Genome-wide H3K9 Histone Acetylation Profiles Are Altered in Benzopyrene-treated MCF7 Breast Cancer Cells*\textsuperscript{1}

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Current toxicogenomic approaches generate transcriptional profiles that can identify functional gene expression signatures of environmental toxicants. However, the intricate processes governing transcription are overlaid with a complex set of molecular instructions involving epigenetic modifications. These commands regulate both gene expression and chromatin organization through coordinated sets of histone modifications and inheritable DNA methylation patterns. Although the effects of specific environmental toxicants on gene expression are the subject of much study, the epigenetic effects of such compounds are poorly understood. Here we have used human promoter tiling arrays along with chromatin immunoprecipitation to identify changes in histone acetylation profiles because of chemical exposure. Chromatin from cells exposed to the polyaromatic hydrocarbon benzo(a)pyrene was immunoprecipitated with antibodies against acetylated histones. Affymetrix promoter tiling microarrays were probed to generate epigenomic profiles of hypo- and hyperacetylated chromatin localized to gene promoter regions. Statistical analyses, data mining, and expression studies revealed that treated cells possessed differentially acetylated gene promoter regions and gene-specific expression changes. This chromatin immunoprecipitation-on-chip approach permits genome-wide profiling of histone acetylation patterns that can identify chromatin-related signatures of environmental toxicants and potentially determine the molecular pathways these changes target. This approach also has potential applications for profiling histone modifications and DNA methylation changes during embryonic development, in cancer biology, and in the development and assessment of cancer therapeutics.

The development of microarray and bioinformatic technologies has provided new tools to address the biological mechanisms of development and disease. These tools also allow assessment of biological responses to environmental stimuli at the single gene or pathway level or across the entire genome. This capability to study chemical effects on the expression of thousands of genes either simultaneously or sequentially within regulatory pathways encompasses the discipline of toxicogenomics (1). Current toxicogenomic research has generated transcriptional profiles resulting from specific environmental contaminants, with the aims of characterizing their potential mechanisms of action and to develop biomarkers of chemical exposures (2). Such approaches have the potential to provide a wealth of mechanistic information related to gene expression as well as uncover adverse reactions to compounds at levels of toxicity that are beyond the limits of conventional assays (3).

The intricate process governing transcriptional regulation of gene expression is overlaid with a complex set of molecular instructions that regulate both gene expression and chromatin organization through two coordinated sets of molecular alterations. First, the reversible addition of methyl groups at cytosines within CpG dinucleotides can directly prevent the binding of proteins to their binding motifs as well as recruit protein complexes that promote transcriptional repression (4, 5). The dynamic and plastic epigenetic patterning of DNA methylation within the cell plays key roles in development. These roles include the regulation of tissue-specific gene expression, the maintenance of chromatin organization, and the maintenance of X chromosome inactivation and genomic imprinting (5, 6). The consequences of inappropriate methylation contribute to cancer progression. Aberrant hypermethylation of gene promoter regions can repress tumor suppressor genes, whereas the mis-timed hypomethylation of chromatin regions is implicated in genomic instability. Both types of alterations are well defined characteristics of cancer progression (6, 7).

In addition to DNA methylation patterns, nucleosomal histone proteins are targets for a wide array of post-translational modifications. These modifications occur primarily along the amino-terminal histone tails and include acetylation, methylation, phosphorylation, and ubiquitylation events (5, 6). The complex biological effects of these changes are only partially understood, although in general, lysine acetylation usually correlates with chromatin accessibility and transcriptional activity, whereas lysine methylation has both activating and repressive effects (4–6). For example,
Benzopyrene Alters H3K9 Histone Acetylation

High resolution genome-wide mapping with ChIP4-on-chip assays has revealed high levels of histone H3 acetylation in gene-rich regions with chromatin accessibility, and in the promoter regions of transcribed genes (8, 9). These results demonstrate the involvement of acetylation and other histone marks at functional regulatory elements across the human genome. Together, DNA methylation and histone modifications modulate a landscape that organizes and maintains the integrity of the nuclear architecture while establishing and enabling (or in some cases, impeding) the expression patterns of specific genes within chromatin regions.

The dynamic nature of DNA methylation and histone modifications means that alterations in their placement or removal can have significant health consequences. In fact, such abnormalities are contributing factors in autoimmune diseases and aging, as well as causative factors in genetic disorders, pediatric syndromes, and cancer (7). Cancer progression is a particularly well defined paradigm for epigenetic alterations, primarily in the context of CpG hypermethylation events that inactivate tumor suppressor genes (6, 7, 10). Changes in the histone modifications are less well documented, although DNA methylation has been correlated with deacetylation of histones 3 and 4, along with shifts in histone methylation patterns (11, 12), suggesting that these changes may be part of the defining signature in tumorigenesis (6). It is the dynamic nature of these histone modifications that renders them particularly susceptible to environmental influences. For example, dietary fluctuations, such as low folate levels, can alter methyl metabolism, shift methylation patterns, and change gene expression (13, 14). Similarly, exposures to environmental pollutants such as vehicular exhaust, tobacco smoke, and heavy metals elicit epigenic changes and affect genes involved in DNA repair, apoptosis, or chemical detoxification pathways (15, 16).

Here we describe a ChIP-on-chip approach using human promoter tiling arrays to identify genome-wide changes in histone acetylation patterns because of the environmental pollutant benzo(a)pyrene (BaP). BaP is a common polyaromatic hydrocarbon and an environmental toxicant present in vehicle emissions, environmental tobacco smoke, certain foods, and occupational exposures (17, 18). BaP activates multiple signal transduction pathways and directly affects transcription through the aromatic hydrocarbon receptor (19). Metabolites of BaP damage DNA by forming stable DNA adducts at methylated CpG dinucleotides (20) and induce epigenetic events and cell cycle disruptions (21). Mechanistically, these DNA adducts, if not recognized and repaired, may increase the risk over time of creating pre-tumorigenic cells harboring DNA damage, which then proliferate into a larger at-risk cell population that is susceptible to later mutational events. We addressed our hypothesis that BaP would cause genome-wide histone acetylation changes, and we provide the first evidence that BaP exposure induces hypoacetylation changes as well as hyperacetylation events that correlate with gene-specific expression changes in some genes. This ChIP-on-chip approach has practical applications as a method to generate histone acetylation profiles of environmental toxicants and to determine their mechanisms of action.

MATERIALS AND METHODS

Cell Treatments—Breast carcinoma MCF-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 20 mM HEPES Buffer Solution (Invitrogen), and penicillin/streptomycin in humidified tissue culture incubators at 37 °C and 5% CO2. Cells (2 × 106) were plated on 15-cm tissue culture plates in 20 ml of growth media. After 24 h the cells were treated with 0.05% Me2SO (Fisher) or 0.5 μM BaP (Sigma) suspended in Me2SO to a total 30-ml volume and exposed for 4 days.

ChIP-on-Chip—Chromatin immunoprecipitation followed by the microarray hybridization (ChIP-on-chip) was performed with the Affymetrix Human Promoter 1.0R Arrays using a modification of the Affymetrix chromatin immunoprecipitation assay protocol. Sequences used in the design of this microarray platform were selected from the NCBI human genome assembly (Build 34), with repetitive elements removed by RepeatMasker and containing probes targeted to regions proximal to transcription start sites and representing 59% of the CpG islands annotated by University of California Santa Cruz. Chromatin was cross-linked in 1% formaldehyde while shaking the plates for 5 min at room temperature. The cells were harvested in phosphate-buffered saline with 0.5 mM phenylmethylsulfonyl fluoride (Sigma), Cell pellets were suspended and lysed using 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, and mammalian protease inhibitor mixture (Sigma). The chromatin was sonicated to reduce the size of DNA fragments to 200–1000 nucleotides, and 100 μl of chromatin (3 μg/μl) was aliquoted as input, immunoprecipitated (IP), and nonspecific precipitation using 2.5 μl of normal rabbit IgG (Santa Cruz Biotechnology; sc2027) overnight. The antibody-associated DNA fragments were recovered using 45 μl of protein A-agarose solution (Upstate), followed by immunoprecipitation using 2.5 μl of chip-grade anti-acetyl-H3K9 antibody (Abcam; ab4441; IP), or 2.5 μg of normal rabbit IgG (Santa Cruz Biotechnology; sc2027) overnight. The antibody-associated DNA fragments were recovered using 45 μl of protein A-agarose and washed using Buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 100 mM NaCl), Buffer 2 (Buffer 1 with 500 mM NaCl), Buffer 3 (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl), and 2X TE Buffer. Chromatin was eluted by vortexing and rotation at room temperature in 1% SDS and 0.1 M NaHCO3, de-cross-linked by adding NaCl (to 0.2 M), and inclu-

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4 The abbreviations used are: ChIP, chromatin immunoprecipitation; ATRX, α-thalassemia/mental retardation syndrome X-linked; BaP, benzo(a)pyrene; BAX, BCL2-associated X protein; BCA3, breast cancer anti-estrogen resistance 3; BRMS1, breast cancer metastasis suppressor 1; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; MeSO, dimethyl sulfoxide; GADD45B, growth arrest and DNA-damage-inducible, beta; GADD45B, growth arrest and DNA-damage-inducible, beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3, histone H3; H3K9, histone H3 lysine 9; HDAC1, histone deacetylase 1; IP, immunoprecipitation; MB2D, methyl-CpG-binding protein 2; MB3D, methyl-CpG-binding protein 3; METT5D1, methyltransferase 5 domain containing 1; MTA3, metastasis-associated 1 family, member 3; NAB2, NGFI-A-binding protein 2; TFII1, TATA element modulatory factor 1; USCS, University of California Santa Cruz.
bated at 65 °C overnight. The DNA was purified using the QIAquick PCR purification kit (Qiagen).

Random priming reactions, followed by the genomic PCR, were performed as per the Affymetrix chromatin immunoprecipitation assay protocol using the following PCR conditions: 94 °C, 2 min; 94 °C, 1 min, 52 °C, 2 min, 72 °C, 3 min for 5 cycles; 94 °C, 0.5 min, 52 °C 2 min, 72 °C, 1.5 min for 25 cycles; and 72 °C, 5 min. Probes for hybridization were generated as per Affymetrix chromatin immunoprecipitation assay protocol: 7.5 μg of double-stranded DNA was fragmented with uracil DNA glycosylase and apyrimidinic/apurinic endonuclease for 1 h. Targets were then labeled with Affymetrix labeling reagent and terminal deoxynucleotidyltransferase for 1 h. Mixtures were hybridized to the GeneChips for 16 h at 45 °C at 60 rpm. GeneChips were stained with streptavidin/phycoerythrin, followed by a biotin-conjugated anti-streptavidin and a second streptavidin/phycoerythrin solution, with all liquid handling performed by a GeneChip Fluidics Station 450. GeneChips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA) at the London Regional Genomic Centre (Robarts Research Institute, London, Ontario, Canada).

Data Analysis—Data from .cel files (GCOS 1.3 software) were imported into the Partek Genomic Suite Software. The imported data were pre-normalized using the Robust Multichip Averaging algorithm (22) and converted to log2. To obtain “true” antibody-specific intensities, we subtracted the mean of the signal from the IgG arrays directly from each IP array, across all 4.2 million probes. All probes with positive signal after this subtraction represent regions of antibody-specific binding. To identify significant signals, we used the predefined ChIP-on-chip workflow tool, and set our statistical parameters at \( p < 0.05 \) (single-sided \( t \) test) with a window of 250 nucleotides. This analysis detected all significantly acetylated regions for both control and treated cells, which satisfied the statistical criteria in 2/2 replicate experiments. These regions were then annotated to their corresponding genes using the Probeset ID annotation file from the Affymetrix U133 Plus 2.0 expression arrays. To detect specifically acetylated genes in control and treated cells, we used the VENN analysis tool from Partek (Fig. 1). Hypoacetylated targets are defined as sequences that were specifically acetylated in control cells and represent genes that lost their signal levels after the BaP treatment. Hyperacetylated targets were those target sequences that were significantly increased in the treated cells relative to the controls. The genes in the cross-section area of the VENN diagram represent significantly acetylated gene promoters in both control and BaP-treated cells. We used the Partek Region View tool to generate heat maps of the acetylation signals in our genes of interest. These heat maps were then converted to .wig files using the Partek Create Gene List tool and were uploaded to the Cruz BLAT genomic browser at UCSC. This approach permitted us to overlay the acetylation signal intensity maps in the context of the UCSC tracks and genomic features of interest, such as CpG islands, repetitive elements, and exon sequences.

ChIP-PCR—The original DNA aliquots from the microarray experiment (input, IP, and IgG) were used to specifically amplify hypoacetylated and hyperacetylated genes of interest using specific primer sets (supplemental Table 1), as described previously (23). DNA (1 μl) was amplified for up to 30 cycles, ensuring log phase amplification. Equal volumes of PCR products were separated on 6% acrylamide gels and visualized using the Bio-Rad Molecular Imager Gel Doc system. The results shown are representative of at least two replicate experiments.

Real Time PCR—Control Me2SO and BaP-treated MCF-7 cells were subjected to RNA isolation using RNaseasy mini kit (Qiagen), and cDNA generation using Superscript II reverse transcriptase (Invitrogen) as per the manufacturer’s instructions. Real time PCRs were performed using SYBR Green PCR mixture (Bio-Rad), PTC 200 Thermocycler, and Chromo 4 continuous fluorescence detector (MJ Research). Signal was quantitated using Opticon Monitor 3.1 software (MJ Research), and reactions were performed in triplicate for each of three DNA aliquots. Signals were normalized to GAPDH expression and presented as BaP/Me2SO ratio.

Network Identification and Canonical Pathway Analysis—The genes significantly acetylated in BaP-treated or control MCF-7 cells were analyzed using Ingenuity Pathways Analysis software version 5.0 (Ingenuity Systems, Redwood City, CA). Those genes (Affymetrix ID) and their corresponding log2 acetylation values were uploaded as the input data set into the software, and analyses were performed with both BaP treatment-specific and control-specific acetylated genes (referred to as hyper- and hypoacetylated genes, respectively). As well, these genes were uploaded for a concurrent analysis, in which case the log2 levels of acetylation for the hypoacetylated genes were converted to negative values. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge base, and a subset of “focus” genes were mapped to their corresponding gene objects in the Ingenuity Pathway Knowledge data base. Networks of these focus genes were then algorithmically generated based on their connectiv- ity, assigned a score, and ranked based on relevance to the input data set. A right-tailed Fisher’s test was used to calculate the \( p \) value for networks. The network identified was then presented as a graph to indicate the molecular relationships between genes/gene products. Each network or pathway was arbitrarily set to have a maximum of 35 focus genes. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one literature reference of direct physical, transcriptional and enzymatic interactions or from canonical information stored in the Ingenuity Pathways Knowledge base. The intensity of the node color indicates the degree of hyper-(blue) or hypoacetylation (yellow). Nodes are displayed using various shapes that represent the functional class of the gene product, and edges are displayed with various labels indicating the nature of the relationship between the nodes as described in figure legends.

Functional analysis of a network identifies the biological functions and/or diseases that were most significant to the genes in the network. Canonical pathways analysis identifies the pathways, from the Ingenuity Pathways Analysis library of canonical pathways, which were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on two parameters as follows: 1) a ratio of the number of genes from the
Hypoacetylated and Hyperacetylated Genes

The primary research question is: how does benzopyrene alter H3K9 histone acetylation? To address this, the authors used the Affymetrix human promoter 1.0R microarray platform to identify gene regulatory regions that are hypo- and hyperacetylated. This single-chip technology is composed of over 4.6 million, 25-bp probes tiled at an average resolution of 35 bp. These probes cover a minimum of 7.5 kb upstream through 2.45 kb downstream of transcription start sites of over 25,500 human promoter regions. Labeled DNA generated from ChIP control and BaP-treated samples was hybridized to the Affymetrix chip, and the IP signals for each sample were normalized by IgG signal subtraction. Statistical detection of the significantly acetylated regions (p < 0.05) in Me2SO- and BaP-treated samples and VENN analysis was performed (Fig. 1c). We detected 775 hypoacetylated and 1456 hyperacetylated gene promoters in response to BaP treatment. There were approximately twice as many hyperacetylated genes compared with hypoacetylated genes as a result of BaP exposure. Also, the majority of significantly acetylated genes in control cells also remained acetylated after BaP treatment (4014 genes in the VENN common region).

Mapping of hypoacetylated and hyperacetylated regions using the Partek Genomic View tool showed a net increase in the number of acetylated genes in BaP-treated cells. We observed a broad distribution of these signals across the genome, as well as a range of diverse signal intensities at particular chromosomal regions (Fig. 2). Annotated gene lists of both hypoacetylated and hyperacetylated genes, including their genomic location, Affymetrix (HGU133_plus 2.0) probe set ID, and mean signal intensity, are provided in supplemental Tables 2a and 3a, respectively. Size, signal intensity, and p values at the significant regions within these gene promoters are also shown in supplemental Tables 2b and 3b, respectively. The larger number of significantly acetylated regions compared with the number of corresponding genes is because of certain genes harboring more than one significantly acetylated region within the tiled 10–12.5-kb target regions.

Gene-specific Verification of Promoter Acetylation—We selected several representative hypoacetylated and hyperacetylated gene targets for further analysis to verify our microarray findings on a gene-specific basis (Table 1). These include chromatin remodeling-associated genes (HDAC1, MBD2, MBD3, METTSD1, and ATRX), genes associated with transcription (NAB2 and TMF1), cancer-associated genes (MTA3, BRMS1L, and BCA3), and DNA damage-associated genes (CYP1B1, GADD45B, and BAX). Six genes were selected on the basis of their hypoacetylated status (MTA3, HDAC1, ATRX, MB2, MBD3, and METTSD1), whereas the remaining genes were identified because of promoter hyperacetylation (NAB2, TMF1, BRMS1L, BCA3, CYP1B1, GADD45B, and BAX). Five of the six chosen hypoacetylated targets are genes with roles related to chromatin remodeling or are involved in transcriptional repression (6), whereas the sixth (METTSD1) encodes a novel, uncharacterized transcript that possesses a putative methyltransferase domain.

We verified the acetylation status of representative hypoacetylated (HDAC1) and hyperacetylated (NAB2) genes as shown in Fig. 3, respectively. Fig. 3 shows a BLAT alignment of the Partek Genomic Suite generated .wig files representing probe-specific, background-corrected signal intensities for each individual probe. The corresponding gene promoter regions and other important genomic elements such as CpG

RESULTS

Probe Generation and Microarray Identification of Hypoacetylated and Hyperacetylated Genes—MCF-7 cells were exposed to 0.5 μM BaP (or as a control to 0.05% Me2SO) for 96 h, as described previously (21), and were immunoprecipitated using H3K9-acetyl-specific (IP) or nonspecific (IgG) antisera. H3K9-acetyl-specific immunoprecipitation (Abcam) was verified by gene-specific PCR amplification of the constitutively active and normally hyperacetylated GAPDH promoter (24) (Fig. 1a). Successful chromatin fragmentation and random-prime amplification of genomic DNA fragments confirmed that high quality DNA was produced for labeling reactions and microarray hybridization (Fig. 1b).

We used the Affymetrix human promoter 1.0R microarray platform to identify gene regulatory regions that are hypo- and hyperacetylated. This single-chip technology is composed of over 4.6 million, 25-bp probes tiled at an average resolution of 35 bp. These probes cover a minimum of 7.5 kb upstream through 2.45 kb downstream of transcription start sites of over 25,500 human promoter regions. Labeled DNA generated from ChIP control and BaP-treated samples was hybridized to the Affymetrix chip, and the IP signals for each sample were normalized by IgG signal subtraction. Statistical detection of the significantly acetylated regions (p < 0.05) in Me2SO- and BaP-treated samples and VENN analysis was performed (Fig. 1c). We detected 775 hypoacetylated and 1456 hyperacetylated gene promoters in response to BaP treatment. There were approximately twice as many hyperacetylated genes compared with hypoacetylated genes as a result of BaP exposure. Also, the majority of significantly acetylated genes in control cells also remained acetylated after BaP treatment (4014 genes in the VENN common region).

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islands or repeat elements are also shown in these figures. The signal intensities for the individual probes in replicate experiments are very similar, demonstrating the high reproducibility of this assay. These alignments, in combination with acetylation status data generated by the Partek Genomic Suite Region View tool (supplemental Figs. 2 and 3), allow for the simple and pre-

![Chromosomal distribution of hypoacetylated and hyperacetylated gene targets, respectively.](image)

**FIGURE 2.** Chromosomal distribution of hypoacetylated and hyperacetylated gene targets, respectively. These heat maps (blue/red scale) represent the mean log₂ intensity of signal in the significantly acetylated region(s) of their corresponding genes, i.e. the average signal intensity for the significant region in a particular gene promoter.

**TABLE 1**

| Chromosome | Start   | End     | Gene title                                             | Gene symbol | Mean of regions | Region(s) | Average p value |
|------------|---------|---------|-------------------------------------------------------|-------------|-----------------|-----------|-----------------|
| Hypoacetylated |         |         |                                                       |             |                 |           |                 |
| 2          | 42760205| 42772813| Metastasis-associated 1 family, member 3              | MTA3        | 0.79            | 3197      | 0.01            |
| 1          | 32250180| 32262679| Histone deacetylase 1                                 | HDAC1       | 0.73            | 84        | 0.02            |
| X          | 75795001| 75805076| α-Thalassemia/mental retardation syndrome X-linked    | ATRX        | 0.78            | 5971      | 0.01            |
| 18         | 50000525| 50013145| Methyl-CpG binding domain protein 2                    | MB2D        | 1.97            | 2856      | 0.01            |
| 11         | 28088646| 28098596| Methyltransferase 5 domain containing 1                | METTD1      | 1.86            | 922       | 0.01            |
| 19         | 15412020| 15536520| Methyl-CpG binding domain protein 3                    | MB2D        | 1.00            | 2871      | 0.01            |
| Hyperacetylated |       |         |                                                       |             |                 |           |                 |
| 12         | 55761656| 55771659| NGFI-A binding protein 2 (EGR1-binding protein 2)      | NAB2        | 3.45            | 1640      | 0.01            |
| 3          | 69031857| 69042002| TATA element modulatory factor 1                      | TMF1        | 3.00            | 4702      | 0.02            |
| 19         | 54139928| 54152447| BCL2-associated X protein                              | BAX         | 0.94            | 3551/2    | 0.01            |
| 14         | 34175655| 34188105| Breast cancer metastasis-suppressor 1-like            | BRMS1L      | 1.71            | 2095      | 0.02            |
| 1          | 93519990| 93529940| Breast cancer anti-estrogen resistance 3              | RCAR2       | 1.29            | 209       | 0.01            |
| 2          | 38275351| 38285327| Cytochrome P450, family 1, subfamily B, polypeptide 1  | CYP1B1      | 0.44            | 3732      | 0.01            |
| 19         | 2417256  | 2429777 | Growth arrest and DNA-damage-inducible, β             | GADD45B     | 1.11            | 3349      | 0.02            |
cise identification of regions of interest within the larger 10-kb promoter regions for ChIP-PCR verification.

Next, we verified by gene-specific chromatin IP the promoter hypoacetylation and hyperacetylation for HDAC1 and NAB2, respectively, along with the 11 other genes shown in Table 1 (Fig. 4). Genes detected as hypoacetylated in our microarray experiments exhibited IP-specific enrichment in the Me2SO controls compared with BaP-treated samples, whereas hyperacetylated gene targets displayed increased IP signals in BaP-treated MCF-7 cells compared with the Me2SO controls. Corresponding BLAT/.wig alignments and Region Views for each of these hypoacetylated and hyperacetylated genes are shown in supplemental Figs. 2 and 3, respectively. Promoter histone acetylation is generally associated with gene activation; therefore, we analyzed the expression status of our differentially acetylated genes in Me2SO- and BaP-treated cells by using real time reverse transcription-PCR (Fig. 4b). BaP exposure-dependent increases in histone acetylation correlated with an increase in gene expression for most genes tested. In contrast, most genes that exhibited decreased acetylation upon BaP exposure, with the exception of MBD2, showed no significant changes in gene expression.

We also performed Ingenuity Pathway analysis to clarify pathways and cellular mechanisms responsive to BaP exposure. Fig. 5a shows biological functions that are affected by changes in histone acetylation of the corresponding genes. The most significant of these biological functions is the regulation of gene expression. Our analyses also identified genes with significant acetylation changes in genes related to canonical pathways (Fig. 5b, supplemental Table 4), with the highest number of differentially acetylated genes (83) in the
extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathway. Furthermore, we identified networks of hyper- and hypoacetylated genes affected by BaP exposure. Fig. 5c shows one such network that included 29 genes with functions related to cell cycle, cell assembly and organization, DNA replication, recombination, and repair. We verified hypoacetylation in five of these genes, including MTA3, HDAC1, ATRX, MBD2, and MBD3 (Fig. 4a), as well as the significant loss of expression in MBD2 (Fig. 4b), which is the most hypoacetylated gene in this network. The top five networks contain genes involved in essential cellular functions, including gene expression, DNA replication and repair, and carcinogenesis (supplemental Table 5, a and b).

DISCUSSION

Microarray approaches have become a tool of choice for large scale toxicogenomic studies (2) with the inherent ability to generate a transcriptional profile of an environmental chemical in a single experiment. Here we undertook a ChIP-on-chip approach to identify genome-wide, environmentally induced histone acetylation changes. Reversible DNA methylation and histone modifications provide an additional level of transcriptional regulation, in their capacity to permit or deny transcription factor access to gene regulatory regions. In fact, these complex epigenetic marks can be considered a “structural adaptation of chromosomal regions so as to register, signal, or perpetuate altered activity states” (25). Such an epigenetic language that presides over the chromatin landscape is only now beginning to be deciphered. However, it is clear that any thorough understanding of environment-genome interactions must take into account the vocabulary of DNA methylation and histone modifications if we are to fully interpret how environmental toxicants elicit their effects.

Our goal was to identify changes in the histone acetylation profile because of the environmental toxicant BaP using the Affymetrix Human Promoter 1.0R microarray platform and chromatin immunoprecipitation to target histone H3K9 acetylation. In contrast to whole genome expression microarrays, these promoter microarrays allow the mapping of sites of protein-DNA interactions that may indicate epigenetic changes associated with gene regulatory regions across the genome.

We have provided the first evidence of genome-wide and gene-specific histone acetylation changes in response to an environmental toxicant. BaP and its cellular metabolite benzo(a)pyrene diol epoxide exert a range of toxic genetic and epigenetic effects that include decreases in global DNA methylation (26), inhibition of DNA methyltransferases in vitro (27), and by interference with recruitment of the methylation machinery (20, 28). In addition, benzo(a)pyrene diol epoxide binding affinity in the context of CpG dinucleotides is increased by cytosine methylation, suggesting a role for BaP metabolites in the deregulation of DNA methylation (29–31). We chose to specifically target histone modifications in our experiments rather than DNA methylation in part because DNA adducts formed by BaP metabolites may bias the ability of microarray-based studies that rely on methylation enzymes to define differential methylation patterns. In particular, BaP metabolites preferentially form adducts at methylated CpGs and have been shown to inhibit methylation-sensitive restriction enzymes that are used to generate differential methylation displays (32).

One challenge presented by any microarray-based technology is the need to assess and evaluate the vast amount of data generated by these arrays as well as the functional relevance of the resulting signals. Histone marks, including acetylation, are signals that disrupt chromatin contacts, dictate higher order chromatin packaging, and orchestrate the recruitment of enzyme complexes that manipulate access to DNA (5). As histone acetylation is associated with an open chromatin configuration that is permissive to transcription, the use of antibodies against acetylated H3K9 allowed us to define modifications that could indicate gene expression changes (5, 6). We identified 775 (hypoacetylated) and 1456 (hyperacetylated) target genes in response to BaP exposure. These targets were distributed across the genome, with some acetylation events concentrated at regions on chromosomes 1p, 4p, 6p, 10q, 11p, 12p, 14q, 15q, 17q, and 19p/q. These regions may represent contiguous regions of chromatin across which complex patterns of histone modifications may be established and maintained (4). Data mining of the microarray results and interfacing with the UCSC Genome Browser data tracks also permitted the visualization and localization of individual tiled signals corresponding to specific CpG islands and gene intron/exon elements, as shown in Fig. 3. We observed high reproducibility between individual replicates at all targets visualized, and we were able to confirm these microarray data with gene-specific ChIP/PCR. Real time PCR was also undertaken to identify functional correlations between acetylation status and gene expression. Positive correlations were identified between gene expression and most of the hyperacetylated targets, whereas expression appeared less well associated with most hypoacetylated targets. These results perhaps reflect the complex mechanisms (both genetic and epigenetic) that can be involved in transcriptional regulation of specific genes. Because specific combinations of histone modifications confer expression status (6), the sites of deacetylated H3K9 that we have identified may be necessary but not sufficient for repression of gene expression of certain genes. As well, nucleosomal stability may be affected by the formation of certain DNA lesions such as BaP-induced adducts, without an immediate direct effect on transcription (33).

A previous report described how in vitro exposure of MCF7 cells to BaP led to expression changes in genes involved in a variety of cell functions and pathways (16). We identified striking similarities between our data and the gene list reported by this group (Table 2). Seven of 18 genes reported by Mahadevan et al. (16) displayed significant acetylation changes in our assays. As well, we identified significant acetylation changes in family members of six other genes previously identified on expression microarrays. Our data agree with this previous work in implicating a broad spectrum of fundamental mechanisms altered by polycyclic aromatic hydrocarbon exposures in cells, rang-
Benzopyrene Alters H3K9 Histone Acetylation

b.

Huntington Disease Signaling 15 37 Total = 229
ERK/MAPK Signaling 23 60 202
Ephrin Signaling 12 28 207
PPARα/RXRα Signaling 11 40 145
Integrin Signaling 16 38 211

-c-log(p-value) vs. percentage
ing from proliferation, apoptosis, and cell cycle regulation to metabolism.

We also performed Ingenuity Pathway Analysis to identify pathways and cellular mechanisms responsive to BaP exposures. Given the role of BaP in DNA adduct formation through the aromatic hydrocarbon receptor pathway, it is not surprising that we identified significant histone acetylation changes in genes and gene networks involved in gene expression, DNA replication and repair, and carcinogenesis (Fig. 5; supplemental Table 5, a and b). We also identified, within these networks, a number of genes involved in the organization and remodeling of chromatin, including MTA3, HDAC1, ATRX, MBD2, and MBD3 (Fig. 5c; supplemental Table 5, a and b). Our laboratory and others have previously shown BaP exposure-related disruptions of DNA methylation profiles (21, 26, 27, 34). In light of our current findings of genome-wide disruption of histone acetylation profiles, it appears that BaP exposure results in genome-wide changes in chromatin at the level of both DNA and histones. Hypoacetylation, as well as the loss of expression of MBD2 (Fig. 4, a and b), is of particular interest as this gene is involved in DNA methylation-dependent transcriptional repression via nucleosome remodeling and histone deacetylation in association with the Mi-NuRD as a part of the MeCP1 complex (35).

This ChIP-on-chip technology has an advantage over conventional microarray-based genome assessments as it can identify sites of interaction between DNA and proteins in specific co-repressor or co-activator complexes (36). These complexes may be involved in controlling gene expression either directly or indirectly by their effects on chromatin organization. Either histone modifications or DNA methylation patterns can be chosen as surrogates for gene expression, but these choices should be made intelligently to avoid bias because of physical changes to the DNA template as described previously. Appropriate targets for chromatin immunoprecipitation would include modified histones (4), such as described in our study, proteins that mark the methylome (including MeCP2 and other methyl binding domain proteins) (6) or 5-methylcytosine itself (37). In addition, the environmental effects of specific endocrine disrupters (38), heavy metals (39), or dietary changes such as folate depletion (40) could lead to stressor-specific profiles that combine genetic and epigenetic alterations (41). In the broader medical context, epigenomic profiles of cancer cells as well as markers of tumor prognosis could be developed, as could whole epigenome displays that predict response to chemotherapy, particularly given the potential reversibility of these histone modifications within cancer cells (6).

In summary, epigenetic patterns have emerged as an essential but environmentally sensitive mechanism regulating gene expression, chromatin organization, and embryonic development. We show first evidence of genome-wide disruption of histone acetylation in response to an environmental carcinogen. Our ChIP-on-chip approach has broad potential for application as a comprehensive, genome-wide profiling of histone modifications. Histone acetylation profiling, in conjunction with expression microarray and other “-omic” technologies, can contribute to a comprehensive portrait of how cells and organisms perceive their environment and how changes in the environment can affect genetic and epigenetic regulation and the response of a cell to those exposures. This approach also has potential applications for profiling epigenetic changes during embryonic development.

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**TABLE 2**

| Gene accession no. | Gene symbol | Gene name | Expression | Gene (average p value) | Related genes (average p value) |
|--------------------|-------------|-----------|------------|------------------------|-------------------------------|
| NM_000499          | CYP1A1      | Cytochrome P450, family 1, subfamily A, polypeptide 1 | 4.7 ± 0.28 | CYP24A1 (0.01)          |                                |
| NM_001924          | GADD45A     | Growth arrest and DNA-damage-inducible, α | 3.1 ± 0.00 | GADD45B (0.01)          |                                |
| NM_000104          | CYP1B1      | Cytochrome P450, family 1, subfamily B, polypeptide 1 | 2.5 ± 0.28 | Yes (0.01)              |                                |
| NM_015516          | EZH2       | Hypothetical protein, estradiol-induced | 2.3 ± 0.35 |                      |                                |
| NM_002083          | GPX2        | Glutathione peroxidase 2 | 2.1 ± 0.14 |                      |                                |
| NM_000903          | NQO1        | NAPDH dehydrogenase, quinone | 2.0 ± 0.07 | Yes (0.01)              |                                |
| NM_015869          | PPARγ       | Peroxisome proliferative activated receptor, γ | 1.7 ± 0.21 |                      | PPARG C1A (0.01); CIB (0.01) |
| NM_004633          | UGT1A10     | UDP-glucuronosyltransferase 1 family, polypeptide A10 | 1.7 ± 0.14 | Yes (0.02)              |                                |
| NM_001355          | AKR1C1      | Aldo-keto reductase family 1, member C1 | 1.7 ± 0.07 |                      |                                |
| U05598             | AKR1C2      | Aldo-keto reductase family 1, member C2 | 1.6 ± 0.07 | Yes (0.01)              |                                |
| NM_003899          | CDKN1A      | Cyclin-dependent kinase inhibitor 1A (p21, Cip1) | 1.5 ± 0.00 |                      |                                |
| NM_0000120         | EPHX1       | Epoxide hydrolase 1 | 1.4 ± 0.07 | EPH4 (0.01); A1 (0.02); B4 (0.01) | BCL2L1 (0.04); 9L (0.02); 10 (0.01) |
| AA029050          | BCL2L1 Europeans | BCL2-like 1 (apoptosis facilitator) | 1.3 ± 0.21 |                      |                                |
| U19599             | BAX         | BCL2-associated X protein | 1.3 ± 0.00 | Yes (0.01)              |                                |
| X06399             | CYP2B6 Europeans | Cytochrome P450, family 2, subfamily B, polypeptide 6 | 1.1 ± 0.14 |                      |                                |
| NM_001066          | TNFRSF1A    | Tumor necrosis factor receptor superfamily, member 1A | 1.1 ± 0.00 | TNFRSF12A (0.01)        |                                |
| NM_013370          | ORL1B       | Pregnancy-induced growth inhibitor | 1.0 ± 0.00 | Yes (0.01)              |                                |
| NM_000107          | DDB2        | Damage-specific DNA-binding protein 2 | 1.0 ± 0.00 |                      |                                |

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**FIGURE 5.** a, top eight biological functions of differentially acetylated genes in response to BaP exposure. The horizontal line (T) indicates the threshold of –log(p value) greater than 2.0. The relative numbers of hypo- and hyperacetylated genes are shown for each category. The acetylation status of genes comprising the pathways is presented in the histograms, b, canonical pathways analysis identified the top five pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to our data set. For each canonical pathway, hypo- (yellow) and hyperacetylated (blue) genes are represented by the corresponding bars. The percent of genes per pathway is shown on the bottom axis, and significance (square data points) can be read from the bottom axis. Fisher’s exact test was used to calculate a p value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone. c, network of BaP exposure-related differentially acetylated genes centered around HDAC1. Node (gene) symbols were described in the bottom part of the figure. The intensity of the node color indicated the degree of hyper- (blue) or hypoacetylation (yellow). The biological functions that were most significant to the genes in this network included cell cycle, cell assembly and organization, DNA replication, recombination, and repair.
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