Molecular Basis for Effects of Carcinogenic Heavy Metals on Inducible Gene Expression

Joshua W. Hamilton, Ronald C. Kaltreider, Olga V. Bajenova, Michael A. Ihnat, Jennifer McCaffrey, Bruce W. Turpie, Erin E. Rowell, Jannet Oh, Michael J. Nemeth, Carrie A. Pesce, and Jean P. Lariviere

Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire, and Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Lebanon, New Hampshire

Certain forms of the heavy metals arsenic and chromium are considered human carcinogens, although they are believed to act through very different mechanisms. Chromium(VI) is believed to act as a classic genotoxic and mutagenic agent, and DNA/chromatin appears to be the principal target for its effects. In contrast, arsenic(III) is considered nongenotoxic, but is able to target specific cellular proteins, principally through sulfhydryl interactions. We had previously shown that various genotoxic chemical carcinogens, including chromium(VI), preferentially altered expression of several inducible genes but had little or no effect on constitutive gene expression. We were therefore interested in whether these carcinogenic heavy metals might target specific but distinct sites within cells, leading to alterations in gene expression that might contribute to the carcinogenic process. Arsenic(III) and chromium(VI) each significantly altered both basal and hormone-inducible expression of a model inducible gene, phosphoenolpyruvate carboxykinase (*PEPCK*), at nonovertly toxic doses in the chick embryo *in vivo* and rat hepatoma H4IIE cells in culture. We have recently developed two parallel cell culture approaches for examining the molecular basis for these effects. First, we are examining the effects of heavy metals on expression and activation of specific transcription factors known to be involved in regulation of susceptible inducible genes, and have recently observed significant but different effects of arsenic(III) and chromium(VI) on nuclear transcription factor binding. Second, we have developed cell lines with stably integrated *PEPCK* promoter–luciferase reporter gene constructs to examine effects of heavy metals on promoter function, and have also recently seen profound effects induced by both chromium(VI) and arsenic(III) in this system. These model systems should enable us to be able to identify the critical *cis* (DNA) and *trans* (protein) cellular targets of heavy metal exposure leading to alterations in expression of specific susceptible genes. It is anticipated that such information will provide valuable insight into the mechanistic basis for these effects as well as provide sensitive molecular biomarkers for evaluating human exposure. — *Environ Health Perspect* 106(Suppl 4):1005–1015 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-4/1005-1015hamilton/abstract.html*

Key words: chromium, arsenic, mitomycin C, phosphoenolpyruvate carboxykinase, transcription factors, genetic constructs, transfection

This paper is based on a presentation at the Symposium on the Superfund Basic Research Program: A Decade of Improving Health through Multi-Disciplinary Research held 23–26 February 1997 in Chapel Hill, North Carolina. Manuscript received at *EHP* 11 December1997; accepted 27 March 1998.

The authors dedicate this manuscript to the memory of the late Dr. Karen E. Wetterhahn, who was an invaluable scientist, mentor, colleague, and friend. Dr. Wetterhahn and her laboratory provided reagents, laboratory resources, and valuable advice on this project. Most important, Dr. Wetterhahn was the original director and the major impetus behind the Dartmouth Superfund Basic Research Program Project which made this research possible. The authors also wish to thank A. Barchowsky and his lab for their advice and assistance in the gel shift analysis. This work was supported by grants to JWH from NIH-NIEHS (ES07373), NIH-NCI (CA49002), and the Norris Cotton Cancer Center. JWH was also partially supported by the Norris Cotton Cancer Center; RCK and OVB were supported by fellowships from NIH-NIEHS (ES07373) and AJW was partially supported by the Dartmouth College Chemistry Department. Support for the Dartmouth College Molecular Biology Core Facility was provided by NIH-NIEHS (ES07373) and the Norris Cotton Cancer Center Core grant (CA23108).

Address correspondence to J.W. Hamilton, Department of Pharmacology and Toxicology, 7650 Remsen, Dartmouth Medical School, Hanover NH 03755-3835. Telephone: (603) 650-1316. Fax: (603) 650-1129. E-mail: josh.hamilton@dartmouth.edu

Abbreviations used: EMSA, gel electrophoretic mobility shift assay; MMC, mitomycin C; MMS, methyl methanesulfonate; PEPCK, phosphoenolpyruvate carboxykinase; TNFα, tumor necrosis factor α; TNFβ, tumor necrosis factor β; U.S. EPA, U.S. Environmental Protection Agency; UV, ultraviolet.

Introduction

The heavy metals chromium(VI) and arsenic(III) are considered human carcinogens and share several properties in regard to their carcinogenicity. They are both associated with certain tumors in humans based principally on epidemiologic evidence that is quite strong and that preceded animal carcinogenicity data by many years (*1–3*). Both are considered lung carcinogens whose effects are synergistic with cigarette smoking or exposure to other lung carcinogens such as benzo[*a*]pyrene or other carcinogenic metals. Human exposure to these metals occurs principally in occupational settings and through environmental contaminations such as from toxic waste sites, including numerous Superfund sites, or from naturally contaminated drinking water. Chromium and arsenic are listed as 2 of the 8 heavy metals that are among the top 22 compounds of particular concern to the U.S. Environmental Protection Agency (U.S. EPA) Office of Solid Waste and Emergency Response concerning Superfund sites. Thus, there is now increasing concern about human health effects from exposure to heavy metals found in high concentrations at these sites, such as the high levels of chromium and arsenic at waste sites in Woburn, Massachusetts, and northern New Jersey. However, potential human exposures and health risk from these sites are less well defined. Thus, understanding the overall human health impact of environmental exposure to these heavy metals is of paramount concern.

Chromium(VI) is a known human lung carcinogen and may also contribute to nasopharyngeal cancers (*1,2,4,5*). A strong association between chromium exposure and an increase in lung cancer was first observed in the chrome ore industry in Germany in the 1920s. Since then, there have been numerous studies linking inhalation exposure to chromium, particularly chromium(VI) compounds, with increases in lung and nasal cancers. However, early recognition of this association and significant decreases in exposure through industrial hygiene practices have reduced these incidences. Arsenic is considered to be a probable human lung, skin, and bladder carcinogen (*3,6*). Lung exposure has been principally through occupational settings in the mining, processing, and smelting of arsenic-containing ores, and in the manufacture of arsenic-containing pesticides, whereas skin exposure has been through

many of these same occupational settings as well as topical application of arsenical medications. There is also an association between environmental arsenic exposure, e.g., through arsenic in well water, and an increased risk of certain skin and possibly bladder cancers (*3,7*). Arsenic exposure has also been implicated in certain other tumor incidences including scrotal cancer (copper smelters) and hepatic angiosarcomas (vineyard workers) (*3,7*). As with the other carcinogenic metals, there is a strong synergistic association between arsenic exposure and cigarette smoking for risk of lung cancer. However, in the case of arsenic, this is complicated by the fact that there were high levels of arsenic in tobacco prior to the 1970s as a result of use of arsenical pesticides in tobacco agriculture (*3*). Arsenic has only recently been shown to be carcinogenic in animal models (*3*).

The form and valence of chromium is a major factor in assessing its biologic effects, and chromium(VI) is the principal form responsible for the mutagenic and carcinogenic properties, as first proposed in the uptake-reduction model by Wetterhahn and co-workers (*1,4,8,9*). Chromium(VI) has been shown to be genotoxic, mutagenic, and carcinogenic in a large number of short- and long-term assay systems using various end points and systems (*1,2,4,8,9*). Like many organic carcinogens such as benzo[*a*]pyrene and aflatoxin B_1, chromium(VI) appears to act as a classic initiator in these test systems, and this is believed to be the principal mechanism by which chromium(VI) increases carcinogenic risk. Our laboratory has hypothesized that genotoxic carcinogens, including chromium(VI), exert preferential effects on the expression of a specific class of genes, i.e., inducible genes, as a result of nonrandom DNA damage targeted to members of this gene class (*8–12*). Previous studies in our laboratory have shown that a number of different genotoxic chemical carcinogens that induce different types of DNA damage, including the genotoxic metals chromium and nickel, significantly alter both basal and inducible expression of several model inducible genes but have no effect on expression of constitutive genes. Assuming that the effects on gene expression are a result of carcinogen-induced DNA damage, what is their molecular basis?

We have postulated that inducible genes are strongly affected by chemically-induced DNA damage as a result of both targeting of DNA damage and the intrinsic structural and biochemical properties of those genes, and have proposed a model in which these effects occur as a consequence of chromatin structure and/or nuclear architecture (*10,13*). This nuclear model provides a framework for understanding how two different genes in the same nucleus may reside in very different physical and chemical environments, and may therefore have different sensitivities to chemical attack. Thus, even low levels of DNA damage *in vivo* might be sufficient to have significant effects on expression of certain sensitive genes. We previously demonstrated that treatment of 14-day chick embryos with a single administration of chromium(VI), at a dose that produced no overt toxicity but that caused significant levels of DNA damage (*14*), had profound effects on expression of several inducible genes while having no effect on expression of several constitutively expressed genes (*10*). Both the basal and inducible expression of the inducible 5′-aminolevulinate synthase, cytochrome P450 *CYP2H1,* and phosphoenolpyruvate carboxykinase (*PEPCK*) genes were markedly affected by the chromium treatment, whereas the albumin, transferrin, and β-actin genes were refractory to this treatment. The effects on expression of the inducible genes were seen at both the steady-state mRNA and transcriptional levels, and the time courses for these effects closely matched the time course for chromium-induced DNA damage and repair (*10,12,14*). Interestingly, certain effects most closely correlated with chromium–DNA monoadduct formation, whereas other effects were more closely associated with chromium–DNA cross-link formation (*10,12,14*).

In contrast to chromium, the mechanistic basis for the carcinogenic effects of arsenic is still poorly understood. Arsenic has generally been shown to be negative in bacterial and mammalian mutagenesis assays, and there is little evidence of DNA damage induced by arsenic, although it has been demonstrated to be a clastogen and weak inducer of sister chromatid exchanges in some systems (*3,6*). Arsenic causes morphologic transformation, anchorage independence, aneuploidy, and gene amplification in mammalian cell culture assays (*6*). These effects may be related to its ability to alter DNA synthesis and DNA repair, as well as fidelity of chromosome segregation. Arsenite [As(III)] is approximately 4-fold more toxic than arsenate [As(V)], although these two forms can be inter-converted by redox pathways *in vivo* (*3*). Arsenic(III) is readily methylated *in vivo*, and the methylated forms have a low interaction with macromolecules and are readily excreted. However, the rate of methylation differs among tissues and species. The primary mechanism of arsenic(V) toxicity is uncoupling of oxidative phosphorylation, by substitution for inorganic phosphate. Arsenic(III) also can cause oxidative bursts in endothelial and other cells, and is heat shock-mimetic, inducing the classic heat shock and stress responses of cells (*15*). Thus, its biologic effects are more closely associated with cytoplasmic rather than nuclear effects, and DNA does not appear to be the primary target for arsenic toxicity or carcinogenesis. In this regard arsenic appears to act more as a classic promoter such as the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate or hydrogen peroxide, although its specific effects may be more complex. Arsenic displays distinct interactions with specific proteins *in vivo,* which may be the basis for much of its specific toxic effects as well as its carcinogenicity.

Most of the intracellular effects of arsenic are attributed to arsenic(III) rather than arsenic(V). Arsenic(III) binds avidly to thiols in cells, both to small molecule thiols such as glutathione as well as thiol-containing proteins (*3,16,17*). Arsenic has a particular avidity for hydrolases and can compete with phosphate to inhibit enzymes such as phosphoraldehyde and glucose dehydrogenase (*3*). Many of the cellular effects of arsenic can be ascribed to its ability to elicit a heat-shock response (*15,16*). Arsenic induces phosphorylation of hsp27, increases expression of hsp27, hsp70, and hsp90 through the heat-shock factor, induces heme oxygenase, *mdr1,* and quinone reductase gene expression, and induces metallothionein expression by both a transcriptional and posttranscriptional mechanism, although arsenic is not a ligand for metallothionein protein binding (*3,18,19*). Arsenic exhibits a strong preferential binding to the vicinal dithiol of the glucocorticoid receptor, inhibiting binding of glucocorticoid hormone but not altering hsp90 binding (*16,17*). This effect is extremely specific, as there is not a similar effect on the closely related receptors for androgen, estrogen, mineralocorticoids or progesterone. Because at least one of the model inducible genes we have used in our studies, i.e., *PEPCK,* is regulated by glucocorticoids, we predicted that this induction pathway would also be markedly affected by arsenic treatment. Other effects on *PEPCK* expression are also predicted to be likely, as a result of arsenic effects on other

specific response pathways and regulatory proteins within the cell.

Thus, there is reason to believe that both chromium and arsenic can have very selective effects on activation of regulatory pathways and expression of certain genes *in vivo*, albeit through different mechanisms. We are particularly interested in testing the hypotheses that specific DNA regulatory regions within the promoters of targeted genes are responsible for mediating the *cis* effects, and that specific transcriptional factors are responsible for mediating the *trans* effects of these heavy metals on inducible gene expression. Such effects may be very important in understanding the overall effects of these and other heavy metals on the carcinogenic process. The purpose of these studies is to explore these hypotheses in detail.

Materials and Methods

Animal and Cell Treatment and Sample Preparation

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Fertile White Leghorn chicken eggs (Truslow Farms, Inc., Chestertown, MD) were incubated as previously described (*20*). Test compounds were administered in solution onto the inner shell membrane as previously described (*21*). Chromium(VI) was administered as sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) at a dose of 50 µmol/kg in 100 µl H_2O. Arsenic(III) was administered as sodium arsenite ($NaAsO_2$) at a dose of 100 µmol/kg in 100 µl H_2O. Dexamethasone was administered as dexamethasone-21-phosphate in 10 µl H_2O at a dose of 5 µmol/kg embryo weight. For steady-state mRNA studies, after embryo treatment the livers were removed and immediately frozen at –75 °C. H4IIE rat hepatoma and MDA-MB-435 (MDA) cells were grown and treated essentially as previously described (*22*). UV-C (ultraviolet C) doses (254 nm) were administered to cells in 150-mm cell culture dishes with a UV Stratalinker (Stratagene, LaJolla, CA) or using a calibrated germicidal lamp at the desired dose. Total cellular RNA was subsequently isolated using a guanidine isothiocyanate–cesium chloride gradient technique exactly as previously described (*20*). For the electrophoretic mobility shift assays (EMSA), cells were washed with ice-cold phosphate-buffered saline (pH 7.4), containing 1.0 mM EDTA, protease inhibitor mix (4 µg/ml leupeptin; 4 µg/ml soybean trypsin inhibitor; 1 mM benzamidine), 7 µg/ml L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone, and 0.1 mM dithiothreitol (DTT). Nuclear protein soluble extract was isolated by a procedure of Dignam et al. (*23*), as modified by Barchowsky (*24*).

Measurement of mRNA Levels and EMSA Analysis

Steady-state mRNA levels were measured by a quantitative solution hybridization assay using gene-specific 5′-[^{32}P]-end-labeled synthetic oligonucleotide probes, exactly as previously described (*20*). This assay has been shown to provide a sensitive, linear, and quantitative measure of specific steady-state mRNA levels (fmol of mRNA per milligram of total RNA), and can accurately determine differences in mRNA expression of less than 20% (*10,20,25,26*). The c-*fos*, c-*jun*, *gadd45*, *gadd153*, and *PEPCK* solution hybridization probes we used are synthetic cDNA oligonucleotides corresponding to nucleotides 222–248 (*27*), 622–648 (*28*), 697–719 (*29*), 517-540 (*30*), and 268–291 (*31*), respectively, of the published sequences. Double-stranded DNA consensus sequences for AP-1 and nuclear factor-kappa B (NF-κB) for use as EMSA probes were obtained from Promega (Madison, WI). The Sp1 double-stranded probe was synthesized based on the Sp1/EGR1 site within the *mdr1* gene promoter. Protein concentrations were determined by a standard absorbance assay (bicinchoninic acid [BCA] assay; Pierce, Rockford, IL). The EMSA analysis was performed essentially as previously described (*32*) with the following modifications (*33*): a high ionic loading buffer was used containing 10 mM Tris, 20 mM KCl, 0.2 mM DTT, 0.2 mM EDTA, 4% glycerol, 0.2 µg/µl bovine serum albumin, 0.02 µg/µl poly d(IC) (GIBCO-BRL, Gaithersburg, MD), and 0.04 µg/µl salmon sperm DNA (pH 8.0). A high ionic-strength running buffer was also used containing 50 mM Tris, 380 mM glycine, 2.0 mM EDTA (pH 8.0).

Generation and Analysis of *PEPCK*-Luciferase Genetic Constructs

All DNA-modifying enzymes and lipofectamine were purchased from GIBCO-BRL. The pGL3 vectors and the luciferase assay system were purchased from Promega. The *PEPCK*–luciferase reporter vectors were constructed by cloning a region of the *PEPCK* promoter into the multiple cloning site of the pGL3 basic vector. Rat genomic DNA from H4IIE cells was isolated and used as a template for the PCR amplification of the PEPCK promoter. The sequences for the upper (forward) primers were 5′-CTAGCTAGCCAATCACCCCT (NheI, –592 of the rat *PEPCK* promoter region) and 5′-CTAGCTAGCCATGT CCCTGCCCCC (NheI, –1162 of the rat *PEPCK* promoter region); and the sequence of the lower (reverse) primer was 5′-GGAAGATCTCTACCTTTCTT (Bgl II, +87 of the rat *PEPCK* promoter region). All plasmids used were grown in *Escherichia coli* JM109 strain. The *PEPCK* promoter fragments were digested with NheI and BglII enzymes and ligated into pGL3. Plasmids containing inserts were sequenced with an ABI PRISM DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). All sequences were checked against GenBank *PEPCK* sequences using the Blast sequence analysis program to ensure sequence specificity. Plasmid DNA was purified using Qiagen columns (Qiagen, Valencia, CA) and transfected into H4IIE rat hepatoma cells using the lipofectamine technique (GIBCO-BRL). To obtain stable transfectants, cells were cotransfected with two types of plasmids: pGL3 reporter vectors with different promoters plus pSV2neo that confers resistance to neomycin. Cells were selected in G418 and resistant colonies were subcloned and checked for *PEPCK*–luciferase insertion. For the luciferase assay, cells were lysed in the luciferase reporter buffer in the wells according manufacturer's instructions (Luciferase assay system, Promega). Luciferase activity was measured using a 96-well plate luminometer (Dynatech Laboratories, Chantilly, VA). Protein concentrations were determined by a standard absorbance DC (detergent compatible) protein assay (Bio-Rad, Hercules, CA).

Statistical Analysis

Data were analyzed for statistical significance by ANOVA, Student's *t*-test, and nonparametric analysis, where appropriate, using the Instat and Prism software programs (GraphPad Software, San Diego, CA).

Results

Initial experiments were conducted to examine whether there were preferential effects of chromium and arsenic on expression of a model-inducible gene, *PEPCK*, in 14-day chick embryo livers. The chick embryo represents a simple, inexpensive, and easily manipulated *in vivo* model and has been an excellent system for examining effects of carcinogens on gene expression

based on our previous studies (*10–13, 34–36*). Chick embryo development has been extensively characterized, providing a large background of information about its embryology, physiology, and biochemistry. This model has been used to investigate the genotoxicity of over 50 chemicals using a wide variety of genetic end points, including chromosomal damage, sister chromatid exchange, inhibition of DNA synthesis, unscheduled DNA synthesis, carcinogen-DNA binding, and DNA alkaline elution (*10,14,21,37–39*). The chick embryo also possesses an active, highly inducible hepatic mixed-function oxidase enzyme system capable of metabolizing indirect-acting mutagen–carcinogens to active forms (*20,21,26,37,38,40*). The chick embryo can also be used to specifically examine the developmental effects of agents on an embryonic system and mimics the human fetus in many important aspects with regard to its toxicology (*21,37,38,40,41*). Thus, this system represents an excellent nonmammalian whole animal toxicology model.

The highest doses of chromium(VI) and arsenic(III) that caused no overt toxicity or lethality in dose–response experiments were determined to be 50 µmol/kg and 100 µmol/kg, respectively (*10,12,42*). At these doses there is little or no effect on overall DNA, RNA, or protein synthesis, and the embryos can be hatched normally with no obvious toxic effects (*10,12,42*). Using these doses, 14-day embryos were treated with a single administration of chromium or arsenic, and mRNA expression of various inducible and constitutive genes was measured over time (Figure 1A–C). Chromium (Figure 1A) or arsenic (Figure 1B) treatment significantly increased the basal expression of *PEPCK*, but had no effect on expression of β-actin (Figure 1C). Chromium and arsenic also significantly altered the response of the *PEPCK* gene to glucocorticoid induction (Figure 1A,B). Chromium increased the induction response of *PEPCK* to glucocorticoids initially (1–2 hr) but at later times almost completely suppressed the response of *PEPCK* to its normal induction signal (Figure 1A). Arsenic had similar effects, although the magnitude of the response was greater and occurred over a more protracted time than the response to chromium (Figure 1B).

These effects were then examined in the H4IIE rat hepatoma cell line to investigate the molecular basis for this phenomenon. This cell line expresses *PEPCK* in a basal and hormone-inducible manner similar to that of the liver *in vivo*, and has been used extensively to examine *PEPCK* regulation [reviewed by Granner et al. (*43*) and Lucas and Granner (*44*)]. Initial experiments established the toxicity dose–response to chromium and arsenic using a colony-forming assay as a measure of cell survival (Figure 2). These experiments established the maximal noncytotoxic doses as well as the minimal doses which produced complete cell death. These were used in subsequent experiments to examine responses to low (i.e., noncytotoxic) versus high (i.e., cytotoxic) doses of heavy metals. Using these low doses, the effects of chromium and arsenic on PEPCK expression were examined in the H4IIE cells, and compared to the effects of mitomycin C (MMC), an organic DNA cross-linking agent with similar genotoxic properties as chromium(VI). Figure 3 shows that

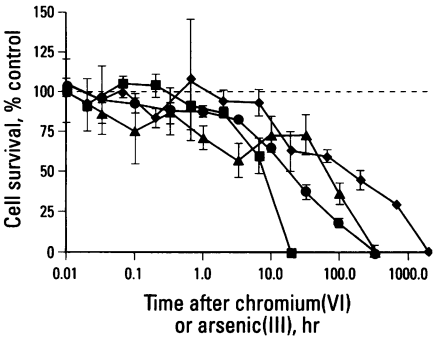

Figure 2. Cytotoxicity of chromium(VI) and arsenic(III) in rat hepatoma H4IIE and human breast carcinoma MDA-MB-435 cell lines. Cells were treated with chromium or arsenic for 4 hr and cytotoxicity was assessed by a colony-forming assay as described in "Materials and Methods." Data are expressed as a percent of control colonies formed. Each data point represents the mean ± SD of values from three separate plates. ●, MDA cells treated with arsenic; ■, MDA cells treated with chromium; ▲, H4IIE cells treated with arsenic; ◆, H4IIE cells treated with chromium.

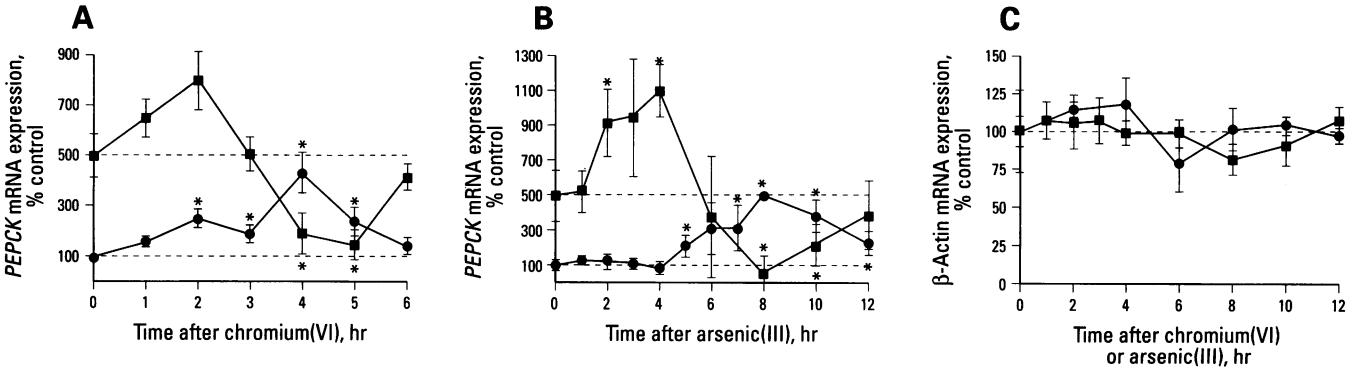

Figure 1. Effects of chromium(VI) and arsenic(III) on basal and inducible *PEPCK* mRNA expression and on constitutive β-actin mRNA expression in 14-day chick embryo liver *in vivo*. Embryos were treated for various times with a single dose of either 50 µmol/kg chromium(VI) as sodium dichromate or 100 µmol/kg arsenic(III) as sodium arsenite, and expression of *PEPCK* and β-actin mRNA was measured in total liver RNA samples by solution hybridization using a gene-specific oligonucleotide probe as described in "Materials and Methods." Alternatively, embryos were treated with chromium or arsenic and beginning at various times afterward were treated with 5 µmol/kg dexamethasone for exactly 3.5 hr to measure effects on hormone-inducible *PEPCK* expression. Data are expressed as a percent of the basal control value (0 hr, 100%) and are graphed as time of total chromium or arsenic treatment (basal, constitutive) or time of chromium or arsenic treatment before the beginning of dexamethasone induction (induced). Asterisks denote values that were significantly different from the appropriate control (basal or induced) at $p<0.01$. (*A*) Effects of chromium(VI) on basal (●) and inducible (■) *PEPCK* expression. Each data point represents the mean ± SEM of values from four to eight individual animals [data reprinted from J. McCaffrey et al. (*12*) with permission]. (*B*) Effects of arsenic(III) on basal (●) and inducible (■) *PEPCK* expression. Each data point represents the mean ± SEM of values from four to six individual animals. (*C*) Effects of chromium(VI) (●) and arsenic(III) (■) on β-actin expression. Each data point represents the mean ± SEM of values from four to six individual animals [chromium data reprinted from Hamilton and Wetterhahn (*10*) with permission].

chromium and arsenic significantly suppressed basal expression of PEPCK in a manner similar to MMC. We had previously shown that chromium, arsenic, and MMC also suppressed expression in these cells of the inducible gene *mdr1*, which codes for P-glycoprotein, but has no effect on several constitutive genes (*22,42*). Thus, this appeared to be a good model for examining in detail the mechanism by which these metals selectively affect inducible gene expression in cells.

We then examined the effects of chromium and arsenic on the nuclear levels of various transcription factors that might be associated with such changes in gene expression. It has been postulated that toxins can alter gene expression by activation of cell-signaling pathways leading to increases in nuclear transcription factor levels and/or activities leading to altered mRNA expression. Cells were treated with low and high doses of metals and nuclear extracts were isolated. Transcription factor binding was determined using EMSA analysis. An example of data generated in these studies is shown in Figure 4, which demonstrates a significant effect of a 1-hr noncytotoxic chromium treatment on nuclear binding levels of the transcription factor Sp1. Using this approach, we then examined in detail the effects of chromium and arsenic on nuclear levels of AP-1, NF-κB, and Sp1 transcription factors, since these factors have been implicated in the regulation of both *PEPCK* and *mdr1* genes, which are affected by these agents (*3,43,45–49*). As summarized in Table 1, there were specific effects of each agent on transcription factor binding that were agent-, dose-, factor- and cell line-dependent. Both chromium and arsenic generally caused significant increases in AP-1 binding in both cell lines at both doses, with the exception that the high dose of chromium suppressed AP-1 binding in the MDA cells. In contrast, the effects on Sp1 binding were quite variable. Chromium significantly increased Sp1 binding in the MDA cells, whereas it had no effect on Sp1 binding levels in the H4IIE cells. Conversely, arsenic had no effect on Sp1 binding in the MDA cells, whereas it decreased Sp1 in the H4IIE cells at the high dose. Metal effects on NF-κB binding were very cell-type specific. Arsenic had little or no effect on NF-κB binding in the MDA cells, whereas chromium increased binding, and neither agent had any effect on NF-κB in the H4IIE cells. Taken together, these results suggest that effects of chromium and arsenic on nuclear levels of specific transcription factors may be a component of tissue-specific alterations in gene expression caused by these metals. These effects may contribute to the overall biologic effects of these metals in humans and may play a role in long-term effects of low-dose exposures such as occur in metal-induced carcinogenesis.

Previously, it had been demonstrated that high-dose UV or γ irradiation as well as treatment with certain chemical carcinogens can stimulate a characteristic response pattern in cells that has been called the UV response (*50–52*). Since activation of certain transcription factors, particularly AP-1 and NF-κB, has been shown to be a component of the UV response pathway

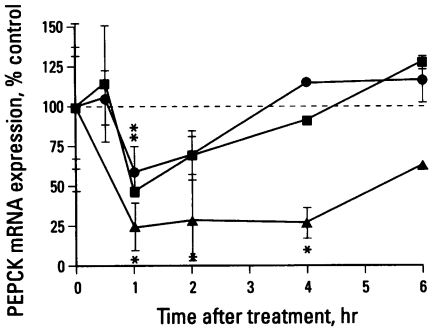

Figure 3. Effects of chromium(VI), arsenic(III), and mitomycin C on basal *PEPCK* mRNA expression in rat hepatoma H4IIE cells in culture. Cells were treated with 2 μM chromium(VI) (●); 0.33 μM arsenic(III) (■); or 0.1 μM MMC (▲) for 4 hr in incomplete medium and *PEPCK* mRNA expression was measured as described in "Materials and Methods" and in Figure 1. Each data point represents the mean ± SD of values from three to four separate flasks. Asterisks denote values that were significantly different from the appropriate control (basal or induced) at $p<0.01$.

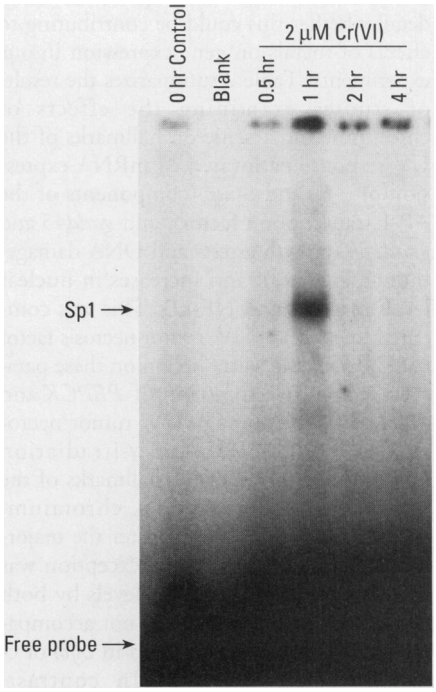

Figure 4. Effects of chromium(VI) on levels of nuclear binding to the Sp1 transcription factor recognition sequence in rat hepatoma H4IIE cells in culture. Cells were treated with 2 μM chromium(VI) for the indicated times, and total nuclear protein extracts were isolated as described in "Materials and Methods." Electrophoretic mobility shift analysis was performed using a radiolabeled duplex oligonucleotide fragment corresponding to the Sp1 recognition sequence from the human *MDR1* promoter region and levels of Sp1 binding (arrow) were analyzed as described in "Materials and Methods." Blank, no nuclear extract added; 0 hr control, extract from cells treated with solvent alone.

Table 1. Summary of effects of chromium(VI) and arsenic(III) on nuclear transcription factor binding levels in H4IIE rat hepatoma and MDA-MB-435 human breast carcinoma cell lines.

	AP-1		Sp1		NF-κB	
Cells and treatment[a]	Change[b]	Time	Change	Time	Change	Time
MDA cells						
Cr low	10-fold	1 hr	17-fold	1 hr	10-fold	1 hr
Cr high	0.3-fold	2 hr	25-fold	1 hr	4-fold	1 hr
As low	3-fold	1 hr	No effect		3-fold	2 hr
As high	8-fold	1 hr	No effect		2-fold	1 hr
H4IIE cells						
Cr low	12-fold	4 hr	No effect		No effect	
Cr high	12-fold	4 hr	No effect		No effect	
As low	9-fold	2 hr	No effect		No effect	
As high	7-fold	0.5 hr	0.5-fold	4 hr	No effect	

[a]Cells were treated with chromium(VI) as sodium dichromate at low (2 μM) or high (20, 100 μM) doses, or with arsenic(III) as sodium arsenite at low (0.33–1 μM) or high (100, 333 μM) doses for up to 4 hr followed by EMSA analysis of nuclear protein binding as described in "Materials and Methods" and in Figure 4, using probes for AP-1, Sp1, and NF-kB binding. [b]Significant changes in binding are expressed as the fold increase or decrease in nuclear binding levels relative to the solvent-treated controls at the maximal time point indicated after the beginning of heavy metal treatment.

(*50,51,53,54*), we investigated in more detail whether this could be contributing to effects of metals on gene expression in our experiments. Table 2 summarizes the results of studies examining the effects of chromium and arsenic on hallmarks of the UV response pathway, i.e., mRNA expression of c-*fos* and c-*jun* (components of the AP-1 transcription factor), and *gadd45* and *gadd153* (growth arrest- and DNA damage-inducible genes), and increases in nuclear levels of AP-1 and NF-κB. This was compared to effects of UV, tumor necrosis factor α (TNF-α), and γ-irradiation on these parameters and on expression of *PEPCK* and *mdr1* mRNA. Although UV, tumor necrosis factor β (TNFβ), and γ-irradiation induced most or all of the hallmarks of the UV response in these cells, chromium, arsenic, and MMC did not alter the majority of these parameters. The exception was an increase in nuclear AP-1 levels by both metals, but this response was not accompanied by the predicted increases in c-*fos* or c-*jun* mRNA expression. In contrast, chromium, arsenic, and MMC all altered mRNA expression of *PEPCK* and *mdr1*, whereas UV, TNFα, and γ-irradiation did not. These results demonstrate conclusively that the effects of noncytotoxic doses of chromium and arsenic (and MMC) on gene expression do not involve activation of the UV response pathway per se.

We then examined whether specific genetic elements within the promoters of target inducible genes could be responsible for mediating the effects of chromium and arsenic on their expression. Constructs consisting of portions of the rat *PEPCK* gene promoter were fused with the luciferase reporter gene and transfected into H4IIE cells to generate stably transfected cell lines with integrated copies of the transgene in their genome. The *PEPCK*–luciferase gene was basally expressed and fully hormone responsive in these cell lines in a manner similar to that of the native *PEPCK* gene (Figure 5A,B). In addition, the constructs containing either 1.2 or 0.6 kb of normal rat *PEPCK* promoter were fully responsive to chromium, arsenic, and MMC, suggesting that this region of the *PEPCK* promoter is sufficient to confer carcinogen sensitivity to another gene and therefore contains a target(s) for their effects. It further suggests that integration into nuclear chromatin structure is important for eliciting these effects. Interestingly, the two wild-type constructs differed qualitatively from each other in their basal response. The 1.2-kb construct exhibited a significant decrease, whereas the 0.6-kb construct exhibited a significant increase in *PEPCK*–luciferase expression in response to each of the three agents (Figure 5A). This suggests that an element(s) between –0.6 and –1.2 kb of the *PEPCK* promoter can contribute qualitatively to the basal response of this promoter to these agents. It had previously been shown that the proximal 0.6 kb of the rat *PEPCK* promoter was sufficient to confer normal hormone responsiveness to heterologous constructs (*43*). Both the 1.2- and 0.6-kb construct cell lines exhibited a normal dexamethasone response (Figure 5B), and both constructs also exhibited a complete inhibition of their dexamethasone response following treatment with each of the three agents (Figure 5B).

Constructs were then transfected with a 1.2-kb portion of the *PEPCK* promoter in which either the two tandem glucocorticoid response elements or the two adjacent accessory factor elements had been mutated using a site-directed mutagenesis technique that leaves the surrounding sequences intact. Mutation of these elements by deletion had previously been shown to significantly decrease or abolish hormone-inducible response of this promoter (*43,44*). As expected, although these constructs displayed a normal basal expression (Figure 5A), they had a significantly reduced responsiveness to induction by dexamethasone (Figure 5B). These constructs did not show any effect of treatment with chromium, arsenic, or MMC followed by dexamethasone (Figure 5B). Interestingly, their basal expression was also observed to be completely refractory to

Table 2. Comparison of effects of chromium(VI), arsenic(III), and mitomycin C on inducible gene expression and on aspects of the UV response pathway in rat hepatoma H4IIE cells in culture.

	UV	TNFα	γ-irradiation	MMC	Cr(VI)	As(III)
UV response[a]						
c-*fos*/c-*jun* mRNA	+	+	+	0	ND	ND
gadd45/*gadd153* mRNA	+	0	+	0	0	0
Nuclear AP-1 binding	+	+	+	0	+	+
Nuclear NF-κB binding	+	+	+	0	0	0
Inducible genes						
PEPCK mRNA	0	0	0	–	–	–
mdr1 mRNA	0	0	0	–	–	–

ND, not determined. [a]Cells were treated and analyzed as described in "Materials and Methods" and in Figure 4 and Table 1. UV, 20 J/m^2 UV-C 1–4 hr; TNFα, 20 U/ml 1–4 hr; γ-irradiation, 300 rad γ-irradiation 1–4 hr; MMC, 0.1 μM MMC 1–6 hr; Cr(VI), 2 μM chromium(VI) 1–6 hr; 0.33 μM arsenic(III) 1–6 hr; +, 2-fold or greater increase; –, 2-fold or greater decrease; 0, no significant change from control (see "Materials and Methods" and text).

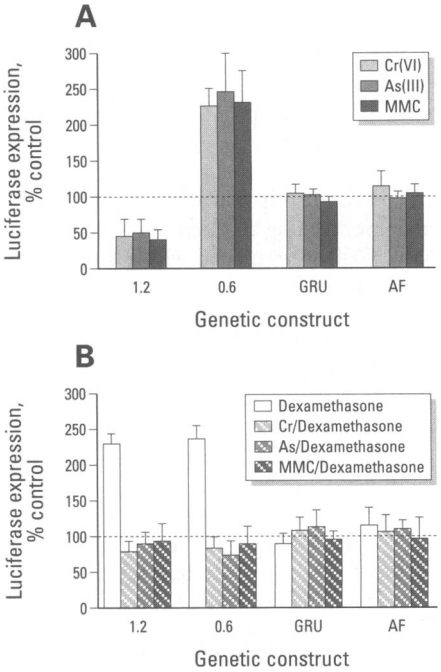

Figure 5. Effects of chromium(VI), arsenic(III), and mitomycin C on basal and hormone-inducible expression of a *PEPCK*–luciferase genetic construct transiently and stably transfected into rat hepatoma H4IIE cells in culture. Abbreviations: 1.2, 1.2-kb wild-type promoter; 0.6, 0.6 response elements; GRU (glucocorticoid response unit) 1.2 kb promoter with site-directed mutations in thge two glucocorticoid response elements; AF (accesory factor region), 1.2-kb promoter with site-directed mutations in the two accessory factor elements (see text). Constructs containing 0.6 or 1.2-kb of the rat *PEPCK* promoter region fused to the luciferase reporter gene were generated and cells were transfected as described in "Materials and Methods." Cells were treated with 2 μM chromium(VI), 0.33 μM arsenic(III), or 0.1 μM MMC as indicated in the legend for 4 hr in incomplete medium and basal *PEPCK*–luciferase expression was measured 20 hr later by a luminometer assay for luciferase activity as described in "Materials and Methods" (*A*). Alternatively, cells were treated with chromium, arsenic, or MMC, and beginning 4 hr later, cells were treated with 0.1 μM dexamethasone for 20 hr to measure effects on hormone-inducible *PEPCK*–luciferase expression (*B*). Data are expressed as a percent of the basal control values. Each bar represents the mean ± SD of values from three individual wells.

chromium, arsenic, or MMC treatments (Figure 5A). These results suggest that the responsiveness of the *PEPCK* gene to glucocorticoid induction is an important component of its ability to respond to chromium and arsenic treatments at both the basal and hormone-inducible level.

Discussion

The principal purpose of these studies was to develop systems to enable us to determine at the molecular level the basis for the preferential effects of the carcinogenic heavy metals chromium(VI) and arsenic(III) on inducible gene expression. We had previously examined the preferential effects of 16 different chemical carcinogens, including the genotoxic metals chromium(VI) and cisplatin, and other organic genotoxic agents that induce different types of DNA damage, on the expression of several different model inducible genes. Those studies demonstrated that treatment of 14-day chick embryos with a single administration of chromium(VI), at a dose that produced no overt toxicity but which caused significant levels of DNA damage (*14*), had profound effects on expression of several inducible genes but had no effect on expression of several constitutively expressed genes (*10*). Both the basal and inducible expression of the inducible ALA synthase, *CYP2H1* and *PEPCK* genes were markedly affected by the chromium treatment, whereas the albumin, transferrin, and β-actin genes were refractory to this treatment. The effects on expression of the inducible genes were seen at both the steady-state mRNA and transcriptional levels, and the time courses for these effects closely matched the time course for chromium-induced DNA damage and repair (*10,12,14*). Interestingly, certain effects most closely correlated with chromium–DNA monoadduct formation, whereas other effects were more closely associated with chromium–DNA cross-link formation (*10,12,14*). This same general phenomenon, i.e., preferential effects on inducible genes and correlation with DNA damage and repair, has also been observed in this system with a large number of other genotoxic and carcinogenic agents. These include the cross-linking agents cisplatin (*11*), and MMC (*35*); the direct-acting, simple alkylating agents methyl methanesulfonate (MMS), methylnitrosourea, ethyl methanesulfonate, and ethylnitrosourea (*34*); several agents that induce bulky monoadduct lesions in DNA, including benzo[*a*]pyrene, aflatoxin B_1, 7,12-dimethylbenz[*a*]anthracene, and 2-acetylaminofluorene (*13*); a chemical that induces strand breaks, bleomycin (*11*); and several synthetic acridine-based mono- and bis-intercalating agents (*42*). The results of many of these studies have recently been summarized (*11*).

The preferential effects of each of these agents strongly correlated with DNA damage, which supports the general hypothesis that alterations in gene expression can be used as a marker for DNA damage *in vivo*. Although the inducible genes were all responsive to DNA-damaging agents, the time course, direction, and magnitude of each response was both agent specific and gene specific. However, similarities in specific effects among groups of agents were based on the type of DNA damage induced, rather than the chemical structure, metabolism, lipophilicity, etc., of the agents themselves, which lends further support to the hypothesis that induction of DNA damage was the basis for the preferential effects on inducible gene expression. Others have also examined effects on gene expression by some of these agents as well as other carcinogens (*55–58*) that cause a variety of DNA lesions, including simple alkylations, bulky lesions, and cross-links. All of the carcinogens tested to date have been demonstrated to selectively alter inducible gene expression. We and others (*55–57*) have examined a number of different inducible genes; all responded to carcinogen treatment. In contrast, none of the constitutively expressed genes tested (β-actin, transferrin, albumin, and α-tubulin) were responsive. Effects on inducible gene expression have been observed in both the rat and chick embryo *in vivo* (*10,13,34,55,59*) and in primary chick embryo, adult rat, and rat embryo hepatocytes and rat hepatoma cell lines in culture (*56–58*). All of these systems show similar responses. Thus, carcinogen-induced alterations in inducible gene expression are a general phenomenon, and responsiveness appears to be independent of the specific system, carcinogen, gene, or induction pathway studied.

In contrast to these organic and inorganic carcinogens that are believed to principally target DNA as their mechanism of action, the mechanism of action of nongenotoxic carcinogens such as the heavy metals arsenic and cadmium is not well understood. However, it is known that these agents interact with specific subsets of proteins, principally through sulfhydryl interactions, and it has been postulated that they may act through epigenetic mechanisms to act as carcinogens. We had hypothesized that arsenic may also specifically and preferentially alter inducible gene expression much like chromium, but through a different mechanism than that of chromium or the other genotoxic agents. The goal of these studies was to examine this hypothesis in detail. Our results indicate that chromium and arsenic both preferentially alter inducible gene expression, and in a similar manner. Each agent had significant effects on basal and hormone-inducible expression of a model inducible gene, *PEPCK*, in both the chick embryo *in vivo* system and in rat hepatoma H4IIE cells in culture. Each agent also altered expression of *PEPCK*–luciferase genetic constructs in which their effects on basal and inducible expression were strongly correlated with the responsiveness of the transgene to glucocorticoids, suggesting that this pathway is important for the response and may be the primary target. These results indicate that this genetic approach will be useful for identifying specific regulatory pathways that mediate these responses.

It is interesting to note that the effects of chromium and arsenic on the integrated constructs containing 1.2 kb of promoter region were very similar to those on the native rat *PEPCK* gene in the H4IIE cells, i.e., a suppression in both basal and hormone-inducible expression, suggesting that this region of the promoter is sufficient to replicate the metal effects on the normal gene. The effects of chromium and arsenic on the 0.6 kb construct, in contrast, were more similar to those seen for *PEPCK* in the chick embryo liver, i.e., an increase in basal expression and a decrease in hormone responsiveness, suggesting that the distal 0.6 kb provided additional elements that qualitatively change the response of the rat *PEPCK* gene to these agents. A complete loss of basal responsiveness of the rat *PEPCK* gene promoter to chromium and arsenic was observed in mutated constructs in which there was also a loss of hormone responsiveness. This is very similar to what had previously been observed in the chick embryo system, in which the responsiveness of the native chicken *PEPCK* gene was intimately associated with its response to dexamethasone (*12*). In that study, we showed that the *PEPCK* gene lost its chromium sensitivity concomitant with the normal developmental loss of liver *PEPCK* hormone responsiveness that occurs between 14 and 17 days of embryonic development (*12,60*), even though the liver *PEPCK* gene was still basally expressed and still

responsive to induction by cAMP at 17 days (*12*). In addition, we have recently observed over that same developmental time period that the kidney *PEPCK* gene gained hormone responsiveness, and concomitantly gained sensitivity to chromium treatment (*42*). However, in a similar developmental study, we observed that the chick embryo liver *PEPCK* gene was refractory to the effects of MMC at 8 days of development even though it was fully glucocorticoid inducible (*61*). Thus, hormone responsiveness appears to be necessary but insufficient by itself to elicit a carcinogen response in this gene. In that study, the *PEPCK* gene became MMC sensitive by day 10 of embryonic development, concomitant with a major change in the chromatin structure of the *PEPCK* gene promoter region (*61*). These results suggest that both glucocorticoid responsiveness and chromatin structure are critical for the selective responsiveness of the *PEPCK* gene to carcinogen treatment.

Chromium and arsenic also altered nuclear transcription factor binding, although the patterns were different for the two agents and were also dose- and cell-type specific. Previous studies by Fornace and coworkers (*52,53,62*) and others (*50,51,54*) reported that treatment of cells with high doses of UV irradiation or other cytotoxic agents causes a characteristic UV response in cells. The response to UV irradiation itself was best characterized and involved a rapid increase in expression of mRNAs for c-*fos* and c-*jun* (components of AP-l), growth arrest and DNA damage-inducible (*gadd*) genes, especially *gadd45* and *gadd153*, as well as other genes (*53,54,63*). There was also a rapid increase in binding of AP-1 and NF-κB to their target DNA regulatory sequences (*50,51*). Other agents that were reported to mimic aspects of this response included X-irradiation, oxidative stress (e.g., by hydrogen peroxide), and certain DNA-damaging agents, including MMS, cisplatin, and MMC, although only at high, i.e., overtly cytotoxic, doses. We investigated whether our treatments stimulated aspects of the UV response as a number of inducible genes have been shown to be regulated by AP-1 and/or NF-κB, including *PEPCK* (*64*) and *mdr1* (*65*) that have been shown to be sensitive to chromium and arsenic in our studies. However, our results clearly indicate that noncytotoxic doses of chromium and arsenic are not acting through the mammalian UV response pathway to exert their preferential effects on inducible gene expression.

We observed a significant increase in both AP-1 and Sp1 binding by low-dose chromium treatments, whereas arsenic increased AP-1 but had little or no effect on Sp1 at low doses. Chromium significantly increased NF-κB binding and arsenic induced modest increases in NF-κB at low doses in the MDA cells, although neither agent had a significant effect on NF-κB binding at low or high doses in the H4IIE cell line. However, chromium and arsenic had no effect on other hallmarks of the UV response pathway. In addition, positive controls such as UV or TNFα that stimulate the UV response did not alter *PEPCK* expression. Thus, effects of chromium and arsenic on the UV response may involve apoptotic or other mechanisms that occur only at very high doses and represent late events, but which are separate from the pathways leading to alterations in gene expression that occur at lower doses. The best chemical DNA-damaging agent to induce the UV response to date is the simple direct-acting alkylating agent MMS (*66,67*). However, in addition to DNA damage, MMS also induces large amounts of protein adducts including protein cross-links (*68*), suggesting a possible alternative mechanism for stimulation of the UV pathway (*69,70*). It is possible that chemical stimulation of the UV pathway requires a certain threshold of nonspecific protein adducts that can only be obtained at very high doses of these agents but that are not critical to the carcinogenicity of these agents at lower doses. Similarly, arsenic has been shown to be heat-shock mimetic, although this also occurs at much higher doses than those which led to alterations in *PEPCK* gene expression in this study. However, lower doses did not significantly alter heat-shock protein expression in preliminary studies (*42*). Thus, it is also unlikely that this pathway is the principal mediator of these effects at the lower doses.

Figure 6 summarizes our current model for the actions of chromium and arsenic on *PEPCK* gene expression. Inducible gene expression ultimately involves the convergence of cell signaling pathways with DNA–chromatin at the level of the gene promoter. Thus, it is possible that two different agents such as chromium and arsenic, which may act at different points of the same pathway, might lead to similar effects on expression of a targeted inducible gene. Chromium is proposed to act principally through its action as a DNA-damaging agent. We hypothesize that chromium-induced DNA lesions lead to alterations in

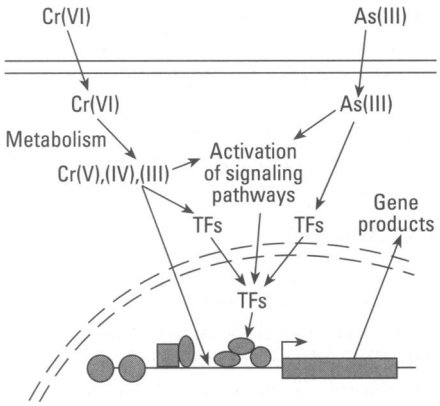

Figure 6. Model for preferential effects of chromium(VI) and arsenic(III) on inducible gene expression. TFs, transcription factors. Chromium(VI) enters the cell as CrO_4^{-2} through nonspecific anion transport pathways and is metabolized through a variety of small molecule and enzyme-reductive pathways to Cr(V), Cr(IV), and Cr(III) species that can interact directly with DNA–chromatin to form monoadducts, DNA interstrand, intrastrand and DNA–protein cross-links, and other forms of DNA damage. These intermediates may also activate or repress specific transcription factors either directly or indirectly through signaling pathways. Arsenic(III) can also enter the cell and activate transcription factors directly or indirectly. Arsenic does not directly interact with DNA but may also indirectly alter chromatin structure through interaction with chromosomal proteins. These chromium and arsenic effects on transcription factors and DNA–chromatin ultimately converge at the promoters of targeted inducible genes such as *PEPCK*, causing alterations in specific DNA–protein interactions that ultimately lead to changes in transcription and gene product expression.

specific DNA–protein interactions within the chromatin of an inducible gene promoter, leading to changes in transcription of that gene. Inducible genes such as *PEPCK* may be preferentially susceptible to these effects because of their large DNase-hypersensitive regions which represent areas of nonnucleosomal decondensed chromatin and regions of complex DNA-transcription factor interactions. In addition, chromium also has significant effects on nuclear levels of specific transcription factors, and these are likely to contribute to the overall effects of chromium on inducible gene expression. We further propose that arsenic acts principally through direct or indirect effects on specific transcription factors and other signaling pathways rather than on DNA per se. Arsenic may also be able to affect chromatin structure within an inducible gene promoter through mechanisms involving alterations in histone and/or nonhistone chromosomal proteins. Collectively, these effects may ultimately lead to alterations in specific DNA–protein interactions within

inducible gene promoters in a manner similar to that of chromium. We have demonstrated that chromium and arsenic have similar effects on expression of the hormone-regulated *PEPCK* gene, both in the chick embryo *in vivo* and in the H4IIE cell line in culture. These effects are closely correlated with glucocorticoid regulation of this gene through the glucocorticoid receptor pathway and its DNA recognition elements within the *PEPCK* promoter region. Examining the details of how chromium and arsenic specifically alter this regulatory pathway will be the focus of future studies.

In summary, we have examined the mechanistic basis for the strong preferential effects of the heavy metals and human carcinogens chromium(VI) and arsenic(III) on inducible gene expression. Our studies indicate that each of these agents has specific effects on transcription factor binding and expression of certain genetic constructs that may provide insight into the cell signaling and gene regulatory pathways that mediate their carcinogenic effects *in vivo*. A combination of both *cis* effects involving specific regions of the *PEPCK* promoter and their chromatin structure, and *trans* effects involving effects on specific transcription factors appear to work in concert to contribute to the overall effects of these heavy metals on inducible gene expression. Determining the mechanisms by which these carcinogenic metals may selectively alter gene expression would have important implications for understanding the molecular basis for the impact of these agents on the carcinogenic process and overall human cancer incidence in exposed populations. Although each of these agents may have specific effects and act through independent mechanisms, they may also have profound but different effects when present in different combinations in the environment. Understanding these interactions at the molecular level is critical for an accurate assessment of the overall health effects of these substances on the human population. Elucidating these molecular events may also eventually provide sensitive biomarkers for evaluating human exposures, for example, by defining a specific transcription factor, signaling pathway, or sentinel gene that might be indicative of prior arsenic or chromium exposure, much as metallothionein expression can serve as an indicator of cadmium exposure. This will first require a more detailed understanding of the basis for these effects.

REFERENCES AND NOTES

1. Hamilton JW, Wetterhahn KE. Chromium. In: Handbook on Toxicity of Inorganic Compounds (Seiler HG, Sigel H, eds). New York:Marcel Dekker, 1987;239–250.
2. Fan AM, Harding-Barlow I. Chromium. In: Genotoxic and Carcinogenic Metals: Environmental and Occupational Occurrence and Exposure (Fishbein L, Furst A, Mehlman MA, eds). Princeton, NJ:Princeton Scientific Publishing, 1987;87–126.
3. Bencko V. Arsenic. In: Genotoxic and Carcinogenic Metals: Environmental and Occupational Occurrence and Exposure (Fishbein L, Furst A, Mehlman MA, eds). Princeton NJ:Princeton Scientific Publishing, 1987;1–30.
4. De Flora S, Wetterhahn KE. Mechanisms of chromium metabolism and genotoxicity. Life Chem Rep 7:169–244 (1989).
5. Langard S. The carcinogenicity of chromium compounds in man and animals. In: Chromium: Metabolism and Toxicity (Burrows D, ed). Boca Raton, FL:CRC Press, 1983;13–30.
6. Landolph JR. Neoplastic transformation of mammalian cells by carcinogenic metal compounds: cellular and molecular mechanisms. In: Biological Effects of Heavy Metals. Vol II: Metal Carcinogenesis (Foulkes EC, ed). Boca Raton, FL:CRC Press, 1990;1–18.
7. IARC. Arsenic and Arsenic Compounds (Group 1). In: IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol 7. Lyon:International Agency for Research on Cancer, 1987;100–106.
8. Wetterhahn KE, Hamilton JW, Aiyar J, Borges KM, Floyd R. Mechanism of chromium (VI) carcinogenesis: reactive intermediates and effect on gene expression. Biol Trace Elem Res 21:405–411 (1989).
9. Wetterhahn KE, Hamilton JW. Molecular basis of hexavalent chromium carcinogenicity: effect on gene expression. Sci Total Environ 86:113–129 (1989).
10. Hamilton JW, Wetterhahn KE. Differential effects of chromium(VI) on constitutive and inducible gene expression in chick embryo liver *in vivo* and correlation with chromium(VI)-induced DNA damage. Mol Carcinogen 2:274–286 (1989).
11. Hamilton JW, McCaffrey J, Caron RM, Louis CA, Treadwell MD, Hunt SR, Reed MJ, Doherty KA. Genotoxic chemical carcinogens target inducible genes *in vivo*. Ann NY Acad Sci 726:343–345 (1994).
12. McCaffrey J, Wolf CM, Hamilton JW. Effects of the genotoxic carcinogen chromium(VI) on basal and hormone-inducible phosphoenolpyruvate carboxykinase gene expression *in vivo*: correlation with glucocorticoid- and developmentally-regulated expression. Mol Carcinogen 10:189–198 (1994).
13. Hamilton JW, Louis CA, Doherty KA, Hunt SR, Reed MJ, Treadwell MD. Preferential alteration of inducible gene expression *in vivo* by carcinogens that induce bulky DNA lesions. Mol Carcinogen 8:34–43 (1993).
14. Hamilton JW, Wetterhahn KE. Chromium(VI)-induced DNA damage in chick embryo liver and blood cells *in vivo*. Carcinogenesis 7:2085–2088 (1986).
15. Chang WC, Chen SH, Wu HL, Shi GY, Murota S, Morita I. Cytoprotective effect of reduced glutathione in arsenical-induced endothelial cell injury. Toxicology 69:101–110 (1991).
16. Sanchez ER. Heat shock induces translocation to the nucleus of the unliganded glucocorticoid receptor. J Biol Chem 267:17–20 (1992).
17. Lopez S, Miyashita Y, Simons SS Jr. Structurally based, selective interaction of arsenite with steroid receptors. J Biol Chem 265:16039–16042 (1990).
18. Thompson DJ. A chemical hypothesis for arsenic methylation in mammals. Chem Biol Interact 88:89–114 (1993).
19. Yanez L, Carrizales L, Zanatta MT, Mejia JJ, Batres L, Diaz-Barriga F. Arsenic-cadmium interaction in rats: toxic effects in the heart and tissue metal shifts. Toxicology 67:227–234 (1991).
20. Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Wetterhahn KE. Expression of 5-aminolaevulinate synthase and cytochrome P-450 mRNAs in chicken embryo hepatocytes *in vivo* and in cell culture: effect of porphyrinogenic drugs and haem. Biochem J 255:267–275 (1988).
21. Hamilton JW, Denison MS, Bloom SE. Development of basal and induced aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity in the chicken embryo *in ovo*. Proc Natl Acad Sci USA 80:3372–3376 (1983).
22. Ihnat MA, Lariviere JP, Warren AJ, La Ronde N, Blaxall JRN, Pierre KM, Turpie BW, Hamilton JW. Suppression of P-glycoprotein expression and multidrug resistance by DNA cross-linking agents. Clin Cancer Res 3:1339–1346 (1997).
23. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl Acid Res 11:1475–1489 (1983).

24. Janssen YM, Barchowsky A, Treadwell MD, Driscoll KE, Mossman BT. Asbestos induces nuclear factor kappa B (NF-κB) DNA-binding activity and NF-κB dependent gene expression in tracheal epithelial cells. Proc Natl Acad Sci USA 92:8458–8462 (1995).
25. Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA, Wetterhahn KE. Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. Arch Biochem Biophys 289:387–392 (1991).
26. Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA, Wetterhahn KE. Inhibition of protein synthesis increases the transcription of the phenobarbital-inducible *CYP2H1* and *CYP2H2* genes in chick embryo hepatocytes. Arch Biochem Biophys 298:96–104 (1992).
27. Curran T, Gordon MB, Rubino KL, Sambucetti LC. Isolation and characterization of the c-*fos* (rat) cDNA and analysis of post-translational modification *in vitro*. Oncogene 2:79–84 (1987).
28. Lamph WW, Wamsley P, Sassone-Corsi P, Verma IM. Induction of proto-oncogene *JUN/AP-1* by serum and TPA. Nature 334:629–631 (1988).
29. Papathanasiou MA, Kerr NC, Robbins JH, McBride OW, Alamo I Jr, Barrett SF, Hickson ID, Fornace AJ Jr. Induction by ionizing radiation of the *gadd45* gene in cultured human cells: lack of mediation by protein kinase C. Mol Cell Biol 11:1009–1016 (1991).
30. Park JS, Luethy JD, Wang MG, Fargnoli J, Fornace AJ Jr, McBride OW, Holbrook NJ. Isolation, characterization and chromosomal localization of the human *GADD153* gene. Gene 116:259–267 (1992).
31. Beale EG, Chrapkiewicz NB, Scoble HA, Metz RJ, Quick DP, Noble RL, Donelson JE, Biemann K, Granner DK. Rat hepatic cytosolic phosphoenolpyruvate carboxykinase (GTP): structures of the protein, messenger RNA, and gene. J Biol Chem 260:10748–10760 (1985).
32. Chodosh LA. Mobility shift DNA-binding assay using gel electrophoresis. In: Current Protocols in Molecular Biology (Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds). New York:John Wiley & Sons, 1993;12.2.1–12.2.10.
33. Read MA, Whitley MZ, Williams AJ, Collins T. NF-κB and I-κB alpha: an inducible regulatory system in endothelial activation. J Exp Med 179:503–512 (1994).
34. McCaffrey J, Hamilton JW. Comparison of effects of direct-acting DNA methylating and ethylating agents on inducible gene expression *in vivo*. Environ Mol Mutagen 23:164–170 (1994).
35. Caron RM, Hamilton JW. Preferential effects of the chemotherapeutic DNA crosslinking agent mitomycin C on inducible gene expression *in vivo*. Environ Mol Mutagen 25:4–11 (1995).
36. Alcedo JA, Misra M, Hamilton JW, Wetterhahn KE. The genotoxic carcinogen chromium(VI) alters the metal-inducible expression but not the basal expression of the metallothionein gene *in vivo*. Carcinogenesis 15:1089–1092 (1994).
37. Hamilton JW, Bloom SE. Correlation between mixed-function oxidase enzyme induction and aflatoxin B_1-induced unscheduled DNA synthesis in the chick embryo, *in vivo*. Environ Mutagen 6:41–48 (1984).
38. Hamilton JW, Bloom SE. Correlation between induction of xenobiotic metabolism and DNA damage from chemical carcinogens in the chick embryo *in vivo*. Carcinogenesis 7:1101–1106 (1986).
39. Bloom SE. Avian and aquatic systems for *in vivo* detection of sister chromatid exchange. In: Sister Chromatid Exchange (Sandberg AA, ed). New York:Alan R. Liss, 1982;249–277.
40. Hamilton JW, Bloom SE. Developmental differences in basal and induced aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity in chick embryo liver and lung *in vivo*. Biochem Pharmacol 32:2986–2988 (1983).
41. Denison MS, Okey AB, Hamilton JW, Bloom SE, Wilkinson CF. Ah receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: ontogeny in chick embryo liver. J Biochem Toxicol 1:39–49 (1986).
42. Hamilton JW, Caron RM, Ihnat MA, Warren AJ, Kaltreider RC. Unpublished observations.
43. Granner D, O'Brien R, Imai E, Forest C, Mitchell J, Lucas P. Complex hormone response unit regulating transcription of the phosphoenolpyruvate carboxykinase gene: from metabolic pathways to molecular biology. Rec Prog Horm Res 47:319–346 (1991).
44. Lucas PC, Granner DK. Hormone response domains in gene transcription. Annu Rev Biochem 61:1131–1173 (1992).
45. Cornwell MM, Smith DE. SP1 activates the MDR1 promoter through one of two distinct G-rich regions that modulate promoter activity. J Biol Chem 268:19505–19511 (1993).
46. Madden MJ, Morrow CS, Nakagawa M, Goldsmith ME, Fairchild CR, Cowan KH. Identification of 5′and 3′ sequences involved in the regulation of transcription of the human *mdr1* gene *in vivo*. J Biol Chem 268:8290–8297 (1993).
47. Chin K-V, Pastan I, Gottesman MM. Function and regulation of the human multidrug resistance gene. Adv Cancer Res 60:157–180 (1993).
48. Cohen D, Piekarz RL, Hsu SI, DePinho RA, Carrasco N, Horwitz SB. Structural and functional analysis of the mouse *mdr1b* gene promoter. J Biol Chem 266:2239–2244 (1991).
49. Hanson RW, Patel YM. Phosphoenolpyruvate carboxykinase (GTP): the gene and the enzyme. Adv Enzymol Rel Areas Mol Biol 69:203–281 (1994).
50. Sachsenmaier C, Radler-Pohl A, Muller A, Herrlich P, Rahmsdorf HJ. Damage to DNA by UV light and activation of transcription factors. Biochem Pharmacol 47:129–136 (1994).
51. Herrlich P, Sachsenmaier C, Radler-Pohl A, Gebel S, Blattner C, Rahmsdorf HJ. The mammalian UV response: mechanism of DNA damage induced gene expression. Adv Enzyme Reg 34:381–395 (1994).
52. Fornace AJ,Jr., Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, Holbrook NJ. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol Cell Biol 9:4196–4203 (1989).
53. Hollander MC, Fornace AJ Jr. Induction of fos RNA by DNA-damaging agents. Cancer Res 49:1687–1692 (1989).
54. Devary Y, Gottlieb R, Lau LF, Karin M. Rapid and preferential activation of the c-*jun* gene during the mammalian UV response. Mol Cell Biol 11:2804–2811 (1991).
55. Wogan GE, Friedman MA. Inhibition by aflatoxin B_1 of hydrocortisone induction of rat liver tryptophan pyrrolase and tyrosine transaminase. Arch Biochem Biophys 128:509–516 (1968).
56. Miller MS, Wogan GN. Inhibition of steroid-inducible tyrosine aminotransferase gene expression by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine in a rat hepatoma cell line. Carcinogenesis 7:1273–1278 (1986).
57. Gayda DP, Pariza MW. Effects of carcinogens on hormonal regulation of gene expression in primary cultures of adult rat hepatocytes. Carcinogenesis 4:1127–1131 (1983).
58. Yeoh GCT, Toia RF, Godfrey ML, Adler B. The development of mixed function amine oxidase in cultured foetal rat hepatocytes and its relation to 3′-methyl-4-*N,N*-dimethylaminoazobenzene effects on tyrosine aminotransferase accumulation. Carcinogenesis 4:1499–1501 (1983).
59. Huang D-P, Maine AB, Chiu J-F. Changes in hepatic levels of tyrosine aminotransferase messenger RNA during chemical carcinogenesis. Cancer Lett 22:143–149 (1984).
60. McCaffrey J, Hamilton JW. Developmental regulation of basal and hormone-inducible phoshoenolpyruvate carboxykinase gene expression in chick embryo liver *in vivo*. Arch Biochem Biophys 309:10–17 (1994).
61. Caron RM, Hamilton JW. Developmentally specific effects of the DNA crosslinking agent mitomycin C on phosphoenolpyruvate carboxykinase gene expression *in vivo*: correlation with changes in chromatin structure within the promoter region of the gene. J Biochem Mol Toxicol (in press).
62. Fornace AJ Jr, Alamo I Jr, Hollander MC. DNA damage-inducible transcripts in mammalian cells. Proc Natl Acad Sci USA 85:8800–8804 (1988).

63. Hollander MC, Alamo I, Jackman J, Wang MG, McBride OW, Fornace AJ Jr. Analysis of the mammalian *gadd45* gene and its response to DNA damage. J Biol Chem 88:3729–3733 (1993).
64. DeLuca LM. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J 5:2924–2933 (1991).
65. Yu L, Cohen D, Piekarz RL, Horwitz SB. Three distinct nuclear binding sites in the promoter of the murine *mdr1b* gene. J Biol Chem 268:7520–7526 (1993).
66. Luethy JD, Holbrook NJ. Activation of the *gadd153* promoter by genotoxic agents: a rapid and specific response to DNA damage. Cancer Res 52:5–10 (1992).
67. Jackman J, Alamo I Jr, Fornace AJ Jr. Genotoxic stress confers preferential and coordinate messenger RNA stability on the five *gadd* genes. Cancer Res 54:5656–5662 (1994).
68. Farmer PB, Bailey E, Campbell JB. Use of alkylated proteins in the monitoring of exposure to alkylating agents. IARC Sci Publ 59:189–198 (1984).
69. Kuo ML, Meng TC, Lin JK. Involvement of glutathione in induction of c-*jun* proto-oncogene by methyl methanesulfonate in NIH 3T3 cells. Carcinogenesis 17:815-820 (1996).
70. Carrier F, Gatignol A, Hollander MC, Jeang KT, Fornace AJ Jr. Induction of RNA-binding proteins in mammalian cells by DNA-damaging agents. Proc Natl Acad Sci USA 91:1554–1558 (1994).