

MECHANISMS OF ARSENITE-MEDIATED DECREASES IN BENZO[k]FLUORANTHENE-INDUCED HUMAN CYTOCHROME P4501A1 LEVELS IN HEPG2 CELLS

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ABSTRACT:

Polycyclic aromatic hydrocarbons (PAHs) and heavy metals are often environmental cocontaminants that could interact to alter PAH carcinogenicity. The heavy metal, arsenite, and the PAH, benzo[k]fluoranthene, were used as prototypes to investigate, in human HepG2 cells, mechanisms whereby the bioactivation of benzo[k]fluoranthene by human CYP1A1 could be diminished by arsenite-mediated decreases in CYP1A1 induction by benzo[k]fluoranthene. To determine whether arsenite down-regulates CYP1A1 transcription, quantitative real-time reverse transcriptase-polymerase chain reaction assays and luciferase reporter gene expression assays were used with HepG2 cells treated with benzo[k]fluoranthene and arsenite, separately and as a mixture. Benzo[k]fluoranthene (0.5 μ M) and arsenite (5 μ M) markedly decreased benzo[k]fluoranthene-mediated induction of CYP1A1 mRNA by 45%. Plasmids containing the CYP1A1 promoter region (pHu-1A1-FL) were induced 7.4-fold over vehicle by benzo[k]flu-

oranthene (0.5 μ M), whereas arsenite (1, 2.5, or 5 μ M) decreased reporter gene expression by 46%, 45%, and 61%, respectively. The plasmid, pHu-1A1- Δ 100-FL, lacked xenobiotic response element (XRE) sites at -1061 and -981 and showed greater responsiveness relative to pHu-1A1-FL, by 1.7-fold. Benzo[k]fluoranthene (0.5 μ M) and arsenite (1, 2.5, or 5 μ M) decreased reporter gene expression by 0%, 27%, and 39%, respectively, relative to expression levels produced by benzo[k]fluoranthene alone. Arsenite is stable for at least 48 h in the HepG2 cell medium with respect to its ability to diminish CYP1A1 benzo[k]fluoranthene induction. Arsenite did not affect benzo[k]fluoranthene induction directly through XRE sites, nor did it affect the stability of CYP1A1 mRNA. Thus, arsenite affects the transcriptional regulation of the benzo[k]fluoranthene-mediated induction of CYP1A1 and could diminish PAH carcinogenicity by decreasing bioactivation by CYP1A1.

Polycyclic aromatic hydrocarbons (PAHs) and heavy metals occur as environmental cocontaminants at hazardous waste sites and are released together from sources such as fossil fuel combustion, municipal waste incineration, and cigarette smoke (Maier et al., 2000). PAHs and heavy metals are ranked high among the 275 compounds that comprise the priority list of the most hazardous environmental substances published by the Agency for Toxic Substances and Disease Registry and the Environmental Protection Agency. Prioritization of the compounds within the list is based on frequency of occurrence in the environment, toxicity, and potential for human exposure [Agency for Toxic Substances and Disease Registry (1999) CERCLA List of Priority Hazardous Substances, available from: <http://www.atsdr.cdc.gov/99list.html>]. The PAH, benzo[k]fluoranthene, and the heavy metal, arsenic (as arsenite), used in this study to determine mechanisms whereby metals affect PAH induction of cytochrome P4501A1 (CYP1A1), were selected based, partially, on these hazard priorities.

P450 enzymes are heme-containing proteins that biotransform numerous xenobiotics, including PAHs. PAHs are bioactivated by the

human CYP1 family of enzymes, which includes CYP1A1, CYP1A2, and CYP1B1, to a wide range of metabolites, some of which are highly carcinogenic (Shimada et al., 1996, 1999). PAHs, including benzo[k]fluoranthene, also inductively enhance levels of CYP1 enzymes, thereby facilitating the production of bioactivated forms of PAHs (Vakharia et al., 2001a,b). Heavy metals, such as arsenite, affect PAH bioactivation pathways (Jacobs et al., 1998, 1999, 2000) and could consequently affect carcinogenic risks associated with bioactivated PAHs. Another selection criterion for benzo[k]fluoranthene as the prototypic PAH for these studies is its high inductive capacity for CYP1A1 and CYP1A2 (Vakharia et al., 2001a,b).

PAH-mediated induction of CYP1A1 is regulated primarily at the level of transcription through the aryl hydrocarbon receptor (AhR) pathway (Fisher et al., 1990; Whitlock, 1999). CYP1A1 is not constitutively expressed in human tissue; it is maintained in a silent state by negative regulatory mechanisms, involving negative regulatory elements upstream of the *CYP1A1* promoter region. Thus, the expression of CYP1A1 depends on the interaction of positive and negative regulatory factors (Boucher et al., 1995).

Induction of CYP1A1 is down-regulated by inflammatory cytokines, heavy metals, such as arsenic (Jacobs et al., 1998, 1999, 2000),

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ABBREVIATIONS: P450, cytochrome P450; DMSO, dimethyl sulfoxide; RT-PCR, reverse transcriptase-polymerase chain reaction; PAH, polycyclic aromatic hydrocarbon; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EROD, 7-ethoxyresorufin O-deethylase; BAP, benzo[a]pyrene; NRE, negative regulatory element; NF1, nuclear factor 1; BTE, basic transcription element; FL, firefly luciferase; XRE, xenobiotic response element.

and oxidative stress (Morel and Barouki, 1998); however, the mechanisms are not well defined. Arsenite was shown to decrease 3-methylcholanthrene-mediated induction of CYP1A4 and CYP1A5 mRNA (Jacobs et al., 2000), but not phenobarbital (PB)-mediated induction of CYP2H1 mRNA in primary cultures of chick hepatocytes (Jacobs et al., 1998). In primary cultures of rat hepatocytes, arsenite decreased 3-methylcholanthrene-mediated induction of CYP1A1 mRNA, PB-mediated induction of CYP2B1 mRNA, and dexamethasone-mediated induction of CYP3A23 mRNA but had no effect on PB-mediated induction of CYP3A23 mRNA (Jacobs et al., 1999).

It has been suggested that transcriptional regulation of *CYP1A1* by oxidative stress is via factors acting at a characterized oxidative response region upstream of the promoter (Morel and Barouki, 1998; Morel et al., 1999) and that reactive oxygen species produced during the catalytic cycle of CYP1A1 limit the levels of induced CYP1A1 mRNA in a negative-feedback autoregulatory loop, by limiting *CYP1A1* gene promoter activity (Morel et al., 1999). Basal and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced human 5' upstream promoter reporter activity was strongly decreased with H₂O₂ exposure or glutathione depletion.

The current study investigates the mechanisms of arsenite modification of benzo[*k*]fluoranthene regulation of CYP1A1 in human HepG2 cells. The human hepatoma cell line HepG2 was used throughout these studies as a model for investigation for human CYP1A1 and has been used by others (Hines et al., 1988; Boucher et al., 1993, 1995; Kress et al., 1998; Morel and Barouki, 1998; Morel et al., 1999; Vernhet et al., 2003). HepG2 cells retain endogenous bioactivation capacity, including PAH-mediated induction of CYP1A1, a P450 form that is not constitutively expressed in these cells. HepG2 cells are thus suitable for determination of the mechanisms of interaction of arsenite with the benzo[*k*]fluoranthene-mediated induction of CYP1A1 enzymes in human cell lines (Vakharia et al., 2001b). To identify transcriptional effects, quantitative real-time RT-PCR was used to construct mRNA time courses. To assess the effect of arsenite on CYP1A1 transcriptional regulation, plasmids containing portions of the human CYP1A1 gene were constructed. Finally, to determine the effect of arsenite on CYP1A1 mRNA stability, studies were conducted with actinomycin D.

Materials and Methods

Materials. Benzo[*k*]fluoranthene, 99 to 100% pure, was purchased from AccuStandard Inc. (New Haven, CT), and a stock solution (10 mM) was prepared in dimethyl sulfoxide (DMSO) from Sigma-Aldrich (St. Louis, MO). Sodium arsenite (NaAsO₂) was purchased from Sigma-Aldrich; a 10 mM stock solution was prepared in deionized water. These stock solutions were stored at room temperature and dilutions were prepared just before use. Poly-D-lysine, 7-ethoxyresorufin, dicoumarol, and Serum Replacement 2 were obtained from Sigma-Aldrich. HepG2 cells and cell medium, made up of Dulbecco's modified Eagle's medium (DMEM) containing Phenol Red, penicillin (100 U/ml), and streptomycin (100 µg/ml), supplemented with 10% fetal bovine serum (FBS) obtained from Atlanta Biologicals (Norcross, GA), were obtained from the Media Department of the Wadsworth Center (Albany, NY). Serum-free hepatocyte incubation medium (IVT Medium) was purchased from In Vitro Technologies (Baltimore, MD). Serum-free, low glucose DMEM and LipofectAMINE 2000 were purchased from Invitrogen (Carlsbad, CA) and TRI Reagent from Molecular Research Center (Cincinnati, OH). Oligonucleotide primers were synthesized in the Molecular Genetics Core of the Wadsworth Center. SYBR Green 1 nucleic acid stain was purchased from Molecular Probes (Eugene, OR). QIAGEN One-Step RT-PCR kits were obtained from QIAGEN (Valencia, CA). The BCA protein assay kit, albumin, and the Super Signal-West Pico chemiluminescent substrate kit were purchased from Pierce Chemical (Rockford, IL). Immobilon membrane was obtained from Owl Separation Systems (Portsmouth, NH). pGL3-Basic and promoter luciferase reporter vectors, pRL-CMV vector, and dual-luciferase reporter assay system

were obtained from Promega (Madison, WI). QuikChange site-directed mutagenesis kits and pBluescript KS⁺ were purchased from Stratagene (La Jolla, CA).

HepG2 Cells. Cells were grown and maintained in DMEM containing Phenol Red, supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in sterile 75-cm², 250-ml tissue culture flasks in a 5% CO₂, 95% air incubator at 37°C. Single-cell suspensions were prepared from confluent cultures by repeated passage of cell suspensions through 18-gauge needles. HepG2 cells were plated in poly-D-lysine-precoated (0.1 mg/ml in phosphate-buffered saline) 6- and 24-well plates, where they formed monolayers. HepG2 cells were plated in triplicate for each exposure to DMSO, benzo[*k*]fluoranthene, benzo[*k*]fluoranthene and arsenite, or arsenite alone.

7-Ethoxyresorufin-*O*-Deethylase (EROD) Activity Assay. EROD activity was measured by a modification of the method of Donato et al. (1993). HepG2 cells were seeded in triplicate for each exposure, at a density of 1.0×10^6 cells/well in poly-D-lysine-precoated six-well plates for 24 h. Medium was removed after 24 h, and triplicate subsets of cells were exposed for an additional 24 h to serum-free IVT Medium, containing DMSO (0.1%) or benzo[*k*]fluoranthene (0.25, 0.5, 1.0, 2.5, 5, or 10 µM in 0.1% DMSO) in a total volume of 2 ml, and the cells were incubated in 5% CO₂ and 95% air at 37°C. The culture medium was replaced after the 24-h exposure with 2 ml of IVT Medium containing 8 µM 7-ethoxyresorufin and 10 µM dicoumarol, a NAD(P)H quinone oxidoreductase inhibitor. Cells were further incubated for 45 min in 5% CO₂ at 37°C, after which time 90 µl of culture medium from each well was transferred to a white, opaque 96-well plate and was read using a PerkinElmer Life and Analytical Sciences (Boston, MA) LS50B luminescence spectrometer, fitted with 530-nm excitation and 590-nm emission filters (Vakharia et al., 2001a,b).

Quantitative Real-Time RT-PCR. mRNA analysis was conducted by real-time RT-PCR using a Roche Molecular Biochemicals (Indianapolis, IN) LightCycler instrument and a QIAGEN One-Step RT-PCR kit, containing SYBR Green 1 nucleic acid dye.

HepG2 cells were seeded in six-well plates for 24 h as described above. After 24 h, medium was removed and triplicate subsets of cells were exposed, according to the time course, to serum-free IVT Medium containing DMSO (0.1%), benzo[*k*]fluoranthene (0.5 µM), benzo[*k*]fluoranthene (0.5 µM) and arsenite (5 µM), or arsenite (5 µM) alone. At selected times, total RNA was isolated using TRI Reagent according to the manufacturer's instructions. RNAs were dissolved in diethylpyrocarbonate-treated water, and the concentrations were determined from the ratio of 260:280 nm absorbance, with an extinction coefficient of 40 mg/ml per absorbance unit at 260 nm. For the amplification of the human *CYP1A1* nucleotide sequences, a primer set (GenBank accession number NM_000499), shown in Table 1, was used, giving a 367-bp product, as was previously described (Fasco et al., 1995). Quantitation of 28S rRNA levels was used to verify that equivalent amounts of RNA sample were being assayed. Primers for 28S rRNA (GenBank accession number X00525) are given in Table 1 and yielded a 100-bp product (Simpson et al., 2000). Master mixes were used throughout for one-step RT-PCR. Amplifications were performed in 20-µl aliquots that contained 4 µl of a 5× buffer (supplied), 0.8 µl of deoxynucleoside-5'-triphosphate mixture (10 mM each base in stock solution, supplied), 0.8 µl of One-Step Enzyme Mix (supplied), 0.4 µl of primer mixture (25 µM each in stock solution), 1 µl of SYBR Green 1 (previously diluted to 1:5000 in water), and 1 µl of diluted total RNA sample in H₂O. RNA samples were diluted to 0.1 µl of total RNA in H₂O for CYP1A1 mRNA analysis and to 0.001 µg/µl total RNA in H₂O for 28S rRNA analysis; 1 µl of diluted sample was added to 19 µl of the master mix. Mixtures were reversed-transcribed by heating at 50°C for 30 min. DNA polymerase was activated by heating at 95°C for 15 min, followed by 45 cycles of amplification including 95°C denaturation for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s. Amplification of the PCR products was monitored by fluorescence of SYBR Green 1 dye over 45 cycles at 83°C for CYP1A1 mRNA and 80°C for 28S rRNA. The PCR products from each primer set were verified by melting curve analysis followed by agarose gel electrophoresis.

Standard curves for CYP1A1 mRNA and 28S rRNA were generated using dilutions of total RNA from benzo[*k*]fluoranthene-treated and untreated HepG2 cells, respectively, and using software supplied with the LightCycler. Relative levels of target gene expression were calculated from the standard curve. Data were routinely normalized to expression levels in DMSO control.

TABLE 1
Primer sequences for RT-PCR and plasmid studies

GenBank Accession No.	Gene/Plasmid	Sequence
NM_000499	CYP1A1	5'-TTCCGACACTCTTCCTTCGT-3' (forward primer) 5'-ATGGTTAGCCCATAGATGGG-3' (reverse primer) (Fasco et al., 1995)
X00525	28S	5'-TTGAAAATCCGGGGGAGAG-3' (forward primer) 5'-ACATTGTTCACATGCCAG-3' (reverse primer) (Simpson et al., 2000)
AF253322	pHu1A1-FL 600-bp fragment	5'-GATGGCGCGTAACGATGT-3' (forward primer) 5'-AGTGCTTGATGGCAGAGC-3' (reverse primer)
AF253322	pHu1A1-FL 1000-bp fragment	5'-GCCCCTAGAGGGATGTCG-3' (forward primer) 5'-GATCAGGGAAGGTTCCAAGG-3' (reverse primer)
AF253322	pHu1A1-FL	5'-GATGGCGCGTAACGATGT-3' (forward primer) 5'-GATCAGGGAAGGTTCCAAGG-3' (reverse primer)
AF253322	pHu1A1-Δ100-FL	5'-CTCCCTCCCCCTCGTGGGCACCGCCTC-3' (forward primer) 5'-GAGGCGCGGTGCCACGAGGGGGAGGGAG-3' (reverse primer)
AF253322	pHu1A1-Δ1000-FL	5'-GATGGCGCGTAACGATGTCTCCGTACCCTCTC-3' (forward primer) 5'-GAGAGGTACGGGAAGCATCGTTACGCGCCATC-3' (reverse primer)
	pXRE-FL	5'-GATCTGAGCTC-GGAGTTGCGTGAGAAGAGCCG-3' (forward oligonucleotide) ^a 5'-GATCCGGCTCTTCTCAGCAACTCCGAGCTCA-3' (reverse oligonucleotide) ^a (Denison et al., 1988)

^a XRE core consensus sequence is underlined.

Plasmids and Transient Transfection Assays. The primer sets used to construct *CYP1A1* promoter plasmids were designed using Primer 3 (Rozen and Skaletsky, 2000). The plasmid, pHu1A1-FL, contained approximately 1450 bases from the human *CYP1A1* upstream and promoter region. The insert DNA was prepared as two segments, a 600-bp segment and a 1000-bp segment, which were ligated together at a common *Apal* site. The template was the human *CYP1A1/CYP1A2* BAC clone, accession number AF253322, generously provided by Dr. Frank J. Gonzalez (National Institutes of Health, Bethesda, MD). Primers for the 600-bp segment and for the 1000-bp segment are given in Table 1. The 600-bp and 1000-bp segments were ligated together, and the 1450-bp fragment was amplified using the pHu1A1-FL primers listed in Table 1. The 1450-bp DNA fragment was then ligated into an *Asp718* [Roche Diagnostics, Indianapolis, IN; an isoschizomer of *KpnI/XhoI* double-digested pGL3 Basic Vector (Promega)], upstream of the promoterless firefly luciferase reporter gene, using *Asp718/XhoI* sites that were derived from a TA Cloning Vector (Invitrogen) used in an intermediate step prior to cloning into the pGL3 Basic Vector. DNA was prepared using the Maxi Prep System (Qiagen).

PCR amplifications for making DNA constructs were performed using stepdown PCR in an Ericomp SingleBlock system thermal cycler (Ericomp, Inc., San Diego, CA) and an Advantage-GC Genomic PCR kit (BD-Clontech, Palo Alto, CA) with 1 M denaturant. Amplifications were performed in 25-μl aliquots according to the manufacturer's instructions. Template DNA (1 μl, previously diluted 1:100 in H₂O) was added to the 24-μl master mix. DNA polymerase was activated by heating at 96°C for 5 min followed by 34 cycles of amplification. The basic PCR cycle used a 30-s denaturation at 96°C, a 30-s annealing step, and a 60-s extension step at 72°C. The first three cycles used 66°C for annealing, followed by three cycles at 62°C for annealing and, finally, 28 cycles at 56°C for annealing.

The QuikChange site-directed mutagenesis kit and primers for 5'-pHu1A1-Δ100-FL (Table 1) were used to delete 100 bp from pHu1A1-FL. The resulting plasmid, pHu1A1-Δ100-FL, lacks the XRE sites at positions -1061 and -980 (Jaiswal et al., 1985; Kubota et al., 1991; Corchero et al., 2001). Mutagenesis was performed using an Ericomp SingleBlock system thermal cycler. pHu1A1-FL was diluted to 100 ng/μl and 1 μl was added to a 50-μl master mix. The cycling parameters used were as follows. PfuTurbo DNA polymerase was activated by heating at 96°C for 1 min, followed by 15 cycles of denaturation for 15 s at 96°C, annealing for 30 s at 50°C, and extension for 10 min at 68°C.

The QuikChange site-directed mutagenesis kit was used to delete 1000 bp from the 5' end of the human *CYP1A1* upstream promoter region plasmid, pHu1A1-FL. The resulting plasmid, pHu1A1-Δ1000-FL, contained approximately 450 bp of the 5' upstream flanking region of the *CYP1A1* gene, including core promoter elements. The primers for pHu1A1-Δ1000-FL are provided in Table 1. Mutagenesis was performed as described above.

The primers for pXRE-FL, an XRE-containing plasmid, constructed using complementary pairs of synthetic DNA fragments that include an XRE core

consensus sequence, are given in Table 1. The fragment was inserted into a *BglIII*-digested pGL3-promoter vector, containing an SV40 promoter upstream of the firefly luciferase reporter gene.

All plasmid insert orientations and sequences listed above were verified using an ABI Prism model 3700 sequencer (Applied Biosystems, Foster City, CA) in the Molecular Genetics Core, Wadsworth Center. The pRL-CMV vector containing the *Renilla* luciferase was cotransfected with pHu1A1-FL, pHu1A1-Δ1000-FL, pHu1A1-Δ100-FL, or pXRE-FL, and was used for normalization.

Experimental plasmids and the pRL-CMV vector, used for normalization, were cotransfected into HepG2 cells. In poly-D-lysine-precoated 24-well plates, HepG2 cells were seeded at a density of 100,000 cells in 0.5 ml of medium per well and were immediately cotransfected with the experimental plasmid (480 ng/well) and pRL-CMV (20 ng/well) for 24 h, using LipofectAMINE 2000 (2 μl/well). After the 24-h transfection, medium was changed, and subsets of cells were treated separately. The plasmid, pHu1A1-FL, was exposed to a range of benzo[k]fluoranthene concentrations (0.25–5 μM) for an additional 24 h. The plasmids, pHu1A1-FL, pHu1A1-Δ100-FL, and pHu1A1-Δ1000-FL were treated for 24 h with one of the following: 0.1% DMSO; 0.5 μM benzo[k]fluoranthene; 0.5 μM benzo[k]fluoranthene and 1, 2.5, or 5 μM arsenite together; or 5 μM arsenite alone. Subsets of HepG2 cells, transfected with the plasmid, pHu1A1-FL, were pretreated with 1, 2.5, or 5 μM arsenite for 5, 15, or 30 min, or for 1, 3, 12, or 24 h. Cells were then treated with 0.5 μM benzo[k]fluoranthene for an additional 24 h. Additional subsets of cells were exposed to 0.1% DMSO and 0.5 μM benzo[k]fluoranthene for the duration of the arsenite pretreatment and the subsequent 24-h benzo[k]fluoranthene exposure. A subset of cells was exposed for 24 h to one of the following treatments: 0.1% DMSO; 0.5 μM benzo[k]fluoranthene; or 0.5 μM benzo[k]fluoranthene and 1, 2.5, or 5 μM arsenite. Cells transfected with the plasmid, pXRE-FL, were exposed to one of the following treatments: 0.1% DMSO; 0.5 μM benzo[k]fluoranthene; or 0.5 μM benzo[k]fluoranthene and 1, 2.5, or 5 μM arsenite, each in a total volume of 2 ml of DMEM and Serum Replacement 2 (diluted 50/1). After the appropriate exposure, cells were lysed with Passive Lysis Buffer (Promega), and enzyme activities were determined using a Dual-Luciferase reporter assay system (Promega). Quantification was performed using a Lumat LB 9501 luminometer.

mRNA Decay Rates. HepG2 cells were plated at a density of 1.0×10^6 in poly-D-lysine-precoated six-well plates for 24 h before treatment and yielded near-confluent monolayers. After 24 h, medium was removed and replaced with serum-free IVT medium containing one of the following treatments: 0.1% DMSO; 0.5 μM benzo[k]fluoranthene; 0.5 μM benzo[k]fluoranthene and 5 μM arsenite; or 5 μM arsenite alone, each in a total volume of 2 ml. The cells were then incubated in 5% CO₂ and 95% air at 37°C for 24 h. Total RNA was isolated from cells using TRI Reagent (time 0 was used as the control), and the cells in the remaining wells were treated with the transcriptional inhibitor, actinomycin D (3 μg/ml), to block further transcription. mRNA from actinomycin D-exposed cells was isolated at 3, 6, 12, or 24 h, thereafter. *CYP1A1*

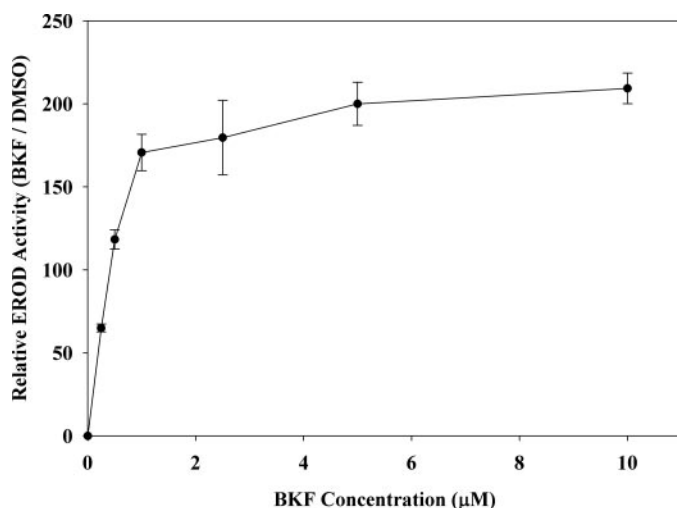


FIG. 1. Dose response of CYP1A1 EROD activities in HepG2 cells following a 24-h exposure to benzo[k]fluoranthene (0.25–10 μ M). EROD activities are reported relative to the corresponding activity in DMSO vehicle-treated cells (28 ± 1 fluorescence units). Values represent mean \pm S.E. for a single experiment ($n = 3$). Additional experimental methods are presented under *Materials and Methods*.

mRNA levels were determined using RT-PCR analysis and were compared with 28S rRNA levels, using the primers and protocol described under real-time RT-PCR.

Statistical Analysis. Results were analyzed by ANOVA and Dunnett's multiple comparisons test, using GraphPad InStat software (GraphPad Software Inc., San Diego, CA).

Results

Dose Response of CYP1A1 to Benzo[k]fluoranthene. An EROD activity assay was used to determine the dose response of benzo[k]fluoranthene induction of CYP1A1 in HepG2 cells. HepG2 cells were exposed for 24 h to benzo[k]fluoranthene over a concentration range of 0.25 to 10 μ M. Activities increased linearly up to 1 μ M benzo[k]fluoranthene and then plateaued (Fig. 1). A benzo[k]fluoranthene concentration of 0.5 μ M was thus used in the following studies to permit determination of possible decreases and increases in expression. None of the cell treatments in these studies produced general cytotoxicity.

Time Course of the Effect of Arsenite on Benzo[k]fluoranthene-Mediated Induction of CYP1A1 mRNA. The effect of 5 μ M arsenite on the benzo[k]fluoranthene-mediated induction of CYP1A1 mRNA in HepG2 cells, as determined by real-time RT-PCR, is shown in Fig. 2A. As indicated in the figure, arsenite alone did not induce CYP1A1 mRNA levels, whereas 0.5 μ M benzo[k]fluoranthene induced mRNA levels over the experimental period (48 h). When HepG2 cells were concomitantly exposed to 0.5 μ M benzo[k]fluoranthene and 5 μ M arsenite, the levels of CYP1A1 mRNA were significantly decreased (by 45% of the maximal level) compared with those in HepG2 cells treated with 0.5 μ M benzo[k]fluoranthene alone, although a similar time course was maintained. The 28S rRNA levels (Fig. 2B) in samples were determined by RT-PCR as a surrogate for total RNA in samples, as an additional check for spectrophotometrically determined values.

Dose Response of the Induction of CYP1A1 Upstream Promoter Firefly Luciferase Reporter Gene Construct, pHu-1A1-FL, by Benzo[k]fluoranthene. To optimize benzo[k]fluoranthene induction of the promoter reporter gene in the plasmid, pHu1A1-FL, containing approximately 1450 bases from the human CYP1A1 upstream and promoter regions (Fig. 3), a dual-luciferase assay was used. HepG2 cells were cotransfected with pHu-1A1-FL, the experimental plasmid containing the firefly luciferase reporter gene, and pRL-CMV, the

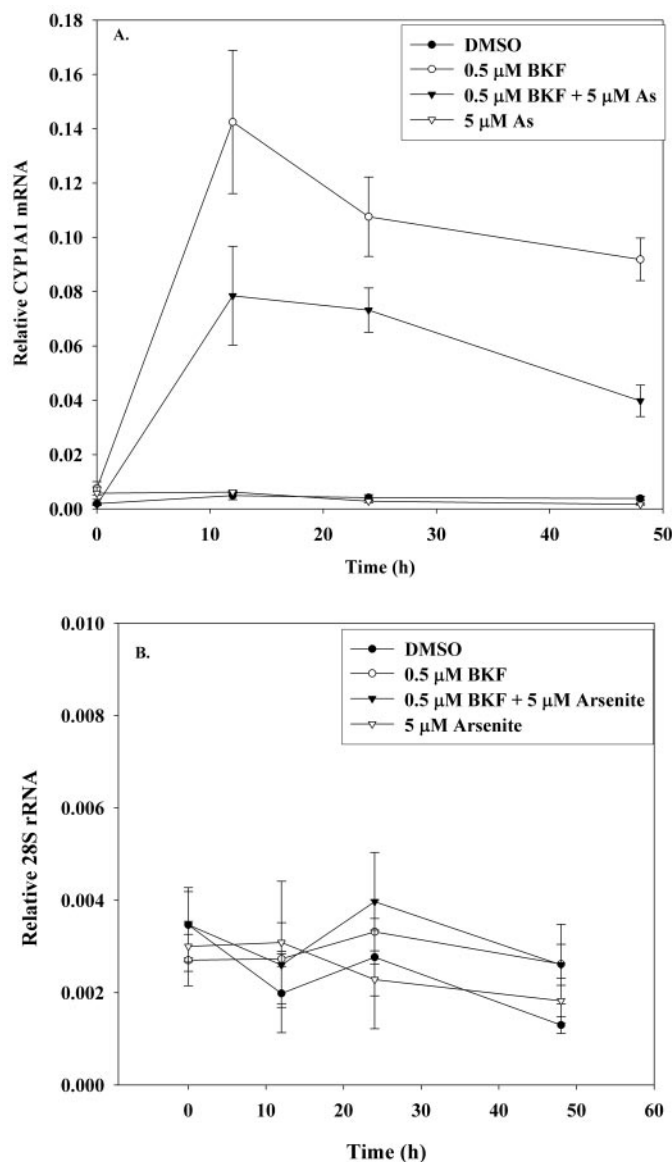


FIG. 2. Time course of the induction of CYP1A1 mRNA (A) and 28S rRNA (B) levels in HepG2 cells by 0.5 μ M benzo[k]fluoranthene, simultaneously administered 0.5 μ M benzo[k]fluoranthene and 5 μ M arsenite, DMSO vehicle, or 5 μ M arsenite. mRNA time courses were determined by real-time RT-PCR analysis of cells cultured in six-well plates at 37°C. The levels of 28S rRNA were used as a measure of total RNA content of the cells. mRNA levels are presented as relative levels of target gene expression to total RNA and were calculated from a standard curve and expressed as the mean \pm S.E. for a single experiment ($n = 3$). CYP1A1 mRNA and 28S rRNA real-time RT-PCR time course products were analyzed by ethidium bromide staining of an agarose gel. Primer sequences for the CYP1A1 mRNA and 28S rRNA assays are given in Table 1. The PCR products from CYP1A1 mRNA and 28S rRNA had approximate sizes of 367 bp and 100 bp, respectively, which are consistent with theoretical values. Additional experimental methods are presented under *Materials and Methods*.

plasmid containing *Renilla* luciferase that was used for normalization. Cells were exposed for 24 h to benzo[k]fluoranthene over a concentration range of 0.25 to 5 μ M. Benzo[k]fluoranthene induction of the construct was maximal at 0.5 μ M benzo[k]fluoranthene (Fig. 4) and, although the dose response was only linear with benzo[k]fluoranthene concentrations up to 0.125 μ M, 0.5 μ M benzo[k]fluoranthene was used for the subsequent study.

Effect of Arsenite on the Benzo[k]fluoranthene Responsiveness of pHu1A1-FL, pHu1A1- Δ 100-FL, or pHu1A1- Δ 1000-FL. To determine the effect of arsenite on the benzo[k]fluoranthene-mediated

CYP1A1 Promoter Constructs

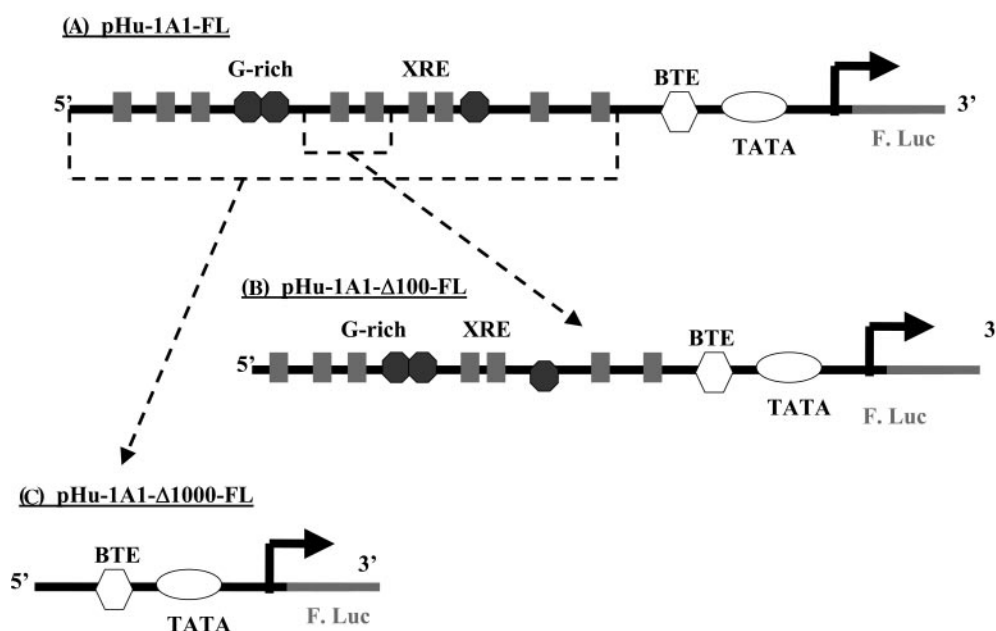


FIG. 3. *CYP1A1* promoter constructs. pHu-1A1-FL represents approximately 1450 bp of the human *CYP1A1* 5' upstream promoter region plasmid, inserted upstream of the firefly luciferase (F. Luc) reporter gene (A). The construct was generated using the human *CYP1A1/CYP1A2* BAC clone, GenBank accession number AF253322. The DNA fragment was inserted into an Asp 718/XhoI double-digested pGL3 basic vector, upstream of the F. Luc reporter gene. Regions of the pHu-1A1-FL were deleted using QuikChange site-directed mutagenesis kits and primers (sequences provided in Table 1), to produce pHu-1A1-Δ100-FL (B), which lacks the XRE sites at positions –1061 and –980 (Corchero et al., 2001), and pHu-1A1-Δ1000-FL (C), in which 1000 bp were deleted from the 5' end of pHu-1A1-FL, resulting in a 450-bp fragment of the 5' upstream region, including core promoter elements. BTE, basic transcription element.

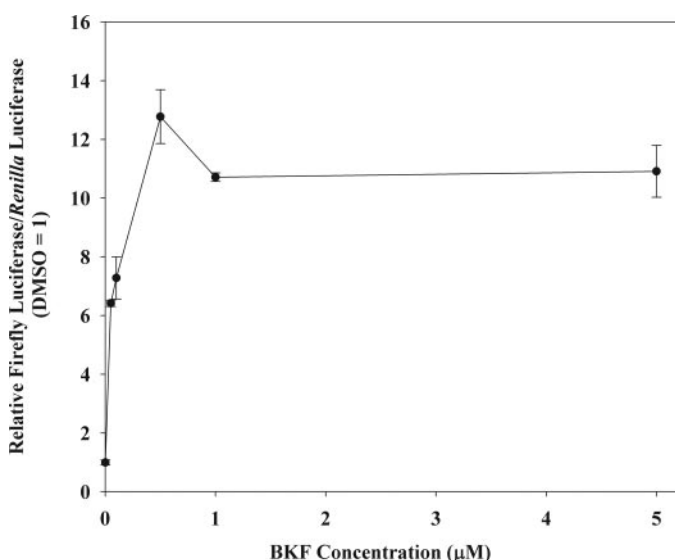


FIG. 4. Dose response of the induction of *CYP1A1* promoter firefly luciferase reporter gene construct (pHu-1A1-FL) by benzo[k]fluoranthene in HepG2 cells cotransfected with pRL-CMV. HepG2 cells were cotransfected for 24 h with pHu-1A1-FL, the experimental plasmid, and pRL-CMV, the plasmid containing *Renilla* luciferase and used for normalization. After 24 h of transfection, cells were treated for an additional 24 h with increasing concentrations of benzo[k]fluoranthene (0.05, 0.1, 0.5, 1, or 5 μM) or DMSO (vehicle control). At 24 h post-treatment, cells were harvested, and cell lysates were used to determine firefly luciferase experimental reporter activity, followed by normalization using *Renilla* luciferase activity. Values are expressed as the mean ± S.E. for a single experiment ($n = 3$). Additional experimental methods are presented under *Materials and Methods*.

responsiveness of the *CYP1A1* promoter region, HepG2 cells were cotransfected with pHu1A1-FL, pHu1A1-Δ100-FL, or pHu1A1-Δ1000-FL (Fig. 3) and pRL-CMV as an internal control. With the

full-length plasmid, pHu1A1-FL, arsenite alone did not induce the reporter gene, whereas the addition of 0.5 μM benzo[k]fluoranthene caused a significant, approximately 7.4-fold, induction. A significant 45% decrease in reporter gene expression occurred when 1 or 2.5 μM arsenite was added simultaneously with 0.5 μM benzo[k]fluoranthene. A significant, approximately 61%, decrease in the expression of the reporter gene occurred upon concomitant exposure of 0.5 μM benzo[k]fluoranthene and 5 μM arsenite to the transfected HepG2 cells (Fig. 5).

Cells transfected with pHu1A1-Δ100-FL, which lacks the XRE sites of pHu1A1-FL at positions –1061 and –980 (Jaiswal et al., 1985; Kubota et al., 1991; Corchero et al., 2001), showed greater responsiveness, by 1.7-fold, when administered 0.5 μM benzo[k]fluoranthene than did cells transfected with pHu1A1-FL. Also, as was observed with pHu1A1-FL, 5 μM arsenite, when administered together with 0.5 μM benzo[k]fluoranthene, significantly reduced, by 39%, the promoter reporter gene response compared with that of benzo[k]fluoranthene-treated cells. In contrast, 1 μM arsenite and 0.5 μM benzo[k]fluoranthene had no effect, compared with benzo[k]fluoranthene alone, on responsiveness; 2.5 μM arsenite significantly reduced by 27% the benzo[k]fluoranthene promoter gene response. Arsenite did not induce the reporter gene pHu1A1-Δ100-FL.

A 1000-bp deletion from the 5' end of pHu1A1-FL yielded the plasmid pHu1A1-Δ1000-FL, which contains approximately 450 bp of the 5' upstream flanking region of the *CYP1A1* gene, including core promoter elements. The inducibility of the pHu1A1-Δ1000-FL reporter gene by 0.5 μM benzo[k]fluoranthene was markedly lower, 31% and 18%, respectively, compared with the inducibility of the pHu1A1-FL and the pHu1A1-Δ100-FL reporter genes by 0.5 μM benzo[k]fluoranthene. Also, in contrast with pHu1A1-FL and pHu1A1-Δ100-FL, coexposure of 0.5 μM benzo[k]fluoranthene and 5

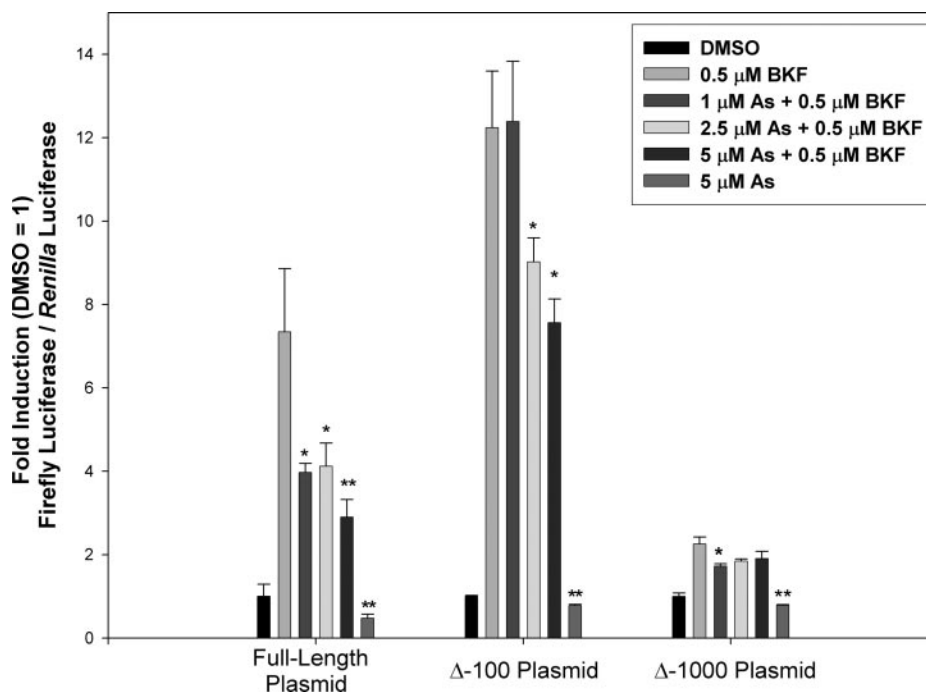


FIG. 5. Benzo[k]fluoranthene responsiveness of pHu1A1-FL, pHu1A1-Δ100-FL (Δ-100), or pHu1A1-Δ1000-FL (Δ-1000) experimental plasmid when cotransfected with pRL-CMV into HepG2 cells. After 24 h of transfection, the cells were treated with DMSO vehicle, 0.5 μM benzo[k]fluoranthene, 0.5 μM benzo[k]fluoranthene and 5 μM arsenite, or 5 μM arsenite alone. Cells were harvested 24 h post-treatment, and firefly luciferase experimental reporter activity was determined in the cell lysates, followed by normalization using *Renilla* luciferase activity. Each transfection was performed in triplicate. Values are reported as the mean ± S.E. for a single experiment ($n = 3$). The mean DMSO values ($n = 3$) were normalized to 1 by multiplying by 4.2 (full-length plasmid), 82.6 (Δ-100 plasmid), or 249.6 (Δ-1000 plasmid). Deletion of the 100-bp fragment and the 1000-bp fragment was performed using QuikChange site-directed mutagenesis kits as described under *Materials and Methods*. *, significantly different from benzo[k]fluoranthene-induced level, $p < 0.05$; **, $p \leq 0.01$.

μM arsenite did not cause a significant decrease in 0.5 μM benzo[k]fluoranthene induction. A significant, but not marked, decrease occurred with coexposure of 0.5 μM benzo[k]fluoranthene and 1 μM arsenite, and a similar, but not significant, decrease occurred with concomitant exposure of 0.5 μM and 2.5 μM arsenite. In this case, arsenite effects were not dose-responsive. However, in light of the low levels of induction of this construct by benzo[k]fluoranthene, little relevance can be placed on the role of arsenite in down-regulating CYP1A1 expression. Arsenite did not induce pHu1A1-Δ1000-FL.

Effect of Arsenite Pretreatment on the Subsequent Induction of the CYP1A1 Promoter Construct, pHu1A1-FL, by Benzo[k]fluoranthene. To determine the effect of arsenite pretreatment of HepG2 cells on the subsequent benzo[k]fluoranthene-mediated induction of CYP1A1, HepG2 cells were cotransfected with the full-length plasmid, pHu1A1-FL, containing the firefly luciferase reporter gene, and with pRL-CMV, containing the *Renilla* luciferase reporter gene that was used as an internal control.

Cells were pretreated with arsenite (1.0, 2.5, or 5.0 μM) for 5, 15, or 30 min, or for 1, 3, 12, or 24 h. Following arsenite pretreatment, 0.5 μM benzo[k]fluoranthene was added to the cells for an additional 24 h. As can be seen in Fig. 6, 1 μM arsenite pretreatment had limited, although sometimes statistically significant, effects on the benzo[k]fluoranthene-mediated induction of the CYP1A1 reporter construct at all time points. Pretreatment of HepG2 cells with 2.5 μM arsenite significantly decreased the corresponding benzo[k]fluoranthene-induced promoter activity at all time points except one (the 5-min time point). A significant, approximately 50% decrease occurred at all time points with the simultaneous addition or pretreatment of 5 μM arsenite.

Arsenite Effects as a Function of XRE Sequence. HepG2 cells were cotransfected with the pXRE-FL plasmid, containing an XRE core consensus sequence upstream of the firefly luciferase reporter

gene, and pRL-CMV, containing *Renilla* luciferase and used for normalization. The pXRE-FL reporter gene was induced 6.0-fold by 0.5 μM benzo[k]fluoranthene; however, the induction was not statistically significantly affected by the coexposure of 0.5 μM benzo[k]fluoranthene with 1, 2.5, or 5 μM arsenite, as was observed with the upstream promoter region constructs. Exposure of 5 μM arsenite alone had no effect on the pXRE-FL reporter gene (Fig. 7).

Effect of Arsenite on the CYP1A1 mRNA Stability. To determine the effect of arsenite on CYP1A1 mRNA stability, HepG2 cells were treated with DMSO (0.1%), 0.5 μM benzo[k]fluoranthene, 0.5 μM benzo[k]fluoranthene and 5 μM arsenite, or 5 μM arsenite, for 24 h. After 24 h, mRNA was isolated from time-0 wells, and actinomycin D was added to the remaining wells. Total RNA was then isolated at the time indicated (Fig. 8), and the levels of CYP1A1 mRNA were determined. As indicated in Fig. 8, benzo[k]fluoranthene and arsenite, either separately or together, did not affect CYP1A1 mRNA decay rates; thus, they did not affect CYP1A1 mRNA stability.

Discussion

Reports that arsenite affects induction of CYP1 enzymes at the level of transcription (Jacobs et al., 1999, 2000) prompted us to investigate the mechanisms of this interaction, to gain insight into how the metal, which is a frequent copollutant with PAHs, could affect the CYP1-mediated carcinogenicity of the PAHs. In particular, we designed studies to resolve questions on the effect of arsenite on CYP1A1 mRNA stability, the potential of arsenite to affect benzo[k]fluoranthene-liganded Ah receptor binding to the CYP1A1 XRE sequence, the stability of arsenite in HepG2 cultures with respect to its capacity to diminish benzo[k]fluoranthene induction of CYP1A1, and the potential of arsenite to affect CYP1A1 induction by benzo[k]flu-

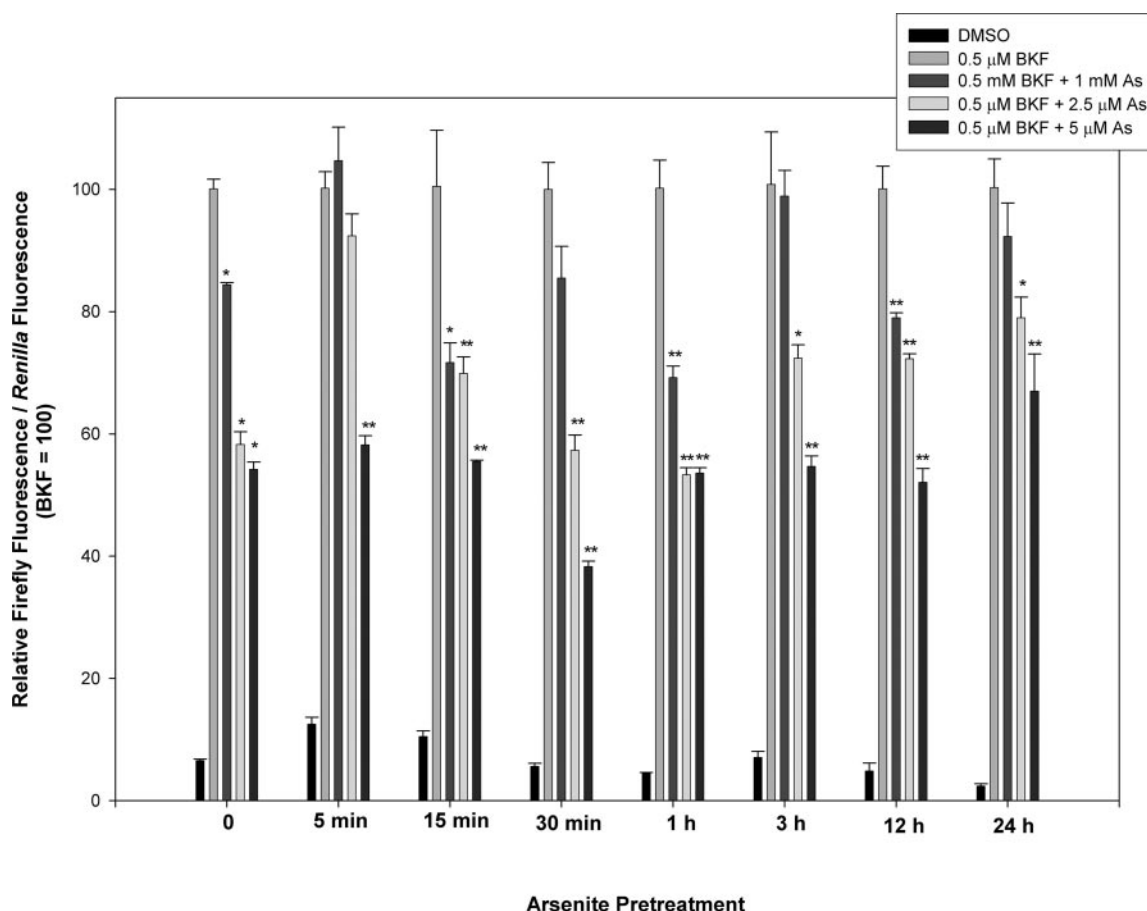


Fig. 6. Time course and dose response of arsenite pretreatment on the subsequent induction of the CYP1A1 promoter construct, pHu-1A1-FL, by benzo[k]fluoranthene. HepG2 cells were cotransfected with pHu-1A1-FL, the experimental plasmid, and pRL-CMV, the plasmid used for normalization. After transfection (24 h), the cells were pretreated with 1, 2.5, or 5 μ M arsenite for 5, 15, or 30 min, or for 1, 3, 12, or 24 h. After arsenite pretreatment, 0.5 μ M benzo[k]fluoranthene was added. A subset of cells was treated with 0.1% DMSO, 0.5 μ M benzo[k]fluoranthene, or 0.5 μ M benzo[k]fluoranthene and 1, 2.5, or 5 μ M arsenite for 24 h. Cells were harvested 24 h after benzo[k]fluoranthene treatment, and cell lysates were used to determine firefly luciferase experimental reporter activity, followed by normalization using *Renilla* luciferase activity. Each transfection was performed in triplicate. For each pretreatment time data set, the benzo[k]fluoranthene group alone was defined as 100%. Values are reported as the mean \pm S.E. for a single experiment ($n = 3$). Experimental methods are described under *Materials and Methods*. *, significantly different from benzo[k]fluoranthene-induced level, $p < 0.05$; **, $p \leq 0.01$.

oranthene by interacting directly with the 5' regulatory sequences of CYP1A1.

In the present study, we initially confirmed in HepG2 cells that arsenite decreases the outcome of benzo[k]fluoranthene-mediated induction of CYP1A1 mRNA. The RT-PCR analysis used to obtain these results does not, however, differentiate between transcriptional and post-transcriptional mechanisms. Similar results were observed in primary cultures of chick hepatocytes (Jacobs et al., 1998), rat hepatocytes (Jacobs et al., 1999), and Hep3B cells (Vernhet et al., 2003), all coexposed to arsenite/PAH mixtures. The current observation of decreased CYP1A1 expression mediated by arsenite is at odds with our previous observations in HepG2 and human hepatocytes that no comparable changes were noted (Vakharia et al., 2001a,b). We believe that this discrepancy is a result of our current use of real-time PCR technology, which permits analysis of the linear amplification phase. However, arsenite did not affect PB-mediated induction of CYP3A23 in rat hepatocytes (Jacobs et al., 1999). Furthermore, arsenite did not affect benzo[a]pyrene (BAP)-inducible CYP1A1 enzymatic activity but did cause an increase in BAP-DNA adduct levels in mouse hepatoma Hep-1 cells (Maier et al., 2002). Arsenite also did not affect BAP-mediated induction of CYP1A1 in the human lung adenocarcinoma cell line (Ho and Lee, 2002).

To determine whether the mechanism of arsenite effects on

CYP1A1 induction involves direct interaction at the CYP1A1 promoter, plasmids were constructed containing the promoter and enhancer regions of the CYP1A1 gene, or truncated versions thereof. The results with the full-length plasmid (Fig. 5), in which arsenite produced decreases similar to those observed with RT-PCR analysis, namely, an approximately 50% decrease in activity, suggest that the effect of arsenite is at the level of transcription and that it is occurring within the promoter or enhancer region of the CYP1A1 gene.

To determine more specifically the site within the CYP1A1 promoter region responsible for the decrease in benzo[k]fluoranthene induction by arsenite, we replaced the full-length plasmid with a truncated plasmid containing approximately 450 bp, including the core promoter. The basal levels of transcription from the truncated plasmid were reduced by more than 60-fold compared with the full-length plasmid, and inducibility by benzo[k]fluoranthene was virtually abolished. Consequently, no dose-responsive effects of arsenite on benzo[k]fluoranthene-mediated expression levels, using this truncated promoter, were detectable. This finding is consistent with reports that CYP1A1 induction occurs through XRE sequences that are located in the deleted enhancer region of the gene (Whitlock, 1999; Ma, 2001).

The truncated plasmid with two XRE sites deleted from positions -1061 and -980 (Corchero et al., 2001) exhibited basal-level ex-

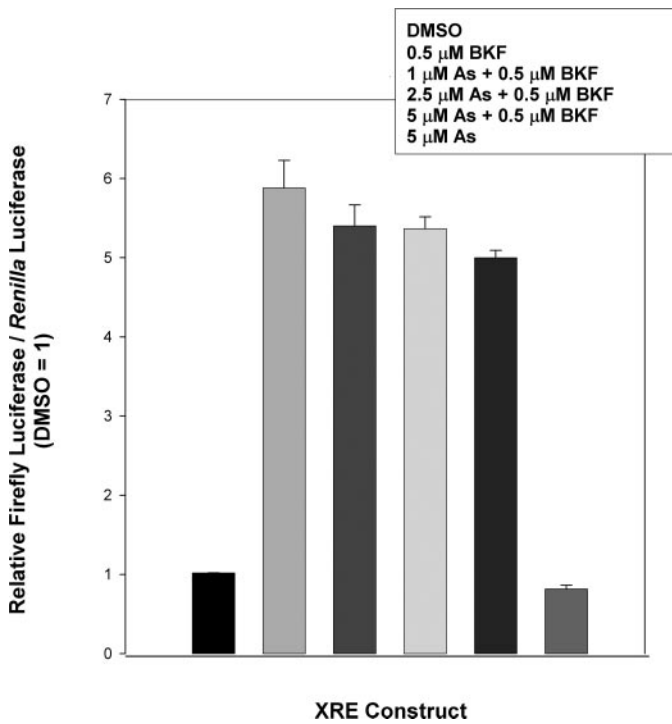


FIG. 7. Benzo[k]fluoranthene responsiveness of the pHuXRE-FL (human XRE consensus sequence) experimental plasmid. The experimental plasmid, pHuXRE-FL, and pRL-CMV were used to cotransfect HepG2 cells. After transfection (24 h), the cells were treated with 0.1% DMSO, 0.5 μ M benzo[k]fluoranthene, 0.5 μ M benzo[k]fluoranthene and 1, 2.5, or 5 μ M arsenite, or 5 μ M arsenite. Control cells were treated with the benzo[k]fluoranthene vehicle, DMSO. Cells were harvested 24 h post-treatment. Cell lysates were used to determine firefly luciferase experimental reporter activity, followed by normalization using *Renilla* luciferase activity. Each transfection was performed in triplicate. Values are reported as the mean \pm S.E. for a single experiment ($n = 3$).

pression that was reduced by 20-fold compared with the full-length plasmid. These XRE sites were selected based on the high specific luciferase activity in two plasmids that contained two identical copies

of each site, individually, from the human *CYP1A1* gene (Kress et al., 1998). Despite this reduction in basal levels, arsenite affected benzo[k]fluoranthene induction to an extent similar to that observed with the full-length plasmid. The extent of induction by benzo[k]fluoranthene with this truncated plasmid over the basal level was significantly greater than the corresponding increase with the full-length plasmid.

Arsenite is methylated in HepG2 cells (Lin et al., 2002), and this was postulated to be a mechanism for detoxification. However, other studies indicate that trivalent methylated arsenic metabolites are toxic (Kitchin, 2001). When HepG2 cells were transfected with the full-length plasmid and were then pretreated with arsenite for up to 24 h, before an additional 24-h exposure to benzo[k]fluoranthene, arsenite retained its capacity to diminish the benzo[k]fluoranthene-mediated induction of the plasmid reporter gene. Thus, for up to a 24-h pretreatment of the cells by arsenite, its effect on benzo[k]fluoranthene induction of *CYP1A1* effectively remained the same as when arsenite and benzo[k]fluoranthene were added simultaneously. This suggests that if any metabolism of the arsenite had occurred in the up-to-24-h pre-exposure period, that metabolism did not affect the role of arsenite in diminishing *CYP1A1* induction by benzo[k]fluoranthene.

To determine whether arsenite-mediated decreases in benzo[k]fluoranthene-induced *CYP1A1* transcription occurred directly through the AhR pathway, a construct containing the core consensus sequence of an XRE site was prepared and tested. Arsenite, when simultaneously administered with benzo[k]fluoranthene, did not significantly decrease the expression levels following treatment with benzo[k]fluoranthene alone. We thus conclude that arsenite's effects are not occurring directly through the AhR pathway, which is consistent with results of published studies (Vernhet et al., 2003).

The studies designed to determine the potential of arsenite to affect the stability of *CYP1A1* mRNA revealed no change in the stability, indicating that arsenite-mediated down-regulation of *CYP1A1* induction by benzo[k]fluoranthene has not contributed to mRNA destabilization. These results are similar to previous findings, in which arsenite treatment did not affect the mRNA stability of *CYP1A4* and *CYP1A5* in chick hepatocyte cells (Jacobs et al., 2000).

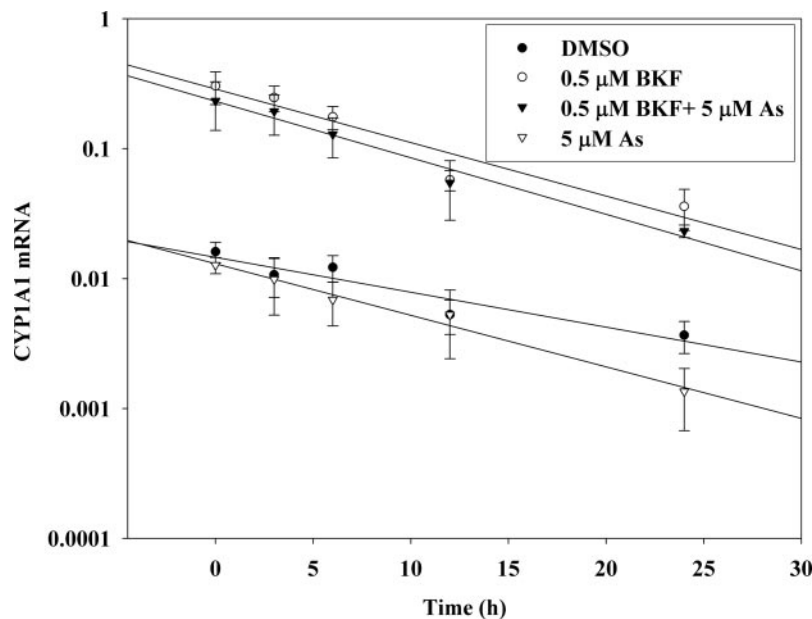


FIG. 8. *CYP1A1* mRNA stability in HepG2 cells exposed to benzo[k]fluoranthene and arsenite, determined using real-time RT-PCR analysis. Results have been normalized to total RNA levels and are expressed as the mean \pm S.E. for a single experiment ($n = 3$). HepG2 cells were exposed to 0.1% DMSO, 0.5 μ M benzo[k]fluoranthene, 0.5 μ M benzo[k]fluoranthene and 5 μ M arsenite, or 5 μ M arsenite alone, for 24 h. After the 24-h exposure, total RNA was isolated from time-0 wells, and 3 μ g/ μ l actinomycin D was added to the remaining wells, to block further transcription. Total RNA was then isolated at 3, 6, 12, or 24 h after the addition of actinomycin D.

In summary, our results indicate that arsenite affects benzo[k]fluoranthene induction, in part, at the level of transcription, as determined by RT-PCR analysis. More specifically, this effect was determined to occur within 1450 bp of the 5' upstream promoter region of the *CYP1A1* gene, which contains the transcriptional control region and includes the core promoter and an enhancer region with internal XRE sites. The two XRE sites (−1061 and −981) were not involved, whereas the result possibly indicates the involvement of a negative regulatory element. Arsenite is stable for at least 48 h in the HepG2 cell medium with respect to its ability to diminish CYP1A1 benzo[k]fluoranthene induction. Arsenite does not affect benzo[k]fluoranthene induction directly through XRE sites, nor does it have an effect on CYP1A1 mRNA stability.

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