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HDAC1 bound to the *Cyp1a1* promoter blocks histone acetylation associated with Ah receptor-mediated transactivation

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Abstract

Metabolic bioactivation of polycyclic aromatic hydrocarbons, such as the environmental procarcinogen benzo[*a*]pyrene, is catalyzed by a cytochrome P450 monooxygenase encoded by the substrate-inducible *Cyp1a1* gene. *Cyp1a1* induction requires *trans*-activation by the heterodimeric transcriptional complex formed by the liganded Ah receptor (AHR) and its partner, ARNT. Previously, we showed that constitutively bound HDAC1 dissociates from *Cyp1a1* promoter chromatin after ligand-mediated induction, concomitantly with the recruitment of AHR/ARNT complexes and p300. Here, we investigated the hypothesis that HDAC1 binding maintains the *Cyp1a1* gene in a silenced state in uninduced cells. We find that *Cyp1a1* induction by the AHR/ARNT is associated with modification of specific chromatin marks, including hyperacetylation of histone H3K14 and H4K16, trimethylation of histone H3K4, and phosphorylation of H3S10. HDAC1 and DNMT1 form complexes on the *Cyp1a1* promoter of uninduced cells but HDAC1 inhibition alone is not sufficient to induce *Cyp1a1* expression, although it allows for the hyperacetylation of H3K14 and H4K16 to levels similar to those found in B[*a*]P-induced cells. These results show that by blocking modification of histone marks, HDAC1 plays a central role in *Cyp1a1* expression and that its removal is a necessary but not sufficient condition for *Cyp1a1* induction, underscoring the requirement for a concerted series of chromatin remodeling events to complete the initial steps of gene *trans*-activation by the Ah receptor.

Keywords

Benzo[*a*]pyrene; *Cyp1a1*; epigenetics; HDAC1; DNMT1; histone modifications

Introduction

The cytochrome P450 *CYP* genes constitute an extended multigene family of heme-containing proteins involved in drug metabolism and phase I detoxification. Through oxygenation of their substrates, these enzymes play a critical role in the metabolism of numerous xenobiotics, environmental chemicals, dietary constituents, endogenous substrates, most drugs of therapeutic value, and the metabolic bioactivation of chemical procarcinogens and toxins. Commonly, the xenobiotic substrates modulate their own detoxification through the induction of the specific *CYP* genes responsible for their metabolism. CYP1 family members, including CYP1A1, CYP1A2, and CYP1B1, are highly inducible enzymes that catalyze the metabolic

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activation of environmental PAHs, such as B[a]P [1]. B[a]P is present in industrial emissions, charbroiled meats, tobacco smoke, and overheated cooking oils for example, and its oxygenation by CYP1A1 and 1B1 enzymes generates genotoxic intermediate metabolites associated with DNA damage responsible for mutagenesis and subsequent carcinogenesis [2]. Induction of *CYP1A1* expression is transcriptionally regulated by the aryl hydrocarbon receptor, a ligand-activated transcription factor of the bHLH/PAS family [3]. The AHR also plays a fundamental role in mediating the activity of many highly toxic HAHs that pose a threat to the health of humans and wildlife. Among these are the polychlorinated-dibenzo-p-dioxins, of which TCDD is the prototype, the polychlorinated-dibenzofurans, the coplanar biphenyls, and various other environmental toxicants [4,5].

The AHR is sequestered in the cytoplasm in a conformation stabilized by chaperone proteins HSP90, XAP-2, and p23 [6]. Ligand binding results in a conformational change that leads to the exposure of a nuclear localization signal recognized by the importin- β transporter that facilitates AHR nuclear translocation [7]. Once in the nucleus, the AHR dimerizes with ARNT (HIF-1 β), and the complex binds to specific DNA recognition sites (5'-TNGCGTG-3'), referred to as xenobiotic, dioxin or AHR response elements (XREs, DREs or AhREs), located in multiple copies in the 5'-flanking region of the *CYP1A1* gene promoter and of a growing number of genes collectively known as the *AHR gene battery* [8]. Following transcriptional activation, the Ah receptor is transported back to the cytoplasm by the CRM-1 protein through the recognition of a nuclear export signal [9], where it undergoes rapid proteasome-dependent degradation [10].

The 5'-flanking region of the *CYP1A1* gene contains a transcriptional control region located upstream of the transcriptional start site and hereafter referred to as the proximal promoter. This promoter has binding sites for several transcription factors, including the TATA-binding proteins, but it does not have AhREs [11]. The AhREs are clustered in an enhancer domain located several hundred base pairs upstream of the proximal promoter. Previous studies have shown that the promoter is silent in the absence of the enhancer region, and that a liganded AHR complex bound to the enhancer region is required to elicit promoter chromatin remodeling needed for gene induction [12,13]. Remodeling leads to destabilization of a nucleosome poised over the promoter, followed by the assembly of the general transcription machinery [14]. These findings suggest that promoter function is under the control of the enhancer region. In the absence of inducer, the promoter exists in an inactive configuration, i.e., associated with the nucleosome, but during transcriptional activation, the enhancer somehow communicates with the promoter and induces its opening to the transcriptional machinery. Recent studies have shown the recruitment of transcriptional complexes to *CYP1A1* promoter/enhancer sequences, although the mechanistic details of these events have not been fully elucidated, even though the overall mechanism is thought to be similar to that of other nuclear receptors. Several nuclear receptor cofactors interact with AHR, including p300/CBP [15], the p160 family members: NCoA-2/SRC-1/p/CIP [16], BRG1 [17], ERAP140 [18], RIP140 [19], and BRCA1 [20]. In vitro experiments have also reported the recruitment of Sp factors and an increase in the enhancer activity of AhREs by the presence of adjacent GC-rich elements in the *Cyp1a1* promoter [21].

The observations summarized above suggest that chromatin structure plays an essential role in *CYP1A1* transcription. In this context, previous work from this laboratory has shown that in the ground state, HDAC1 is bound to the *Cyp1a1* promoter and is released in concert with the recruitment of p300 upon B[a]P stimulation [22]. Prompted by recent findings that HDAC1 inhibition reactivates estrogen receptor- α silencing in ER-negative human breast cancer cell lines [23], we tested whether both HDAC1-mediated repression and histone modifications played a role in *Cyp1a1* activation. In the present study, we have used ChIP assays and real-time PCR to explore the role of HDAC1 in *Cyp1a1* silencing. We find that HDAC1 removal

allows for several histone modifications associated with the Ah receptor mediate induction of *Cyp1a1* expression. Removal of HDAC1 is necessary but not sufficient to activate *Cyp1a1* expression.

Materials and Methods

Cell culture and chemical treatments

Mouse hepatoma Hepa-1c1c7 (Hepa-1) cells were cultured in α -minimal essential medium (α -MEM, Gibco) supplemented with 5% (v/v) fetal bovine serum (Sigma), and 1% (v/v) antibiotic-antimycotic (Gibco) in 5% CO₂ humidified atmosphere at 37°C. When the cells reached 70-80% confluence they were stimulated with 5 μ M B[a]P in a final volume of DMSO not to exceed 0.1% of the total culture medium. 5-Aza-2'-deoxycytidine and sodium butyrate (Sigma) were dissolved in DMSO and sterile deionized water, respectively, prior to use. Cells were seeded 24 h before the beginning of treatments with 2 μ M Aza, 2 mM NaB or both. Medium was supplemented with Aza for a total of 72 h, and at every 12-h interval, spent medium was replaced with fresh medium supplemented with Aza. NaB was added to the medium 16 h prior to termination of the experiments or before treatment with B[a]P.

SiRNA transfection

siRNAs (Ambion) were transfected into Hepa-1 cells using the neofection protocol recommended by the manufacturer. Briefly, 1 h before transfection cells in exponential growth phase were trypsinized, resuspended in complete growth medium at a density of 1×10^5 cells/ml and maintained at 37°C until transfection. siRNA duplexes were used at a final concentration of 25 nM for siGAPDH (Catalogue #4631) and 50 nM for siDNMT1 #1 (ID # 161526), siDNMT1 #2 (ID #161527), HDAC1 #1 (ID #61931), HDAC1 #2 (ID #62023) and the negative control (scrambled, Catalogue #4611). Forty eight hours post-transfection, cells were either used to measure mRNA and protein expression of siRNA-target genes or total RNA was extracted after stimulation with B[a]P.

Preparation of total protein extracts from Hepa-1 cells and Western blotting

Forty eight hours post-transfection with siRNAs, cells were directly lysed in the plate with 2X loading buffer (0.125 M Tris-HCl [pH 6.5], 20% glycerol, 4% SDS, 5% β -mercaptoethanol and bromophenol blue). Lysates were boiled for 5 min, run on a 12% polyacrylamide gel and transferred to HybondTM-P membranes (AP-Biotech). Membranes were blocked in 1X PBS containing 0.1% (v/v) Tween 20 (PBS-T) and 5% fat free milk. Primary antibodies were, rabbit polyclonal anti-DNMT1 (Abcam), mouse monoclonal anti-HDAC1 (Upstate) or mouse monoclonal anti- β -actin (Sigma) all used in PBS-T containing 5% fat-free milk. Proteins of interest were visualized with a chemiluminescent detection reagent (Supersignal[®] West Pico, Pierce).

RNA extraction and cDNA synthesis

Total RNA was extracted using NucleoSpin RNA II columns (Macherey-Nagel) according to the manufacturer's instructions. cDNA was synthesized by reverse transcription of 1 μ g total RNA using SuperScriptTM II RNase H⁻ reverse transcriptase (Invitrogen). An aliquot of the resulting cDNA products was used as template for subsequent quantification by real-time PCR amplification. Samples were amplified with mouse CYP1A1 primers (forward: 5'-GTGTCCTGGTTACTTTGACAAGTGG-3' and reverse: 5'-AACATGGACATGCAAGGACA-3'), HDAC1 primers (forward: 5'-TTCCAACATGACCAACCAGA-3' and reverse: 5'-GGCAGCATCCTCAAGTTCTC-3'), DNMT1 primers (forward: 5'-CTGACCGCTTCTACTTCCTC-3' and reverse: 5'-TCCCTTTCCCCTTCCCTTTC-3'), GAPDH primers (forward: 5'-

AACTTTGGCATTGTGGAAGG -3' and reverse: 5'- GGATGCAGGGATGATGTTCT -3'), and β -actin primers (forward: 5'-CATCCGTAAAGACCTCTATGCC -3' and reverse: 5'-ACGCAGCTCAGTAACAGTCC -3'). Amplification of β -actin cDNA in the same samples was used as an internal control for all PCR amplification reactions.

ChIP and PCR analyses

ChIP was performed with minor modifications of the procedure described by Wells *et al* [24], using approximately $1.5 - 2 \times 10^7$ Hepa-1 cells cross-linked with 1% formaldehyde for 10 min at room temperature. Cell pellets were resuspended in cell lysis buffer (5 mM PIPES [pH 8.0], 85 mM KCl, 0.5% NP-40, plus 1X protease inhibitor cocktail (Roche) and incubated on ice for 10 min. The nuclei were pelleted and resuspended in nuclei lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS plus protease inhibitor cocktail) and incubated on ice for 10 min. Chromatin was sheared to a size range of 0.3-0.8 kbp by sonication in a crushed-ice/water bath with six 30-sec bursts of 200 W with a 30-sec interval between bursts using a Bioruptor (Diagenode). Chromatin was precleared for 1.5 h at 4°C with a 50% gel slurry of protein A-agarose beads saturated with salmon sperm DNA and BSA (Upstate). Precleared chromatin was diluted 3-fold in IP dilution buffer (16.7 mM Tris-Cl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 1.1 % Triton X-100, 0.01% SDS), and 10% of the supernatants were used as inputs. The diluted chromatin was incubated overnight on a rotating platform at 4°C with antibodies specific for the proteins of interest. See supplemental Table S1 for information on the antibodies used in ChIP assays. The immune-complexes were recovered by 2-hour incubation at 4°C with a 50% gel slurry of either protein A-agarose or protein-G-agarose beads (Upstate) depending on the antibody specificity. The agarose beads were pelleted and washed as described [24]. In Re-ChIP experiments, immune-complexes were eluted by incubation for 30 min at 37°C in 10 mM DTT. After centrifugation, the supernatant was diluted 25 times with Re-ChIP buffer (1 % Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1]) and subjected again to the ChIP procedure using a different antibody. Cross-linking was reversed by adding NaCl to a final concentration of 0.3 M and incubated overnight at 65°C in the presence of RNase A. Samples were then digested with proteinase K at 45°C for 1.5 h. DNA was purified by chromatography on QIAquick® columns (PCR purification kit, Qiagen), eluted in ddH₂O, and an aliquot was used for analysis by PCR. Standard end-point and real-time PCR reactions were performed using primers specific for the mouse *Cyp11a1* promoter region (Fig. 1A). For a complete coverage of the region between -4.0 kbp and +0.2 kbp of the mouse *Cyp11a1* promoter, 13 primer sets were designed (see Supplemental Table S2) and tested in PCR reactions with genomic DNA as template. The end-point PCR products were separated by electrophoresis through 15% polyacrylamide gels and visualized after staining with ethidium bromide.

Quantitative real-time PCR analysis

QRT-PCRs were performed at least in duplicate in a reaction mixture containing 1X Power SYBR® Green PCR Master Mix (Applied Biosystems), and 0.1 μ M of each primer. Amplification was performed on an ABI 7500 real-time PCR system (Applied Biosystems) where the reaction was heated to 95°C for 10 min and immediately cycled 35 times through a denaturing step at 95°C for 15 s and an annealing-elongation step at 60°C for 60 s. Melting curve analyses were performed after PCR amplification to ensure that a single product with expected melting curve characteristics was obtained as preliminarily determined during primer tests. For analysis of results, we used the sequence detection software (SDS software version 1.3.1, Applied Biosystems).

Data analyses

Relative differences in QRT-PCR among samples were determined using the $\Delta\Delta C_T$ method as described in the Applied Biosystems instructions. The ΔC_T value for each sample was determined using the C_T value (obtained from the means of replicates) from either the input DNA to normalized ChIP assay results or the β -actin signal to normalized gene expression assays. The $\Delta\Delta C_T$ was calculated by subtracting control ΔC_T values from the corresponding experimental ΔC_T . The resulting values were converted to fold-changes over control by raising 2 to the power of $-\Delta\Delta C_T$.

Results

HDAC1-DNMT1 complexes occupy the Cyp1a1 proximal promoter in uninduced cells

Earlier studies from this laboratory have shown that HDAC1 is constitutively present in the *Cyp1a1* promoter and is released upon binding of the B[a]P-liganded AHR complex and recruitment of p300 [22]. To investigate whether HDAC1-mediated repression mechanisms maintain the *Cyp1a1* gene in a silenced state in unstimulated cells, we used chromatin IP analyses of extracts of Hepa-1 cells treated with DMSO or B[a]P and immunoprecipitated with anti-HDAC1 and anti-AHR antibodies. Specific enrichment in the immunoprecipitated DNA was evaluated by QRT-PCR with specific primers for enhancer (−0.8 kbp) and proximal (−0.1 kbp) *Cyp1a1* promoter regions. Anti-HDAC1 antibodies immunoprecipitated both enhancer and proximal promoter sequences in DMSO-treated but not in B[a]P-treated cells (Fig. 1C), verifying that HDAC1 is constitutively bound to the 5'-flanking region of the *Cyp1a1* gene and is released upon B[a]P stimulation.

RNA polymerase II and AHR were recruited to the promoter only after B[a]P stimulation (Fig. 1B-C). Immunoprecipitation with anti-AHR and anti-RNA polymerase II antibodies showed a significant recruitment of AHR to the enhancer domain and less so to the proximal promoter. Even though no AhRE motifs are present in this promoter domain, AHR recruitment to this region was significant, albeit lower than to the enhancer (Fig. 1B-C), consistent with a looping model of AHR/ARNT transcriptional activation of the *Cyp1a1* gene [25]. Conversely, RNA polymerase II did not bind to the enhancer domain to any appreciable extent, but it bound to the proximal promoter domain.

The main property of HDACs is their ability to repress transcription *via* their histone deacetylase activity [26]. The presence of HDAC1 in the uninduced *Cyp1a1* promoter is consistent with this function and with the silent status of the gene, and raises the question as to the role of this protein in the regulation of *Cyp1a1* gene expression. HDACs are found in large multiprotein complexes, which include transcriptional corepressors, such as SMRT, NCoR, and BCoR [27-29] and with other effectors of epigenetic marks, such as members of the DNMT family of DNA methyltransferases, which play an important role in the epigenetic control of gene expression [30,31]. To determine if HDAC1 and DNMT1 were associated on the silent *Cyp1a1* promoter, we used ChIP assays to measure the binding of DNMT1 to the enhancer (−0.8 kbp) and the proximal promoter (−0.1 kbp) regions of the *Cyp1a1* gene. The results showed that DNMT1 was constitutively associated with both promoter regions and that the association was lost after B[a]P induction (Fig. 1B-C). To determine whether HDAC1 and DNMT1 formed a binary complex that might act as a repressive unit of *Cyp1a1* transcription, we carried out re-CHIP experiments, using chromatin samples of control and B[a]P-treated Hepa-1 cells that were immunoprecipitated with anti-DNMT1, eluted and, without reversal of the cross-links, re-precipitated with anti-HDAC1 or with non-specific IgG antibodies. Immunoprecipitated DNA samples were amplified by PCR with primers targeting the enhancer (−0.8 kbp) and proximal (−0.1 kbp) promoter regions. As a positive control, chromatin samples were sequentially immunoprecipitated with anti-AHR followed by anti-ARNT antibodies and

PCR amplified for the enhancer region. Sequential IP with AHR and ARNT antibodies showed a strong DNA enrichment of the enhancer region in B[a]P-treated samples relative to DMSO controls (Fig. 1B-C). Sequential immunoprecipitation with anti-DNMT1 followed by anti-HDAC1 showed stronger DNA enrichment of the proximal promoter in control DMSO-treated cells relative to the enhancer region. The quantity of immunoprecipitated DNA was much reduced in B[a]P-treated cells (Fig. 1C). Neither enhancer nor promoter regions were significantly immunoprecipitated by sequential ChIP/re-ChIP with anti-DNMT1 and non-specific IgG. These results are consistent with the conclusion that HDAC1 and DNMT1 form a complex that colocalizes to the *Cyp1a1* proximal promoter region and that is released by binding of the liganded AHR/ARNT complex.

We sought to define the extent of HDAC1 localization over the *Cyp1a1* promoter to better understand its role in chromatin structure and *Cyp1a1* regulation. To this end, we mapped the *Cyp1a1* promoter by immunoprecipitation with anti-HDAC1 antibodies and the use of 13 specific PCR primers sets, spanning from -3.6 to +0.6 kbp, to compare DNA enrichment in samples treated with or without B[a]P. As shown in Fig. 1D, the HDAC1 signal was distributed over a narrow segment of the 5'-flanking domain, mainly derived from the region extending from -0.8 to +0.2 kbp with a maximum in the -0.5 - 0.1 kbp domain that is quickly lost after B[a]P treatment. We also mapped the same 5'-flanking region for DNMT1 binding and found that the association of DNMT1 and HDAC1 occurs over the same region of the promoter (Fig. 1D). In this region of the promoter, the *Cyp1a1* promoter has a canonical CpG island that comprises a 520-bp fragment extending from -1.265 kbp to -0.746 kbp, with a G+C content of 62.5% and a CpG observed/expected ratio of 0.61 (supplemental Fig. S1). Bisulfite sequencing of this island showed only background cytosine methylation levels, strongly arguing against the possibility that the association of HDAC1-DNMT1 complexes with the *Cyp1a1* promoter leads to its methylation (Supplemental Fig. S1).

AHR activation induces specific chromatin changes in the *Cyp1a1* promoter

Chromatin marks such as hyperacetylation of histones H3 and H4, and mono-, di- and tri-methylation of Lys4 on histone 3 (H3K4), are generally associated with transcriptionally active genes and accessible chromatin structure, whereas other chromatin configurations, such as histone hypoacetylation and methylation of Lys9 and Lys27 on histone 3 (H3K9 and H3K27, respectively), are generally associated with transcriptionally inactive genes and inaccessible chromatin structures [32]. To search for discrete histone marks associated with remodeling by AHR/ARNT binding that might be suppressed by HDAC1, we carried out ChIP analyses with antibodies specific for histone H3 and H4 modifications, including histone acetylation at Lys9 and Lys14 in histone H3, Lys8 and Lys16 in H4 (H3K9ac, H3K14ac, H4K8ac and H4K16ac), mono-di- and tri-methylation at Lys4, Lys9 and Lys27 of H3 (H3K4me2, H3K4me3, H3K9me, H3K93me, H3K27me, and H3K27me3), and phosphorylation or Ser10 in H3 (H3S10ph and H3K14acS10ph). These assays were associated with a systematic mapping of the promoter, between position -3.6 kbp to +0.6 kbp from the transcription start site, to detect any localized changes that might result from AHR/ARNT or HDAC1 binding. B[a]P treatment strongly resulted in the acetylation of K9 and K14 in histone H3, mostly so over the proximal promoter region, consistent with the release of HDAC1. Similarly, acetylation of K16 in histone H4, involved in prevention of higher-order chromatin compaction [33], was also associated with B[a]P treatment, but in this case, acetylation occurred mainly on the enhancer domain (Fig. 2 A-B). In contrast, no apparent changes could be detected for H4K8 acetylation, nor were methylation modifications of K9 or K27 in histone H3 significantly different in B[a]P-treated cells compared to controls (data not shown). On the other hand, significant differences were clearly evident on the methylation of H3K4, which is highly dimethylated in control cells in both enhancer and proximal promoter domains and becomes trimethylated in B[a]P treated cells (Fig. 2 A-B). Phosphorylation of Ser10 in H3, detected with either anti-H3S10ph or anti-

H3K14acS10ph, was significantly increased only in the enhancer domain of B[a]P-induced cells but not of controls (Fig. 2 A-B). Control immunoprecipitations with non-specific IgG showed no significant differences between treated or control cells over any region of the *Cyp1a1* 5'-flanking sequences.

Quantification of these results by QRT-PCR (Fig. 2B) shows that upon induction by B[a]P, acetylation of both histone H3 and H4 increases, but that their hyperacetylation is specific to one or two positions. For example, a robust increase of H3K14ac is observed in the proximal promoter region, whereas the enhancer region shows a marked increase of H4K16ac. The greatest change observed was the striking negative correlation between H3K4me3 and H3K4me2 signals upon B[a]P stimulation. This change was highly correlated with position in the promoter and proximity to the transcriptional initiation start site. Dimethylation levels decreased significantly and trimethylation increased with B[a]P treatment, resulting from the addition of a third methyl group to already dimethylated Lys4. These histone modifications appear to characterize epigenetic changes in the *Cyp1a1* promoter that take place concomitantly with AHR transactivation.

Knock-down of HDAC1 and DNMT1 with siRNA is not sufficient to derepress *Cyp1a1*

Because of their constitutive localization to the *Cyp1a1* promoter and their release upon induction by B[a]P-liganded AHR, it could be expected that HDAC1 and DNMT1 might play an active role in *Cyp1a1* transcriptional repression. To test whether removal of these proteins from the promoter would lead to *Cyp1a1* derepression in the absence of AHR activation, we knocked-down their expression by transient transfection of siRNA oligonucleotides. Control experiments at 48 h post-transfection showed that mRNA (Fig. 3A) and protein (Fig. 3B) levels of *Hdac1*, *Dnmt1* and a *Gapdh* control were reduced by greater than 80 - 85% in transfected cells by their respective siRNA oligos, but not by a scrambled oligonucleotide control. In contrast, *Cyp1a1* mRNA levels in cells treated for 8h with DMSO or 5 μ M B[a]P were unaffected by any of the siRNAs, singly or in combination, or by inhibition of HDAC1 or DNMT1 with sodium butyrate or 5-aza-2'-deoxycytidine, respectively (Fig. 3C). Knock-down of DNMT1, HDAC1 or both simultaneously in control DMSO-treated cells caused no *Cyp1a1* derepression while B[a]P treatment caused a major 80-fold mRNA stimulation, indicating that transactivation of the gene was unimpaired by the treatments.

Inhibition of HDAC1 with sodium butyrate significantly decreased the binding of HDAC1 to enhancer or proximal promoter domains and did not promote the binding of AHR to either domain in the absence of ligand. Similarly, p300 binding was not increased by HDAC1 inhibition nor were there major differences in trimethylation and phosphorylation marks in H3K4 and H3S10, respectively, which were also unaffected by NaB treatment (Fig. 4A-B). On the other hand, acetylation marks in H3K14 and H4K16 were greatly increased relative to their levels in NaB-untreated cells, increasing to levels comparable to those attained by B[a]P induction (Fig. 4A-B). By blocking modification of these histone marks, HDAC1 functions as an active repressor of basal *Cyp1a1* expression even though its inactivation is not sufficient to accomplish the complete reversal of histone marks associated with activation of gene expression by the AHR complex.

Discussion

In this study, we extend our previous observations and establish evidence to the effect that complexes of HDAC1 and DNMT1 are constitutively bound to the 5'-flanking chromatin of the *Cyp1a1* gene promoter and maintain this gene in a ground, silenced state. Upon ligand stimulation, the AHR complex binds to the enhancer domain, containing the cluster of AHR binding sites, and sets in motion the initiation of transcription. The AHR also binds to the proximal promoter, even though no AHR-binding sites are present in this region where RNA

polymerase II is recruited. These data are consistent with a looping model of AHR/ARNT mediated *Cyp1a1* transcriptional activation, with a tight cooperation between enhancer and proximal promoter regions in the rapid recruitment of the transcriptional machinery, as proposed by Tian and co-workers [25].

Previous work has described the presence of a nucleosome positioned over the proximal promoter region, blocking transcription initiation [14]. It is likely that, as proposed for other genes [34], the HDAC1/DNMT1 complex is associated with this nucleosome and dissociates from it through a chromatin-remodeling event involving the binding of the AHR complex that facilitates the repositioning of the nucleosome and recruitment of RNA polymerase II. In our experiments, maximum recruitment of the AHR occurred between 60 and 100 min after activation (data not shown), consistent with previously published results on maximal transcription rates after TCDD activation [14]. The interaction is stabilized thereafter, slowly decreasing with time rather than cycling on-and-off as described for the metabolizable AHR agonist, β -naphthoflavone [35].

The presence of HDAC1/DNMT1 complexes in the *Cyp1a1* promoter raises the question of their role in its regulation. Our data with siRNA and inhibitors demonstrates that their knock-down does not lead to gene induction in the absence of AHR activation. It could be argued that HDAC1 binding is irrelevant to the regulation of *Cyp1a1* expression. Alternatively, their removal might be a necessary, but not sufficient condition for gene induction, which would require further binding of the activated AHR complex. We propose that the latter possibility is correct, based on our data showing that HDAC1 inhibition is sufficient to cause H3K14 and H4K16 hyperacetylation, two histone marks generally associated with gene *trans*-activation, to levels as high as those reached with B[a]P treatment. Hence, we propose that HDAC1 functions as a repressor of *Cyp1a1* basal expression. This conclusion is supported by reports that, while CYP1A1 and CYP1A2 proteins are not detectable in rat hepatocytes exposed to TSA, another HDAC1 inhibitor, both proteins are expressed after treatment with TCDD, and their levels are further increased when TCDD-treatment was accompanied by TSA [36]. This is in contrast with recent findings of the silencing of the estrogen receptor- α in several ER-negative human breast cancer cell lines, which is reactivated by HDAC1 inhibition concomitant with CpG demethylation [23].

DNA methylation, controlled by the DNA methyltransferase family of enzymes [37], constitutes a critical epigenetic mechanism of regulation of gene expression. Chromatin IP demonstrates the presence of DNMT1 in the unstimulated *Cyp1a1* promoter. In the process of silencing gene expression, HDAC1-DNMT1 complexes initiate the recruitment of methyl-CpG binding domain proteins that mediate gene silencing by facilitating CpG island hypermethylation. Methylation of one of the cytosine residues in the core AhRE motif has been shown to diminish AHR binding to the motif and the transcriptional response to AHR ligand [38]. Also, recent reports have shown that aberrant methylation of the human *CYP1A1* promoter suppressed its dioxin responsiveness in LNCaP prostate cancer cells and in 11 out of 30 prostate tumor samples, but not in non cancerous RWPE-1 cells [39]. It could be argued that the silencing effect resulting from HDAC1/DNMT1 complex association was due to a similar case of promoter methylation. Our results, however, show no evidence of CpG island hypermethylation, strongly ruling out promoter hypermethylation as a major consequence of HDAC1-DNMT1 binding.

Chromatin IP analyses using histone antibodies raised against discrete amino acid modifications, including acetylation, methylation and phosphorylation, showed significant differences between B[a]P-stimulated and -unstimulated cells. Histone marks associated with gene induction, such as 3MeH3K4me3, H3K9ac, H3K14ac and H4K16ac, while already present to some extent in unstimulated cells, were highly inducible by B[a]P treatment, whereas

marks associated with stably silenced heterochromatin, such as H3K9me and H3K27me [40] were not affected by B[a]P treatment (data not shown). Dimethylated Lys4 in H3 decreased after induction by the same amount that trimethylated Lys4 increased. This is a mark found exclusively associated with active genes [32]. Histone H3 Lys4 methylations are catalyzed by trithorax-group histone methyltransferases, which mediate mitotic inheritance of lineage-specific gene expression programs and have key developmental functions [41]. Lys4 methylation positively regulates transcription by recruiting nucleosome remodeling enzymes and histone acetylases [42,43]. Key epigenetic regulatory controls involved in development are clustered in the so-called highly conserved noncoding elements of the mammalian genome, which are enriched for genes encoding transcription factors. During embryonic stem cell differentiation, and in differentiated cells, developmental genes are typically marked by broad regions selectively enriched for either Lys27 or Lys4 methylation [44]. Our observation that B[a]P treatment strongly induces Lys4 trimethylation opens up the interesting possibility that exposure to AHR ligands during development may derail developmental gene expression patterns.

Phosphorylation of S10 in histone H3, absent in untreated cells, is strongly induced by B[a]P treatment, but only in the AHR complex binding region. The phosphorylation of this serine residue, tightly correlated with AHR recruitment, acetylation of histone H3 and *Cyp1a1* transcriptional activation, is also critical for the activation of other genes in other regulatory systems [45,46] where phosphorylation precedes H3 acetylation. Histone phosphorylation is not always related to gene activation, being a distinctive marker of cells undergoing mitosis [47,48]. Unlike in histone phosphorylation events associated with mitosis, the H3S10ph mark in the *Cyp1a1* promoter is not uniformly extended along the chromatin but is specific for the enhancer domain of the promoter where the AHR complex binds, suggesting that it is triggered by a signaling pathway activated during AHR translocation or that the AHR itself or the AHR/ARNT complex, recruit the protein kinase responsible for the phosphorylation. In either case, it would seem reasonable to conclude that phosphorylation of Ser10 in H3 is required for *Cyp1a1* induction. In this context it is worth noting that several inhibitors of protein kinases, such as 2-aminopurine, staurosporine, and SB202190, a JNK inhibitor, block *Cyp1a1* induction by TCDD in Hepa-1 cells [49].

Although the *Cyp1a1* promoter in Hepa-1 cells is in a fully euchromatic state, removal of potential repressor HDAC1-DNMT1 complexes is not sufficient to recruit p300 and cause the constitutive transcription of *Cyp1a1* mRNA. Binding of AHR to the enhancer region is a necessary condition to induce p300 binding and a wave of chromatin modifications, including hyperacetylation, methylation and phosphorylation that establish a level of regulation of *Cyp1a1* expression at the chromatin level capable of modulating the effects of Ah receptor activation. Our results underscore the requirement for a previously uncharacterized concerted series of chromatin remodeling events to complement and complete the initial steps of gene transactivation by the Ah receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AHR	aryl hydrocarbon receptor
AhRE	AHR response element
ARNT	aryl hydrocarbon receptor nuclear translocator
Aza	5-Aza-2'-deoxycytidine
B[a]P	benzo[a]pyrene
BCoR	BCL6 corepressor
bHLH/PAS	basic region/helix-loop-helix/Per-ARNT-Sim
BRG1	brm (brahma)/SWI (singled wings) 2-related gene-1

CBP	CREB (cAMP responsive element binding protein) binding protein
ChIP	chromatin immunoprecipitation
CRM-1	chromosome region maintenance protein-1
DNMT	DNA methyltransferase
DRE	dioxin response element
ERAP140	estrogen receptor-associated protein of 140 kD
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HAH	halogenated aromatic hydrocarbons
HDAC	histone deacetylase
HIF-1β	hypoxia-inducible factor 1 β
HSP	heat-shock protein
NaB	sodium butyrate
NCoA-2	nuclear receptor coactivator 2
NCoR	nuclear corepressor
PAH	polycyclic aromatic hydrocarbons
QRT-PCR	quantitative real-time PCR
RIP140	receptor-interacting protein 140
SMRT	silencing mediator for retinoid acid and thyroid hormone receptors
Sp	specific protein
SRC-1	steroid receptor coactivator-1

TCDD2,3,7,8-tetrachlorodibenzo-*p*-dioxin**XAP-2**

hepatitis B virus X-associated protein-2

XRE

xenobiotic response element

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Fig. 1. HDAC1 and DNMT1 colocalize on the *Cyp11a1* proximal promoter region

(A) Schematic representation of the mouse *Cyp11a1* promoter and the primer positions used to amplify specific regions in ChIP experiments. Position of AhRE motif clusters and primers (black arrows) relative to the transcriptional start site (blue arrow) numbered as +1 are indicated. A nucleosome (dashed circle), probably positioned over the promoter, is localized on the scheme in addition to other previously described elements where transcription factors interact with the promoter. (B) ChIP assays for HDAC1, DNMT1, AHR and RNA pol II and ReChIP assays for HDAC1 and DNMT1 were performed with chromatin from cells treated for 1.5 h with DMSO vehicle (lanes labeled D) or 5 μ M B[a]P (lanes labeled B). The DNA was amplified by PCR using primers spanning the enhancer (–0.8 kbp) and the proximal promoter (–0.1 kbp) regions and PCR products were visualized by ethidium bromide staining after gel electrophoresis. (C) DNA was quantified by real-time PCR and normalized to inputs. (D) HDAC1- and DNMT1-immunoprecipitated DNA samples were used to compare the occupancy by HDAC1 and DNMT1 across the *Cyp11a1* promoter in control and stimulated cells. DNA enrichment was evaluated by real-time PCR, normalized to inputs and compared to IgG non-specific immunoprecipitation. Results shown in gels are representative of three independent experiments and quantification data are means $\pm$ SD of three independent experiments.

Fig. 2. AHR activation by B[a]P induces posttranslational modification of histones in the *Cyp1a1* promoter

(A) Hepa-1 cells were treated for 1.5 h with DMSO (lanes labeled D) or B[a]P (lanes labeled B). Cells were then used for ChIP analysis using specific antibodies raised against post-translationally modified histone amino acids. ChIP DNA was amplified by PCR using primers specific for the indicated regions. PCR products amplified with -3.2, -0.8 and -0.1 kbp primers were resolved by electrophoresis. Results shown are representative of three independent experiments. (B) Results of real time PCR amplification are expressed as percent of total input. Data are the means ($\pm$ SD) of three independent experiments.

Fig. 3. Effect of HDAC1 and DNMT1 siRNAs and inhibitors on constitutive and B[a]P-inducible *Cyp1a1* mRNA expression

Hepa-1 cells were transfected with or without GAPDH siRNA, scrambled-siRNA, DNMT1 siRNA (#1 and #2), HDAC1 siRNA (#1 and #2), and a combination of DNMT1 and HDAC1 siRNAs. Forty eight hours post transfection, the effect of the siRNAs on target gene expression was evaluated for mRNA (A) and protein (B). Cells maintained in medium with or without 2 μ M Aza for 72 h, 2 mM NaB for 16 h or transfected for 48h with siRNAs were then treated for 8 h with DMSO or 5 μ M B[a]P and *Cyp1a1* mRNA expression levels were determined (C). Relative mRNA expression is expressed as fold-induction calculated as the ratio of target signal to β -actin relative to the same ratio in control. Results shown in the Western blot experiment are representative of two independent experiments and data are means $\pm$ SD of two independent experiments.

Fig. 4. Effect of sodium butyrate on histone modifications associated with *Cyp11a1* expression

Hepa-1 cells were maintained in medium with or without 2 mM NaB for 16 h and then treated for 1.5 h with DMSO or 5 μ M B[a]P. ChIP DNA was amplified and quantified by real-time PCR using primers spanning the *Cyp11a1* enhancer (−0.8 kbp) and the proximal promoter (−0.1 kbp) regions. DNA enrichment was expressed as percent of total input. Data are the means ($\pm$ SD) of three independent experiments.