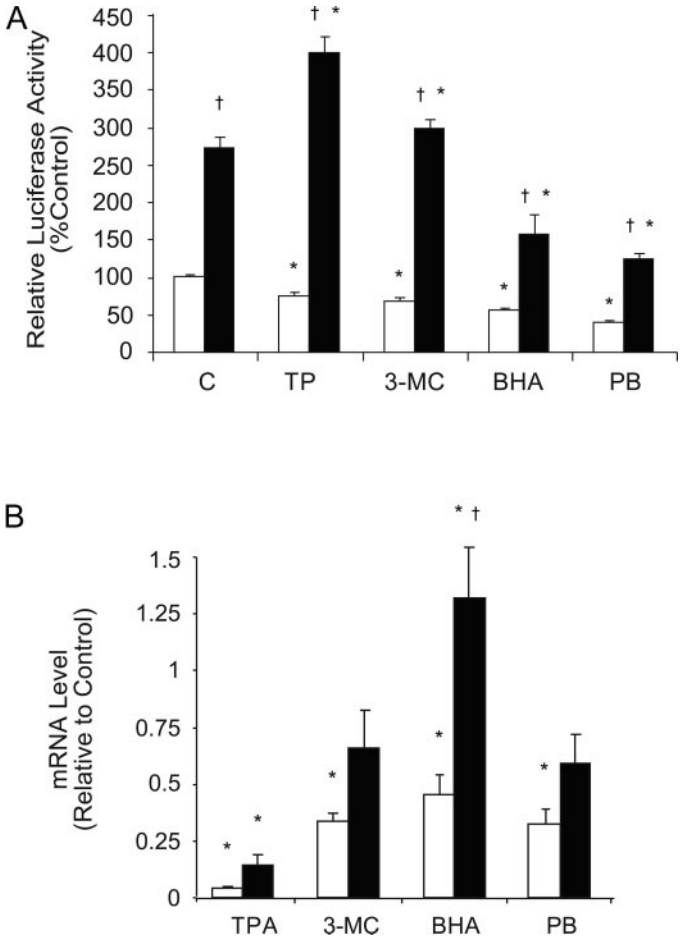

Fig. 7. Overexpression of HNF1α partially reverses GSTA1 transcriptional repression by rodent GST inducers. A, Caco-2 cells were transiently transfected with pGSTA1-1591-luc alone (white columns) and cotransfected with either pHNF1α (black columns) or empty expression plasmid (pcDNA3.1) and then cultured for 24 h in the presence of TPA, 3-MC, BHA, or PB. Cells were harvested and luciferase activities were measured and corrected for differences in transfection efficiency based on *R. reniformis* activity. Luciferase activities (mean ± S.E.) are expressed as a percentage relative to the 100% untreated control values. B, Caco-2 cells were transiently transfected with either pHNF1α (black columns) or an empty expression plasmid (pcDNA3.1) and then cultured for 24 h in the presence of TPA, 3-MC, BHA, and PB. Total RNA was isolated and GSTA1 mRNA levels were quantified by real-time PCR as described under *Materials and Methods*. Messenger RNA values are expressed as -fold differences relative to untreated controls. Results represent the mean of three independent experiments. Significantly different from untreated control; $*$, $P < 0.05$). Significantly different from cells transfected with pcDNA3.1; †, $P < 0.05$.

Discussion

The transcriptional regulation of rodent GSTs by various xenobiotic inducers, including phenobarbital, planar aromatic compounds (Rushmore et al., 1990), antioxidants (Pinkus et al., 1996), indoles (Renwick et al., 1999), isothiocyanates (Hecht, 2000), oltipraz (Kang et al., 2003), and other dithiol-3-thiones (Kensler et al., 2000), has been extensively studied. Indeed, specific *cis*-acting regulatory elements such as the electrophile responsive element (ERE) and the ARE that are responsible for inducible expression of α class GSTs in mice and rats have been identified and well characterized (Rushmore et al., 1991; Friling et al., 1992). In view of the protection against potent chemical carcinogens provided by GST inducers in rodents, there has been considerable interest in extrapolating the chemopreventive properties to humans. However, lack of sequence homology in the 5′-flanking regions of rodent and human α-class GST genes and the absence of functional ARE and ERE regulatory regions suggests that control of human GSTA1 expression may be different, putting into question the rationale of extrapolating results from rodent GST regulation studies to humans. Indeed, the results of this study indicate that human GSTA1 responds in a negative manner to several rodent GST inducers via a mechanism mediated by differential expression of both transactivator and repressor isoforms of HNF1 as well as variable binding of these isoforms to the HRE in the proximal *GSTA1* promoter.

Transcriptional repression of *GSTA1* by TPA, 3-MC, BHA, and PB was manifested in Caco-2 cells as reduced GSTA1 mRNA levels and transcriptional activity. TPA produced the most profound repression with a reduction of GSTA1 mRNA levels to 7% of controls. A similar reduction of GSTα mRNA levels was observed previously in TPA-treated Caco-2 cells and HepG2 cells (Eickelmann et al., 1995). In that study, reduction was attributed to a post-transcriptional event (GSTα mRNA degradation); however, the results of our gene

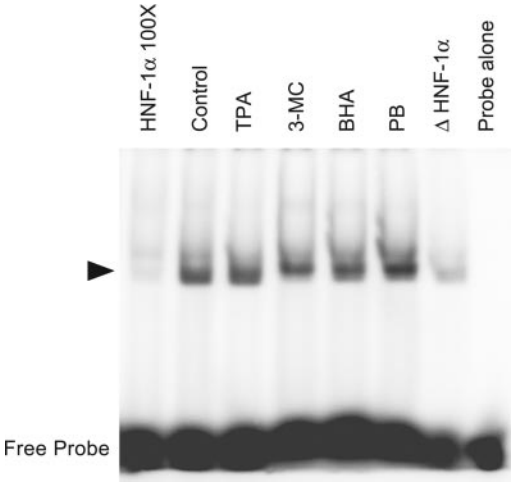

Fig. 8. Gel shift analysis of protein binding to HRE. Nuclear extracts were prepared from cells that were incubated with either TPA, 3-MC, BHA, or PB for 24 h. Twenty micrograms of nuclear extracts was incubated for 20 min with 20 μg of radiolabeled HNF1α-containing oligonucleotide. The arrowhead indicates DNA bound with HNFα. For competition assays, a 100-fold molar excess of unlabeled HNF oligonucleotide was added to the incubation reaction mixture. Nuclear extracts incubated with the mutant HNF1α-containing oligonucleotide (ΔHNF1) are also shown.

reporter assays clearly indicate that TPA represses GSTA1 transcription in Caco-2 cells. In mice, induction of GSTA1 by TPA follows overexpression of c-jun and c-fos and increased binding to the ERE by the Fos/Jun heterodimeric complex (AP-1) (Bergelson et al., 1994). Our reporter assays have eliminated the involvement of AP-1 in transcriptional repression of *GSTA1* by TPA and have implicated HNF1α in the process. Although TPA is known to suppress the expression of other genes (Lim et al., 2005), the involvement of HNF1α has not previously been demonstrated.

Apart from the above-mentioned TPA study, repression of *GSTA1* expression by chemical inducers of rodent GSTs has not previously been demonstrated in cultured human cells. In a study of human hepatocytes in primary culture, GSTA1 mRNA levels were increased by 3-MC, PB, and dithiolethiones; however, induction by 3-MC and PB was extremely variable, occurring at low levels in only one-third of hepatocyte cultures. Interindividual variability in responsiveness of P450 enzymes to inducers has been previously shown in human hepatocytes (Pichard et al., 1991). Lack of induction of *GSTA1* by chemical inducers in Caco-2 cells may be due to differences in regulation between hepatocytes and enterocytes, because we also observed *GSTA1* repression in HT-29 cells (data not shown). In Caco-2 cells, expression of the drug-metabolizing enzyme CYP1A1 is increased by various chemical inducers, including 3-MC and phenobarbital; however, other enzymes, including γ-glutamyltranspeptidase, and various membrane proteins associated with glucose utilization such as sucrase isomaltase and hexose transporters, are down-regulated (Boulenc et al., 1992; Carriere et al., 1998).

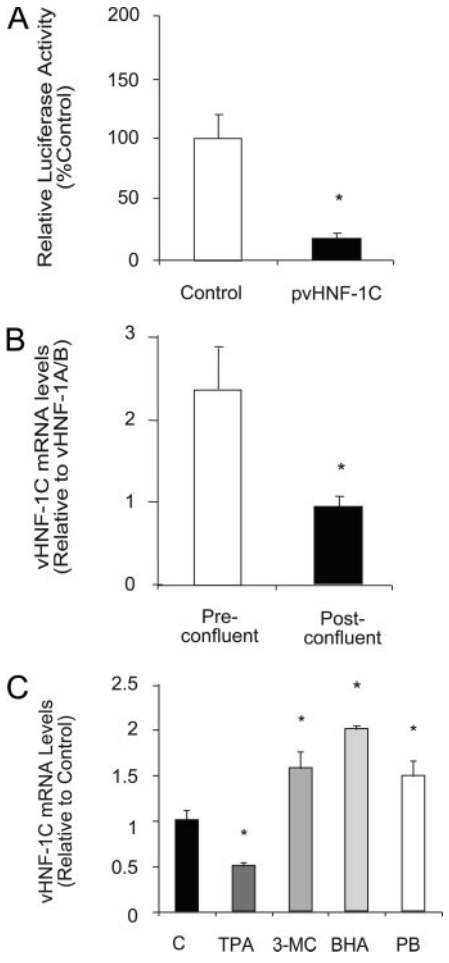

Fig. 9. Expression of vHNF1C in Caco-2 cells. A, overexpression of vHNF1-C represses GSTA1 transcriptional activity. Preconfluent Caco-2 cells were transiently transfected with pGSTA1-1591-luc alone (white columns) and cotransfected with either pvHNF1-C (black columns) or an empty expression plasmid (pcDNA3.1). Cells were harvested, and luciferase activities were measured and corrected for differences in transfection efficiency based on *R. reniformis* activity. Luciferase activities (mean ± S.E.) are expressed as a percentage relative to the 100% untreated control values. B, vHNF1-C is expressed at high levels in preconfluent Caco-2 cells. Total RNA was isolated and vHNF1-C mRNA levels were quantified by real-time PCR as described under *Materials and Methods*. C, 3-MC, BHA, and PB increase vHNF1-C mRNA levels. Preconfluent Caco-2 cells were exposed for 24 h to TPA, 3-MC, BHA, and PB. Total RNA was isolated and vHNF1-C mRNA levels were quantified by real-time PCR as described under *Materials and Methods*. Results are the mean of three independent experiments. Significant differences from control values are designated as $*$, $P < 0.05$.

The HNF1 family of *trans*-acting transcription factors consists of isoforms of both HNF1α and vHNF1 (also called HNF1β) generated by alternative splicing events that result in varying degrees of transactivating properties (Cereghini, 1996). The three isoforms of HNF1α enhance transcription, with isoforms B and C being more potent transactivators than the A isoform. Of the three isoforms of vHNF1 protein, vHNF1A and B enhance transcriptional activity, whereas the vHNF1-C isoform acts as a transdominant repressor when dimerized with other HNF1α proteins (Bach and Yaniv, 1993). The finding that HNF1α overexpression increased GSTA1 reporter activity in luciferase constructs with an intact HRE indicates that constitutive expression of GSTA1 is regulated by HNF1α. Moreover, marked overexpression of *GSTA1* in postconfluent Caco-2 cells correlated with elevated levels of HNF1α at that stage of confluence. These results support the previous association of reduced human *GSTA2* expression with lower levels of HNF1 expression in kidney tissue and renal cell carcinoma (Clairmont et al., 1994). In that study, a functional HRE was identified within the *GSTA2* promoter (GGAACATATTAAC) at the same location and with similar sequence homology to the HRE in the *GSTA1* promoter (GGAACACATTAAC) identified in the present study (single base differences are underlined). The finding that overexpression of vHNF1-C significantly repressed *GSTA1* transcriptional activity emphasizes the repressive role of this isoform. Moreover, the differential expression of vHNF1-C associated with degree of confluence may explain the low and high constitutive *GSTA1* expression

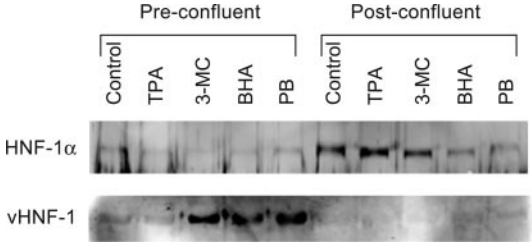

Fig. 10. Immunodetection of HNF1α and vHNF1C binding to HRE. Binding reactions were carried out using 50 μg of nuclear extract from cells incubated with either TPA, 3-MC, BHA, or PB for 24 h and a biotinylated HRE probe identical to that used for the EMSA. The binding reactions were then incubated with streptavidin-agarose, washed, and HNF1 protein bound to biotin-target DNA was collected in 1% SDS, 10 mM DTT and analyzed by Western blot analysis using polyclonal antibodies to HNF1α or HNF1β. The immunoblot shown represents one of two experiments with similar results.

in preconfluent and postconfluent Caco-2 cells, respectively. Indeed, dedifferentiated hepatoma cells express high levels of vHNF1, whereas HNF1α is expressed in highly differentiated cells reflecting the different functional roles of these isoforms during differentiation (Baumhueter et al., 1988; Cereghini et al., 1988).

The repressive effect of chemical inducers on *GSTA1* transcription was observed in preconfluent but not in postconfluent cells. The reason for this discrepancy may be related to the fact that treatment with xenobiotics reduced HNF1α and increased vHNF1-C mRNA levels (with the exception of TPA treatment) in preconfluent cells but not in postconfluent cells. These findings were also reflected in DNA binding assays in which the degree of HRE binding of HNF1α and vHNF1-C was decreased and increased, respectively, in preconfluent cells treated with the xenobiotics. This suggests that *HNF1α* down-regulation and *vHNF1-C* up-regulation results in *GSTA1* repression. It remains to be determined whether the mechanism underlying altered HNF1 expression involves altered transcription or mRNA degradation. The fact that overexpression of *HNF-1α* prevented transcriptional repression of *GSTA1* by rodent GST inducers in luciferase reporter assays further implicates HNF-1α in the mechanism of transcriptional repression. Ceramide, a key regulator of apoptosis, represses rat *GSTA2* transcription in H4IIE cells by reducing nuclear HNF1α levels via ubiquitination (Park et al., 2004). Ceramide also prevents induction of *GSTA2* by oltipraz and *tert*-butylhydroquinone by inhibiting HNF1 DNA binding.

That transcriptional repression by rodent GST inducers does not occur when the HRE is deleted or mutated in luciferase reporter constructs indicates that down-regulation of *GSTA1* is mediated through interaction of HNF1 proteins, and perhaps other nuclear proteins, and the HRE. This supports our previous demonstration of HRE involvement in *GSTA1* repression by interleukin-1β (Romero et al., 2002). Although results of gel shift assays suggest that nuclear protein binding to the HRE is not altered by rodent GST inducers, the identity and composition of the proteins in these complexes were not resolved using this approach. However, Western blot analysis of HRE-bound proteins in DNA binding assays revealed that the relative amount of HNF1α and vHNF1 present in complexes varies considerably in untreated and xenobiotic-treated preconfluent and postconfluent cells. In view of the different transactivator and repressor functions of *HNF* isoforms, the composition of HNF1-HRE complexes probably has a profound impact on *GSTA1* gene regulation. Thus, increased binding of vHNF1-C to HRE may explain repression of *GSTA1* in preconfluent cells treated with 3-MC, BHA, and PB. Moreover, absence of vHNF1-C binding to HRE may explain the lack of repression by xenobiotics observed in postconfluent cells. Our results do not explain the mechanism underlying TPA-mediated repression of *GSTA1* expression because TPA treatment reduced both vHNF1-C mRNA levels and vHNF1-C-HRE binding in preconfluent cells. Moreover, preliminary experiments comparing HRE-bound proteins in TPA-treated and control cells by two-dimensional gel electrophoresis, did not (apart from a reduction in HNF1-C bound to HRE) reveal any treatment-related differences in the profile of HRE-bound proteins. The possibility of TPA-mediated modification of HNF1 function in *GSTA1* transcription merits further investigation.

In conclusion, we have demonstrated that 1) constitutive expression of *GSTA1* is regulated by interactions of HNF1 proteins and an HRE in the proximal promoter of the *GSTA1* gene; 2) *GSTA1* transcription is repressed by chemical inducers of rodent GSTs, including TPA, 3-MC, BHA, and PB; and 3) the mechanism of repression of *GSTA1* expression by 3-MC, BHA, and PB in preconfluent Caco-2 cells is associated with increased binding of vHNF1-C to the HRE.

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