Chromium Inhibits Transcription from Polycyclic Aromatic Hydrocarbon-inducible Promoters by Blocking the Release of Histone Deacetylase and Preventing the Binding of p300 to Chromatin

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Co-contamination with complex mixtures of carcinogenic metals, such as chromium, and polycyclic aromatic hydrocarbons is a common environmental problem with multiple biological consequences. Chromium exposure alters inducible gene expression, forms chromium-DNA adducts and chromium-DNA cross-links, and disrupts transcriptional activator-co-activator complexes. We have shown previously that exposure of mouse hepatoma Hepa-1 cells to chromate inhibits the induction of the Cyp1a1 and Nqo1 genes by dioxin. Here we have tested the hypothesis that chromium blocks gene expression by interfering with the assembly of productive transcriptional complexes at the promoter of inducible genes. To this end, we have studied the effects of chromium on the expression of genes induced by benzo[a]pyrene (B[a]P), another aryl hydrocarbon receptor agonist, and characterized the disruption of Cyp1a1 transcriptional induction by chromium. Gene expression profiling by using high density microarray analysis revealed that the inhibitory effect of chromium on B[a]P-dependent gene induction was generalized, affecting the induction of over 50 different genes involved in a variety of signaling transduction pathways. The inhibitory effect of chromium on Cyp1a1 transcription was found to depend on the presence of promoter-proximal sequences and not on the cis-acting enhancer sequences that bind the aryl hydrocarbon receptor-aryl hydrocarbon receptor nuclear translocator complex. By using transient reporter assays and chromatin immunoprecipitation analyses, we found that chromium prevented the B[a]P-dependent release of HDAC-1 from Cyp1a1 chromatin and blocked p300 recruitment. These results provide a mechanistic explanation for the observation that chromium inhibits inducible but not constitutive gene expression.

Environmental exposures to toxic or carcinogenic compounds rarely result from the presence of single, isolated toxicants.

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More often, the toxic agent is a complex mixture of chemical entities in numbers ranging from a few, such as may occur in occupational exposures, to several hundred, such as in cigarette smoke. Often, these mixtures include a combination of carcinogenic metals and polycyclic aromatic hydrocarbons, as for example those co-released from sources such as fossil fuel combustion or municipal waste incineration. Of these, chromium and B[a]P are among the top 20 hazardous substances in the Agency for Toxic Substances and Disease Registry/EPA priority list (1) and are found as co-contaminants in more than 25% of the National Priority List of Superfund sites (2).

Chromium has been known for over 100 years to be a human carcinogen (3). Epidemiologically, the greatest risk of cancer from chromium exposure is associated with Cr(VI). Cr(VI) enters cells via the sulfite anion transporter system and is reduced to intermediate oxidation states, such as Cr(V) and Cr(IV), in the process of forming stable Cr(III) forms (4, 5). Cr(III) is the most prevalent form of chromium in the environment and in biological tissues, but the vast majority of the evidence indicates that exposure to trivalent chromium does not induce tumors in animals (6).

The available experimental evidence indicates that chromium exposure has little or no effect on constitutive gene expression but alters inducible gene expression, possibly due to the formation of chromium-DNA adducts, chromium-DNA cross-links, or to the disruption of transcriptional activator-co-activator complexes (3, 7–9). For example, chromium was found to block the expression of metal-inducible metallothionein or hormone-inducible phosphoenolpyruvate carboxykinase without affecting the expression of housekeeping genes, such as β-actin or albumin (4, 7, 8, 10, 11). These observations have led to the hypothesis that the chromatin structure of inducible promoters, perhaps by virtue of being more open, may offer a better target for chromium binding than the more closed chromatin of constitutive promoters (12). Chromium-induced DNA-protein cross-links are found preferentially in nuclear matrix DNA (13), where many replication, repair, and transcription proteins associate, suggesting that cross-links between DNA

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and any or several of these proteins may be responsible for effectively blocking their function.

Generation of free radicals from the reduction of Cr(VI) to Cr(IV) and Cr(V) induces an increase of NFκB DNA binding activity but do not cause a concomitant increase in NFκB-dependent gene expression (14–16), possibly because chromium blocks the binding of the p65 subunit to CBP/p300, whose association with p65 is essential for NFκB-enhanced transcriptional activity (9). These data suggest that the intermediate oxidation states of chromium may be critical for its effect on gene expression. The molecular mechanism responsible for this effect is likely to involve the reduction process in the persistent stimulation of regulatory pathways affecting interactions of transcription factors with transcriptional co-regulators and chromatin remodelling factors, more so than binding of the factors themselves to their cognate recognition sites.

Earlier studies from this laboratory have shown that exposure of mouse hepatoma Hepa-1 cells to chromium inhibits the induction of the Cyp1a1 and Nqo1 genes by dioxin, an AHR ligand (17). The AHR is a ligand-activated basic region helix-loop-helix/Per-ARNT-Sim transcription factor that forms heterodimers with ARNT and binds to cis-acting AHR-responsive enhancer elements in the regulatory domains of target genes, such as Cyp1a1 and Nqo1, leading to changes in chromatin structure and activation of gene transcription (18). These changes also include the recruitment to the transcription machinery of associated co-regulator proteins. In recent years several transcriptional co-regulators, including the co-activators CBP/p300, SRC-1, RIP140, ERAP140, the chromatin remodelling factor BRG-1, and the co-repressor SMRT, have been shown to interact with AHR-ARNT complexes and play a role in transactivation (19–26).

We have studied the effects of chromium on the expression of genes induced by B[a]P, another AHR agonist. We have tested the hypothesis that blocking of AHR-dependent gene expression by chromium was not limited to Cyp1a1 or Nqo1 but that it affected globally a large number of genes induced by B[a]P. We have further tested the hypothesis that chromium blocked gene expression by interfering with the assembly of productive transcriptional complexes in the promoters of inducible genes. We find that chromium prevents the B[a]P-dependent release of HDAC-1 from chromatin, preventing the association of p300 with the Cyp1a1 transcriptional complex and hence blocking gene transcription.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemical Treatments—Mouse hepatoma Hepa-1 (27) cells were grown in α-minimal essential medium (Invitrogen) supplemented with 5% fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Cells were treated when grown at 80–90% confluence. Hepa-AhrRDTKLuc cells have been described previously (28); they were derived by stable integration into Hepa-1 cells of a pHaHRDTKLuc3 luciferase reporter plasmid containing the AHR-responsive domain of the mouse Cyp1a1 gene promoter (from −1100 to −869), harboring five AHR-responsive elements fused to the HSV-1 thymidine kinase minimal promoter from −79 to +53 (29) from which the Sp1-binding site had been removed (Fig. 1). Potassium chromate and dichromate (K2CrO4 and K2Cr2O7, referred to here simply as chromium) were freshly dissolved in sterile deionized water prior to use. Sodium butyrate was dissolved in serum-free α-minimal essential medium just prior to use. B[a]P was added to the cells in a final Me2SO volume of 0.1%. Detailed treatment procedures are given in the text and the figure legends.

Plasmid Constructs and Transfections—To assess AHR-dependent gene expression, we used the luciferase reporter plasmid p1646Luc2 (28), containing the mouse Cyp1a1 gene promoter region from nucleotide −1646 to +57. Plasmids expressing the co-activator p300 were from Upstate Biotechnologies Inc.; human BRG-1 expression plasmid and co-regulator plasmids pc3.1-hSRC-1A and pSG5-Tif 2 (SRC-2) were kindly provided by Dr. Erik Knudsen and Dr. Sohaib Khan (University of Cincinnati Medical Center), respectively. For transfection experiments, cells were plated in 24-well plates at a density of 4 × 10^4 cells/well and transfected at 70–80% confluence using LipofectAMINE Plus (Invitrogen). Briefly, 50 ng of reporter plasmid, 200 ng of pBlue-script or co-regulator plasmids, and 25 ng of pCMV-gal plasmid were incubated with Plus reagent for 15 min in serum- and antibiotic-free medium. LipofectAMINE was added, and the mixture was incubated for an additional 15 min. The transfection was carried out for 3 h, and thereafter the medium was changed to normal culture medium containing 5% fetal bovine serum, and transfected cells were allowed to grow overnight before treatment. After treatment, cells were washed twice with PBS and lysed with 100 μl of reporter lysis buffer (Promega, Madison, WI). Aliquots of 50 μl of cell lysate were used to measure luciferase activity. Light units were determined immediately upon addition of 150 μl of luciferase assay buffer (20 mM Tricine, 1.07 mM MgCO3, 2.67 mM MgSO4, 33.3 mM dithiothreitol, 14.8 mg coenzyme A, 530 μM ATP, 0.1 mM EDTA, and 10 μg of luciferin), using a Wallac 1420 Victor plate reader. Luciferase measurements were normalized for transfection efficiency in transient transfections using β-galactosidase activity.

RNA Isolation and Real Time Reverse Transcriptase-PCR—Total RNA was isolated using TRIReagent (Invitrogen) according to the manufacturer's instructions with additional purification steps applied to RNA samples used for microarray analysis. To verify RNA quality prior to labeling for microarray analyses, samples were analyzed using an Agilent 2100 Bioanalyzer. cDNA was synthesized by reverse transcription of 20 μg of total RNA in a total volume of 30 μl containing 1× reverse transcriptase buffer, 2.5 μM random hexamers, 0.25 mM dNTP, 0.1 M dithiothreitol, 20 units of RNasin and 200 units of SuperScript™ II RNase H−reverse transcriptase (Invitrogen). Samples were incubated at 42 °C for 1 h, and the reverse transcriptase was inactivated by heating to 99 °C for 5 min. For real time PCR amplification, 3 μl of cDNA were amplified with mouse Cyp1a1 primers (forward primer, 5′-GCCCTTACTTCTGGAGCGCTTC-3′; reverse primer, 5′-CAATG-GTTCCTCGATGTC-3′), giving a product of 280 bp between exon 5 and 7 of the mouse Cyp1a1 gene. Amplification of luciferase cDNA was with primers 5′-CCACACCGCAGACCTTC-3′ and 3′-CCACACGACGCTTC-3′, giving a product of 182 bp. β-Actin amplification of the same cDNA samples was used as an internal standard. Amplification was conducted in the Smart Cycler (Cepheid, Sunnyvale, CA) in a total volume of 25 μl consisting of 1× Brilliant™ SYBR® Green QPCR Master Mix (Stratagene) and 0.4 μM mouse Cyp1a1 primers. The reaction mixtures were heated to 95 °C for 10 min and immediately cycled 40 times through a 24-s denaturing step at 95 °C, a 60-s annealing step at 55 °C, and a 46-s elongation step at 72 °C. Cycle threshold (Ct) of each sample was automatically determined to be the first cycle at which a significant increase in optical signal above an arbitrary base line set at 30 fluorescence units was detected. All determinations were done in triplicate. The values shown represent the C<sub>t</sub> ratio of experimental to control cells treated with Me2SO, normalized to the β-actin mRNA level in the same sample.

Fluorescent Labeling of Target cDNAs and High Density Microarray Hybridization—Labeling of cDNAs, preparation of microarrays, and hybridization reactions were performed by the University of Cincinnati Functional Genomics Core and are briefly described here. Fluorescence-
labeled cDNAs were synthesized from 20 μg of total RNA using an indirect amino allyl labeling method (30). The cDNA was synthesized by an oligo(T)-primed, reverse transcriptase reaction, and the cDNA was labeled with monofunctional reactive cytidine-3 and cytidine-5 dyes (Cy3 and Cy5; Amersham Biosciences). Specific details of the labeling protocols may be found at microarray.uc.edu.

The hybridization probes were from arrayed mouse oligonucleotide microarrays purchased from the Operon Technologies, Verifor. The arrayed microarrays consisted of 13,433 segments from annotated mouse genes, affixed each in a 100-μm diameter spot to polystyrene-treated microscope slides. The hybridization targets were the paired Cy3- and Cy5-labeled control and test cDNAs, which were mixed in approximately equal proportion and applied to the microarray for hybridization under high stringency conditions. After hybridization and washing unhybridized targets, the Cy3 (red) fluorescent channels were simultaneously scanned with independent lasers at 10 μm resolution. Each comparison was done in triplicate with flipped dye arrays to allow for the removal of gene-specific dye effects. Each comparison consisted of three microarray slides; in two slides, the cDNA was labeled with one fluorescent dye and in one it was labeled with the other dye. Overall, to eliminate labeling bias, cDNA from any one preparation was labeled an equal number of times with Cy3 as with Cy5.

Data Analysis and Normalization—Microarray hybridization data representing raw spot intensities generated by the GenePix software were analyzed to identify differentially expressed genes under different experimental conditions. Data normalization was performed in three steps for each microarray separately. First, channel-specific local background subtraction was subtracted from the median intensity of each channel (Cy3 and Cy5). Second, background-adjusted intensities were log-transformed, and the differences (R) and averages (A) of log-transformed values were calculated as $R = \log_{2}(X1) - \log_{2}(X2)$ and $A = \log_{2}(X1 + \log_{2}(X2))/2$, where $X1$ and $X2$ denote the Cy3 and Cy5 intensities after subtracting local backgrounds, respectively. Third, data centering was performed by fitting the array-specific local regression model of $R$ as a function of $A$ (31). The difference between the observed log ratio and the corresponding fitted value represented the normalized log-transformed gene expression ratio. Normalized log intensities for the two channels were then calculated by adding a half of the normalized ratio to $A$ for the Cy5 channel and subtracting half of the normalized ratio from $A$ for the Cy3 channel.

Identification of Differentially Expressed Genes—The statistical analysis was performed for each gene separately by fitting the following mixed effects linear model (32): $Y_{ijk} = μ + A_k + S_j + C_{ij} + ε_{ijk}$, where $Y_{ijk}$ corresponds to the normalized log intensity on the (th) array ($i = 1, \ldots, 15$), with the (th) treatment combination ($j = 1, \ldots, 5$), and labeled with the (th) dye ($k = 1$ for Cy5, and $k = 2$ for Cy3), is the overall mean log intensity, $A_k$ is the effect of the (th) dye, $S_j$ is the effect of the (th) treatment combination, and $C_{ij}$ is the effect of the (th) dye. Assumptions about model parameters were the same as described elsewhere (32), with array effects assumed to be random, and treatment and dye effects assumed to be fixed. Statistical significance of the differential expression between different treatment combinations, after adjusting for the array and dye effects, was assessed by calculating $p$ values for corresponding linear contrasts. Multiple hypothesis testing adjustment was performed by calculating false discovery rate values (33, 34). Data normalization and statistical analyses were performed using SAS statistical software package (SAS Institute Inc., Cary, NC).

Chromium Speciation—HeLa-1 cells were treated with 25 μM K3[CrCl6] and 5 μM B[a]P for 10 h of incubation. Cells were rinsed twice with cold PBS and collected by scraping in deionized water. Collected cells were sonicated with four 15-s bursts and mixed with an equal volume of 100% ethanol. The precipitate was removed by centrifugation at 10,000 rpm for 2 min. Cell pellets were resuspended in 2 volumes of lysis buffer (50 mM PIPES, pH 7.9, 10 mM KCl, 2 mM MgCl2) and Dounce-homogenized after addition of Nonidet P-40 to a final concentration of 0.2%. All procedures were carried out on ice. Cell lysates were centrifuged in an Eppendorf microcentrifuge at 3,000 rpm for 2 min. The supernatant and the pellet were frozen at −80 °C and saved as the cytoplasmic and nuclear extracts, respectively. For analysis, samples were thawed, mixed with HNO3 to a final concentration of 1%, and digested for 30 min in a Parr Microwave Digestion Bomb. Acid digests were analyzed for chromium concentration by monitoring m/z 53 using the Agilent 7500c ICP-MS and comparing to a chromium standard curve. Experiments were carried out in triplicate.

For measurements of 32Cr incorporation, 32Cr as Na2CrO4 (specific activity 425 Ci/mg; 175175 MBq/mg) was used (PerkinElmer Life Sciences). Incorporation of carrier-free 32P-labeled cDNAs was performed by calculating false discovery rate values (33, 34). Data corresponding linear contrasts. Multiple hypothesis testing adjustment was performed by calculating false discovery rate values (33, 34). Data normalization and run-off assays were conducted essentially as described (17) with the exception that 2 × 106 nuclei and 0.2 mCi of [32P]UTP (3000 Ci/mmol) were used for each reaction. Incubations were carried out for 30 min at room temperature. For those reactions in which chromium was added to the nuclei in vitro, K3Cr2O7 was added before addition of [32P]UTP to a final concentration of 1 mM. At the end of the run-off incubation, nuclei were centrifuged at 15,000 rpm for 10 s, and the supernatant was discarded. Purification of the labeled RNA and hybridization was carried out as described previously (17). Incorporation was quantitated using a PhosphorImager (Storm 860, Amersham Biosciences).

ChIP Assays—Protocols were based on procedures published by others (36, 37) with minor modifications. Hepa-1 cells were grown to 90–100% confluence (~2–3 × 107 cells) in 150-cm plates. Cells were treated with Me2SO vehicle or 5 μM B[a]P for 1 h or pretreated with 50 μM chromium for 0.5 h followed by either no other treatment or with 5 μM chromium for an additional 10 min. Cross-linking was performed with 10–20 μg/ml formaldehyde for a 10-min incubation at room temperature with formaldehyde added directly to the culture media to a final concentration of 1%. The reaction was stopped by adding glycine to a final concentration of 0.125 M. After rinsing twice with ice-cold PBS, cells were scraped from the dishes, pelleted, and washed again with PBS plus 0.5 μg/ml phenylmethylsulfonyl fluoride. Cell pellets were resuspended in cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, plus protease inhibitors) and incubated on ice for 10 min. Cells were homogenized on ice in a Dounce homogenizer using the B-type pestle 20 to aid in nuclei release. The nuclei were pelleted and resuspended in nuclear buffer (50 mM Tris-HCl, pH 8.1, 107 cells/μl EDTA, 1% SDS plus protease inhibitors) and incubated on ice for 10 min. Chromatin was sonicated on ice with four 10-s bursts of 30 watts with a 30-s interval between bursts. Average length of the DNA was 600 bp. For immunoprecipitation, chromatin was first precleared for 15 min at 4 °C with protein A-agarose saturated with bovine serum albumin and salmon sperm DNA (Upstate Biotechnology, Inc., Lake Placid, NY). The supernatant was divided equally among all samples and incubated overnight on a rotating platform at 4 °C with antibodies (1 μg/2 × 107 cells) against AHR (Biomol), ARNT (a gift of Dr. Oliver Hankinson, p300 (Upstate Biotechnology, Inc.) and HDAC-1 (Upstate Biotechnology, Inc.), respectively. Protein A-agarose slurry (20 μl) was added and incubated for 15 min at room temperature to allow it to bind to the antibody. The agarose beads were pelleted and washed twice with 1× dialysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.2% Sarkosyl) and sequentially four times with IP wash buffer (100 mM Tris-HCl, pH 9.0, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid). Immunocomplexes were eluted from the beads with elution buffer (50 mM NaHCO3, 1% SDS). Cross-linking was reversed by heating the eluates at 67 °C for 4–5 h. To allow for quantitation of DNA recovery, a constant known amount of 32P-labeled prokaryotic DNA was added to the eluates before DNA purification. The eluates were digested with proteinase K at 45 °C for 1.5 h. DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. Radioactivity in the resultant pellets was measured by phosphorimaging, adjusting for the recovery of radioactivity during the purification process. PCR amplification of the Cyp1a1 promoter was quantitated by inclusion of a small amount of [α-32P]dCTP during PCR amplification. Amplification of the distal promoter region was accomplished with the
primer sets 5′-CTATCTCTAAAACCCACCCCCA-3′ and 5′-CTAGAGTGTGAGGAAGGGTC-3′, corresponding to the enhancer domain (−1141 to −784 bp, Fig. 1). The proximal promoter region was amplified with the set 5′-ATTATTTCCTCCACCCCCTCC-3′ and 5′-AGGGACTAGTGAAGAGTGC-3′ (−291 to +71 bp, Fig. 1). PCR products were separated in 10% polyacrylamide gels. After electrophoresis, gels were dried and exposed to x-ray film. DNA bands were visualized in a PhosphorImager (Storm 860, Amersham Biosciences) and quantitated using ImageQuant 5.2 software.

RESULTS

Chromium Inhibits Expression of Many B[a]P-inducible Genes—The available experimental evidence indicates that chromium exposure has little or no effect on constitutive gene expression but alters inducible gene expression, possibly due to the formation of chromium-DNA adducts, chromium-DNA cross-links, or to the disruption of transcriptional activator-co-activator complexes (7, 9, 10). Earlier studies from this laboratory have shown that chromium represses the induction of Cyp1a1 and Nqo1 mRNA by TCDD, disrupting the coordinate induction of phase I and phase II gene expression (17). To verify that this was not a unique effect of TCDD, we assessed the effects of chromium on gene transactivation by the Ah receptor using B[a]P, another AHR ligand, as the inducer. In addition, to test the hypothesis that the inhibitory effect of chromium was a generalized phenomenon affecting many inducible genes and not just specific to genes coding for drug-metabolizing enzymes, we determined global expression profiles of Hepa-1 cells treated with B[a]P or with chromium plus B[a]P, using a high density microarray analysis approach. More than 50 genes, including Cyp1a1 and Nqo1 among others, were up-regulated by B[a]P alone, and their induction was markedly inhibited by addition of chromium prior to B[a]P (Table I). The pronounced inhibitory effect of chromium was partly relieved if the cells were first induced with B[a]P and chromium was not added until 1.5–2 h later. These data are in good agreement with previous results from this laboratory showing the partial relief of inhibition when addition of chromium was delayed relative to addition of agonist (17). The list of B[a]P-inducible genes inhibited by chromium includes genes involved in a variety of signaling transduction pathways, such as calcium-dependent regulation, receptor-associated kinases, transcription factors, cell cycle regulation, differentiation, and apoptosis in addition to genes involved in drug metabolism. It is evident that the inhibitory effect of chromium is well generalized, affecting the inducibility of more than just a few drug-metabolizing genes.

Significant Amounts of Cr(III) Are Found in the Nuclei—Our earlier observations on the differential effects of chromium inhibition depending on timing of addition had led us to hypothesize that chromium disrupts gene expression at an early step of transcription (17). If that were the case, it could be expected that extracellular chromium would enter the cells and be readily taken up into the nucleus. We used a combination of high pressure liquid chromatography and ICP-MS analyses to study chromium uptake, speciation, and distribution in Hepa-1 cells. Chromium uptake was very fast, Cr(VI) being very rapidly reduced to Cr(III) in the cells. By t = 1 min, only 15% of the detectable chromium was still found as Cr(VI) (retention time = 10.8 min), whereas by t = 1 h Cr(VI) was less than 5% (Fig. 2A). By 2 h, Cr(VI) was undetectable (data not shown).

Chromium distribution was determined by both uptake of Na$_2$CrO$_4$ and ICP-MS detection of chromate or dichromate uptake. Both methods showed significant amounts of chromium present in both cytoplasmic and nuclear fractions after a 4-h incubation. Very similar levels and distribution patterns were found when chromate or dichromate (Cr$_2$O$_7^{2-}$) treatments were the source of chromium (Fig. 2B). These data indicate that a large fraction of the chromium administered to the cells enters the nucleus either as reduced Cr(III) or in the process of being reduced from Cr(VI) to Cr(III), and might cause its inhibitory effect on gene expression by interacting with the transcriptional machinery.

Chromium Inhibits B[a]P-induced Transcription—As indicated earlier, chromium blocks inducible gene expression at early times of addition more readily than at later times. It is reasonable to speculate that nuclear chromium, either as Cr(III) or as a reduction intermediate, will block transcription at an early step of initiation or elongation. To test this hypothesis, we used transcriptional run-off assays. We measured transcription rates of several B[a]P-inducible genes in the presence or absence of chromium added to cells prior to nuclear isolation or incubated with the nuclei during the run-off assay. The transcription rate of Cyp1a1, Gsta1, and Gspt1 was inhibited several fold by 5 μM B[a]P relative to the transcription of β-actin, used to normalize transcription rates to the rate of an uninduced gene. Induction was inhibited by 50 μM chromium, either when added as Cr(VI) to the cells prior to B[a]P addition or when added to nuclei from B[a]P-treated cells (Fig. 3). These results indicate that chromium blocks the transcription of inducible genes to a greater extent than the expression of the one housekeeping gene, β-actin, used to normalize the data.

Inhibition of B[a]P-induced Gene Expression by Chromium Takes Place at the Level of the Proximal Promoter—None of several antioxidants, such as catalase, superoxide dismutase, and 2,2,6,6-tetramethylpiperidinyl-oxy, could reverse the inhibition by chromium of B[a]P-dependent gene expression induction (data not shown). On the other hand, the combined evidence of our speciation and nuclear run-off studies suggested that Cr(III) or a reduction species intermediate between Cr(VI) and Cr(III) was able to react with an element of the transcriptional machinery and block transcription. Because the effect resulted in transcription repression, we suspected that chromium interfered with the molecular interactions leading to assembly of the transcriptional complex on the Cyp1a1 promoter chromatin. To test this hypothesis, we used the Hepa-AhRDTKLUC cells that carry a stably integrated AhRDTK-Luc3 luciferase reporter plasmid. In this plasmid, luciferase expression is regulated by the AHR-responsive enhancer of the mouse Cyp1a1 gene promoter bearing five AHR-responsive elements. In this plasmid, the enhancer is fused to the HSV-1 thymidine kinase minimal promoter from which the Sp1-binding site had been removed. In these cells, mRNA expression of both luciferase and endogenous Cyp1a1 genes is induced by AHR ligands; however, the replacement of the Cyp1a1 proximal promoter (from position −869 to the transcription start site, see Fig. 1) with the HSV-1 tk minimal promoter facilitates the detection of Cyp1a1 expression changes resulting from molecular events occurring in the proximal promoter domain. To test the role of HDAC in repression by chromium of B[a]P gene induction, we compared Cyp1a1 and luciferase mRNA levels in cells treated with vehicle or with sodium butyrate, an HDAC inhibitor. Cyp1a1 mRNA levels were highly induced by 2 μM BaP treatment, an effect that was practically abolished when cells were treated with 50 μM chromium prior to B[a]P but not when B[a]P was added 2 h before chromium; 2 mM sodium butyrate further reversed the effect of chromium in the latter case (Fig. 4A). Expression of luciferase mRNA was induced by B[a]P as well, but was insensitive to chromium treatment (Fig. 4B), suggesting that control elements in the Cyp1a1 proximal promoter were responsible for the chromium-dependent repression of gene induction by B[a]P. In addition, pretreatment of the cells with 2 mM sodium butyrate also led to a
further elevation of mRNA levels for both chromium plus B[a]P co-treatments (Fig. 4B). These results suggest that inhibition of B[a]P-inducible gene expression was likely to result from interference with chromatin remodeling processes associated with the assembly of transcription factors or the initiation of transcription at the proximal promoter.

Chromium Blocks B[a]P-dependent HDAC Release and Prevents Association of p300 with the Ah Receptor Complex at the Cyp1a1 Promoter—Our previous work has shown that chromium does not block the association of AHR/ARNT heterodimers with their cognate binding AhRE sites in the Cyp1a1 enhancer (17). Hence, it seemed more likely that the transcriptional inhibitory effect of chromium could be exerted through interactions with chromatin occurring after enhanceosome assembly and engagement of the AHR/ARNT-responsive enhancer by the AHR complex. Specifically, given the relief of the chromium-induced block by HDAC inhibition, we hypothesized that chromium might interfere with the association of HAT with chromatin. If this were the case, we would expect that chromium treatment would also repress the superinduction of AHR/ARNT-dependent gene expression resulting from overexpression of the co-activators SRC-1, SRC-2, and p300, which recruit HAT to the transcriptional complex. To determine whether chromium would impair HAT recruitment, we used

| Clone   | Name                                      | B[a]P | Cr+B[a]P | BaP+Cr |
|---------|-------------------------------------------|-------|----------|--------|
| NM009992| Cytochrome P450, 1a1                       | 54.82 | 18.72    | 39.46  |
| U12961  | NAD(P)H-dependent quinone oxidoreductase-1 | 14.18 | 3.04     | 5.74   |
| U12785  | Aldehyde dehydrogenase family 3, subfamily A1 | 10.98 | 3.19     | 6.08   |
| M32032  | Selenium binding protein 1               | 7.86  | 1.25     | 3.55   |
| AK007683| Complement-C1Q TNF-related protein 2 homolog | 5.54  | 2.67     | 5.77   |
| L24118  | Tumor necrosis factor, alpha-induced protein 2 | 5.09  | 3.12     | 3.98   |
| U75215  | Neutral amino acid transporter             | 4.53  | 1.39     | 2.34   |
| L06047  | Glutathione S-transferase, alpha 4        | 3.27  | 1.37     | 1.99   |
| U85498  | Glutamate cysteine ligase, catalytic       | 2.79  | 1.55     | 1.61   |
| NM025797| Cytochrome b-5                           | 2.61  | 1.19     | 1.85   |
| U23021  | Cytosolic stress protein 94 kDa           | 2.60  | 1.59     | 2.04   |
| AK002894| Phosphogluconate dehydrogenase homolog    | 2.57  | 1.38     | 1.85   |
| U38261  | Superoxide dismutase 3, extracellular      | 2.22  | 1.49     | 2.12   |
| AF006462| Ectonucleoside triphosphate diphosphohydrolase 5 | 2.18  | 1.30     | 1.51   |
| U40796  | Excision repair endonuclease (XPG)        | 2.09  | 1.28     | 1.38   |
| NM023665| DNA segment, Chr 4, Wayne State University 53, expressed | 2.06  | 0.98     | 1.02   |
| AB025408| Esterase 10                                | 2.02  | 1.41     | 1.47   |
| U21960  | Calcium modulating ligand                 | 1.93  | 0.77     | 1.18   |
| NM026494| RIKEN cDNA 6330579B17 gene                | 1.90  | 0.69     | 0.83   |
| AF016294| EF4-like factor 3                         | 1.81  | 1.24     | 1.28   |
| AJ297743| Torsin family 1, member B                 | 1.77  | 1.31     | 1.42   |
| BC003962| RIKEN cDNA 1110035H23 gene                | 1.77  | 1.31     | 1.45   |
| U96441  | Numb-like                                 | 1.74  | 1.24     | 1.37   |
| Z19111  | Glucose-6-phosphate dehydrogenase X-linked| 1.73  | 0.94     | 1.01   |
| AF232709| Syntaxin 5                                 | 1.70  | 0.69     | 1.02   |
| X91825  | Small proline-rich protein 1B             | 1.68  | 1.18     | 1.43   |
| NM025964| RIKEN cDNA 2310038H17 gene                | 1.63  | 0.64     | 0.89   |
| AB041657| Hypothetical protein, MNCB-2990           | 1.62  | 0.58     | 0.96   |
| AK005192| Protein translation factor SUF1 homolog GC20 homolog | 1.43  | 0.76     | 0.83   |
| AK004431| Digeorge syndrome-related protein FKSG4 homolog | 1.42  | 0.84     | 0.99   |
| AK008611| Hypothetical protein                      | 1.42  | 0.90     | 1.01   |
| NM026737| DNA segment, Chr 19, ERATO Doi 144, expressed | 1.42  | 0.92     | 1.09   |
| AK010545| BBP-like protein 1 homolog               | 1.40  | 0.93     | 1.21   |
| AF012923| Wild-type p53-induced gene 1             | 1.39  | 0.93     | 1.08   |
| AB041604| Hypothetical protein MNCB-5081           | 1.38  | 0.72     | 0.88   |
| AK010334| Hypothetical protein                     | 1.38  | 1.06     | 1.11   |
| BC003237| ATP/GTP-binding protein                  | 1.37  | 0.87     | 0.75   |
| AK004305| SNAP25 interacting protein 30 homolog    | 1.36  | 0.67     | 0.81   |
| D49439  | TATA box binding protein (TBP)-associated factor, TFIIID subunit | 1.35  | 0.76     | 0.87   |
| NM029385| RIKEN cDNA 2310054H06 gene               | 1.32  | 0.62     | 0.83   |
| AK005691| Similar to zinc finger protein 2 (fragment) | 1.26  | 0.58     | 0.69   |
| NM023658| SCAN-KRA8-zinc finger protein 1          | 1.26  | 0.74     | 0.84   |
| AK009025| Ubiquitin-like protein SB132 homolog     | 1.25  | 0.87     | 1.06   |
| AB055070| Mus musculus Gdi-1 mRNA for RhoGDI-1     | 1.22  | 0.76     | 0.96   |
| NM010590| Ajuba (Jub)                              | 1.22  | 0.77     | 1.01   |
| AK012430| Downstream of tyrosine kinase 5          | 1.20  | 0.68     | 0.80   |
| NM025498| RIKEN cDNA 1700023M09 gene               | 1.20  | 0.93     | 1.04   |
| BC004041| Peptidylprolyl isomerase F (cyclophilin F) | 1.20  | 0.86     | 0.83   |
| AF124725| Apoptotic chromatin condensation inducer in the nucleus | 1.18  | 0.81     | 0.98   |
| NM025982| RIKEN cDNA 270008SA14 gene               | 1.17  | 0.77     | 1.01   |
the p-1646Luc2 luciferase reporter, containing both the enhancer as well as the proximal promoter sequences of the mouse Cyp1a1 gene, in transient co-transfection assays with expression plasmids for SRC-1, SRC-2, p300 and with the chromatin remodeling BRG-1. This reporter will be expected to respond to chromium treatment in a similar manner as the p-1646Luc2 luciferase reporter, containing both the enhancer as well as the proximal promoter sequences of the mouse Cyp1a1 gene, in transient co-transfection assays with expression plasmids for SRC-1, SRC-2, p300 and with the chromatin remodeling BRG-1. This reporter will be expected to respond to chromium treatment in a similar manner as the
indigenous Cyp1a1 gene. All the co-activators tested increased basal luciferase expression levels in MeSO-treated cells and increased B[a]P-induced levels from 2- to 6-fold over those in control cells co-transfected with an empty vector (Fig. 5). In all cases, pretreatment with 25 μM chromium repressed superinduction, which was almost completely abolished by pretreatment with 50 μM chromium (Fig. 5). These results indicated that recruitment of HAT by the co-activators and possibly the concomitant release of HDAC was impaired by chromium. To confirm this conclusion we used ChIP assays, focusing our analyses on the same two regulatory domains of the Cyp1a1 promoter, namely the cis-acting enhancer region, located between coordinates −1200 and −800 and containing five canonical AHR-responsive elements, and the proximal promoter region containing sequences immediately upstream of the transcription start site (Fig. 1), where the general transcriptional factors associated with RNA polymerase II bind. For ChIP assays, Hepa-1 cells were exposed to MeSO vehicle, to 50 μM chromium, to 5 μM B[a]P, or to 50 μM chromium followed 1 h later by 5 μM B[a]P. All cultures were incubated at 37 °C for 90 min, after which time chromatin was prepared and immunoprecipitated with antibodies to AHR, ARNT, p300, and HDAC-1. After cross-link reversal, DNA was purified and amplified by PCR using primers bracketing either the enhancer or the proximal promoter regions (Fig. 1). Immunoprecipitation profiles with anti-AHR and anti-ARNT antibodies were similar. As could be expected, binding of AHR and ARNT to enhancer chromatin was highly induced by B[a]P treatment, as determined by the 10–12-fold increase in enhancer sequences detected in the immunoprecipitates (Fig. 6A). In agreement with our previous electrophoretic mobility shift findings (17), much of the AHR/ARNT binding was still retained in chromium-pretreated cells. On the other hand, B[a]P-induced binding of the AHR/ARNT complex to promoter proximal sequences in the TATA box region was completely abolished by chromium pretreatment (Fig. 6A). Chromium alone had no apparent effect on AHR or ARNT binding to either enhancer or proximal promoter chromatin. These results suggest that chromium causes a transcriptional block by silencing the proximal promoter through interference with the recruitment of enhancer-some complexes to the proximal promoter, as described recently for Cyp1a1 (39, 40). In agreement with this concept, immunoprecipitation with anti-p300 antibodies showed that p300 association with either enhancer or proximal promoter chromatin occurred only in B[a]P-treated cells and that a complete block of binding, to levels even below those in MeSO-treated cells, took place in B[a]P-induced cells pretreated with chromium (Fig. 6B). Conversely, in uninduced cells, immunoprecipitation with anti-HDAC-1 antibodies showed a low level of HDAC-1 binding at the enhancer domain but a high level at the proximal promoter, which was completely eliminated by B[a]P treatment (Fig. 6B). Pretreatment with chromium abolished the release of HDAC from the proximal promoter, which showed levels comparable to those of uninduced cells. In the absence of B[a]P treatment, chromium alone more than doubled the level of promoter proximal chromatin that was bound to HDAC (Fig. 6B). These results strongly suggest that chromium-mediated inhibition of B[a]P-induced gene expression results from the chromium-dependent retention of HDAC at the proximal promoter chromatin and the prevention of p300 entry, in turn, blocking the association of the transcriptional AHR/ARNT complex with promoter proximal TATA box chromatin.

**DISCUSSION**

The results presented in this article show that inhibition of Ah receptor-dependent expression by chromium is a generalized phenomenon that extends to at least 50 B[a]P-inducible genes involved in a variety of cellular processes (Table I). Inhibition occurs at the transcriptional level at least for the three genes, Cyp1a1, Gsta, and Gstp, tested by run-off assays, suggesting that this might be the case for all the B[a]P-inducible genes whose induction was sensitive to inhibition by chromium. These results confirm and extend previous findings that suggest that chromium interferes with an early step of inducible gene transcription (4, 7, 8, 10, 11).

The evidence from our chromium distribution experiments indicates that significant amounts of chromium reach the nucleus and that B[a]P-induced transcription in isolated nuclei is also sensitive to chromium-dependent inhibition, as is the case in whole cells. Several lines of evidence suggest that the critical chromium-sensitive step occurs at the level of the proximal promoter and involves its interaction with the transcriptional assembly machinery. In stably transfected Hepa-AhRDTKLUC cells (Fig. 4), although the presence of the AHR-responsive Cyp1a1 enhancer in the integrated reporter plasmid makes luciferase expression to be inducible by B[a]P, the absence of the proximal promoter sequences may make it insensitive to inhibition by chromium, unlike its effect on the endogenous Cyp1a1 gene. These experiments not only map the chromium-sensitive domain to the proximal Cyp1a1 promoter, but they also reveal a role for HDAC-1 in chromium-dependent inhibition of gene expression, which appears to be blocked in part by inhibition of HDAC with sodium butyrate.
FIG. 6. ChIP assays of Cyp1a1 enhancer and promoter-proximal sequences. Hepa-1 cells were co-transfected with the indicated plasmids and with the reporter p-1646Luc2 as described under “Experimental Procedures” together with the β-galactosidase-expression vector pCMVβ-gal. Transfected cells were allowed to grow overnight before treatment with 5 μM β[a]P, with or without prior treatments for 2 h with 25 or 50 μM chromium. Control cells were treated with an equal volume of Me2SO (DMSO) vehicle. After treatment, aliquots of cell lysates were used to measure luciferase and β-galactosidase activities. Luciferase measurements were normalized for transfection efficiency using β-galactosidase activities. The ordinate represents the fold induction of normalized luciferase activity relative to the activity in empty vector co-transfected, Me2SO treated cells. The experiments were repeated three times with each determination done in triplicate, and the ordinate values represent the average ± S.D. of a representative experiment.
Hexavalent chromium appears to be rapidly reduced by cells to stable trivalent chromium. Within our experimental parameters, we do not observe, nor are our experiments designed to detect, oxidative stress or DNA damage associated with chromium reduction through the reactive intermediates Cr(V) and Cr(IV). It is likely, however, that the intermediate oxidation states of chromium are critical factors not only for the generation of oxidative stress and DNA damage but also, within a more immediate time frame, for its effects on gene expression. Electron spin resonance and spin trapping measurements have shown that generation of free radicals from the reduction of Cr(VI) to Cr(V) and Cr(IV) induces an increase of NFκB DNA binding activity in cultured human and rat cells (14–16); however, NFκB-dependent gene expression is not concomitantly increased, because chromium blocks the binding of the p65 NFκB subunit to CBP/p300, a transcriptional co-activator with intrinsic HAT activity, whose association with p65 is essential for NFκB-enhanced transcriptional activity (9). Furthermore, Cr(VI) has also been found to modify the transactivation potential of MTF-1 without affecting basal or inducible binding to general nuclear cofactors, including the co-activators CBP/p300, p300 binding to either enhancer or proximal promoter chromatin was stimulated 50% of the level in B[a]P-treated cells. In comparison, the interaction of HDAC-1 and p300 with chromatin was much more critically affected by chromium, p300 binding to either enhancer or proximal promoter chromatin was stimulated by 2–3-fold by B[a]P treatment, an effect that was completely blocked by chromium pretreatment prior to B[a]P induction. Conversely, HDAC-1 binding, which was very strong in the proximal promoter of uninduced or chromium-treated cells, was reduced to undetectable levels by B[a]P but remained strong when the cells were treated with chromium prior to B[a]P induction. These results lead us to conclude that chromium blocks the release of HDAC-1 from the Cyp1a1 proximal promoter, maintaining a state of histone deacetylation and transcriptional repression and preventing the recruitment of p300, with subsequent histone acetylation and transcriptional induction. Recent data have shown that HDAC-1 and HDAC-2 are recruited to Sp1/Sp3-binding sites through butyrate-response elements associated with Sp1/Sp3-binding sites (42). A computer analysis of the Cyp1a1 promoter reveals the presence of multiple potential Sp1/Sp3-binding sites, some of which have been shown to be functional and to cooperate with AHR and ARNT in the induction of the Cyp1a1 gene (19). It is attractive to speculate that chromium, known to cross-link DNA and proteins (43–46), may be able to cross-link HDAC to any one of a number of potential partners, including histone, bound Sp factors, or Sp-response DNA elements in the Cyp1a1 promoter. With the advent of new tools developed to map changes in histone acetylation status on a genome-wide basis (37, 47), it will be possible to address this question.

Our ChIP analysis results suggest that in the process of gene activation, the AHR-ARNT complex bound at the enhancer domain makes contact with promoter proximal sequences. These data are consistent with a looping model of AHR/ARNT-mediated Cyp1a1 transcriptional activation and agree with recent results reported by Tian and co-workers (40). It is noteworthy, however, that the basal expression level of this gene is maintained in a silent state not solely by the absence of a bound activated receptor complex but also by the additional repressive effect of HDAC-1 bound at the proximal chromatin. This unexpected finding is worthy of further investigation.

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Chromium Inhibits Transcription from Polycyclic Aromatic Hydrocarbon-inducible Promoters by Blocking the Release of Histone Deacetylase and Preventing the Binding of p300 to Chromatin

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