Identification of Transcription Factors and Coactivators Affected by Dibutylphthalate Interactions in Fetal Rat Testes

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Previous analysis of in utero dibutylphthalate (DBP)-exposed fetal rat testes indicated that DBP’s antiandrogenic effects were mediated, in part, by indirect inhibition of steroidogenic factor 1 (SF1), suggesting that peroxisome proliferator–activated receptor alpha (PPARα) might be involved through coactivator (CREB-binding protein [CBP]) sequestration. To test this hypothesis, we have performed chromatin immunoprecipitation (ChIP) microarray analysis to assess the DNA binding of PPARα, SF1, CBP, and RNA polymerase II in DBP-induced testicular maldevelopment target genes. Pathway analysis of expression array data in fetal rat testes examined at gestational day (GD) 15, 17, or 19 indicated that lipid metabolism genes regulated by SF1 and PPARα, respectively, were overrepresented, and the time dependency of changes to PPARα-regulated lipid metabolism genes correlated with DBP-mediated repression of SF1-regulated steroidogenesis genes. ChIP microarrays were used to investigate whether DBP-mediated repression of SF1-regulated genes was associated with changes in SF1 binding to genes involved in DBP-induced testicular maldevelopment. DBP treatment caused reductions in SF1 binding in CYP11a, STAR, and CYP17a. Follicle-stimulating hormone receptor (FSHR), regulated by SF1 but unaffected by DBP-treatment, also contained SF1-binding peaks, but DBP did not change this compared with control. GD15 and GD19 fetal testes contained PPARα protein–binding peaks in CYP11a, STAR, and CYP17a regulatory regions. In contrast to its repressive effect on SF1, DBP treatment caused increases in these peaks compared with control. PPARα-binding peaks in the FSHR promoter were not detected in GD15 samples. Hence, the repressive effect of DBP on SF1-regulated steroidogenic genes correlates with inhibition of SF1-DNA binding and increased PPARα-DNA binding. The data indicate that PPARα may act as an indirect transrepressor of SF1 on steroidogenic genes in fetal rat testes in response to DBP treatment.

Key Words: transcription; steroidogenesis; dibutylphthalate; testes; dysgenesis.

In utero exposure of male rats to dibutylphthalate (DBP) has been shown to induce reproductive tract abnormalities, due, in part, to decreased testosterone synthesis as a result of gene expression changes in pathways that regulate the synthesis of this hormone (Gray et al., 2000; Liu et al., 2005; Parks et al., 2000). Many of the effects of DBP relevant to inhibition of steroid biosynthesis are thought to occur in the Leydig cells (Liu et al., 2005; Plummer et al., 2007). There is also evidence that in utero DBP treatment can perturb Sertoli cell and gonocyte development (Johnson et al., 2007; Mahood et al., 2005). The possibility that DBP might affect human reproductive development has focused attention on the necessity to understand mechanisms of DBP-induced testicular maldevelopment in the rat and human in the hope that greater understanding of these mechanisms will lead to better tools for risk assessment of this chemical.

This work was dedicated to the memory of our colleague and friend, the late Dr. Sheila MacPherson. We wish to acknowledge the help of the staff at our respective laboratories and the continued support of our funding bodies. A full list of the funding sources supporting this work is available in the online version of this article.
PPARα could therefore starve SF1 of a cofactor essential for its transactivation functions.

The study was designed to test a hypothesis derived from our previous investigation (Plummer et al., 2007) that high doses of DBP affect transcription factor (SF1 and PPARα) and coactivator (CBP) binding to genes involved in DITMD by ChIP-on-chip microarray analysis. We also measured RNA polymerase II (RNA polII) binding to the coding regions of DBP-induced testicular maldevelopment target genes in order to assess how their rate of transcription is affected by DBP treatment. The main aim of this study was to investigate the idea that PPARα is involved in the indirect inhibitory effects of DBP on SF1-regulated steroidogenic genes in fetal rat testes.

MATERIALS AND METHODS

Animals and treatments. Time-mated female Wistar rats (Harlan) aged 10–12 weeks were treated once daily from gestational day (GD) 12 with vehicle (control—corn oil) or DBP at 500 mg/kg (CAS no. 84-74-2, Sigma-Aldrich Co. Ltd, Dorset) in 1 ml/kg corn oil, by oral gavage, until the day prior to sample (Plummer et al., 2007). The time (day) of mating was determined by the presence of a vaginal plug, and this day was designated GD1. The DBP administered was 99% pure according to the supplier. The housing, handling, and treatment of the animals were carried out according to U.K. Home Office guidelines. At the end of the study, either GD15 or GD19, dams were killed 2 h after the last dose of DBP by exposure to an increasing concentration of CO2. Litters were removed and fetuses placed in ice-cold PBS prior to microdissection. Fetal testes were carefully retrieved using a dissection microscope (Leica CMS x100) and either snap frozen in liquid nitrogen or fixed in Bouin’s as previously described (Plummer et al., 2007).

Immunohistochemical staining. In order to confirm that the transcription factors of interest were present in the fetal testis tissue at both ages of interest, immunohistochemistry was conducted for PPARα on GD15 fetal testis tissue samples. GD15 fetal testes from vehicle-exposed litters were fixed in Bouin’s for 2 h, transferred to 70% ethanol, and stored at 4°C. The fixed tissue was processed and embedded into paraffin blocks. Sections of 10μM were dewaxed and rehydrated before being blocked in methanol containing 3% H2O2, for 20 min at room temperature. After rehydration, sections were washed in deionized water for 5 min followed by Tris-buffered saline (TBS) for 5 min before being blocked in 20% normal donkey serum (NDS)/TBS/5% bovine serum albumin (BSA), 20 min at room temperature. Samples were incubated overnight at 4°C with PPARα antibody (Abcam; ab24509) diluted to 1:500 in NDS/TBS/BSA. Sections were washed 2 × 5 min in TBS before incubation with donkey anti-rabbit peroxidase diluted to 1:750 in NDS/TBS/BSA, 30 min at room temperature. Unbound antibody was washed (2 × 5 min) in TBS prior to addition of tyramide-fluorescein isothiocyanate (FITC) diluted to 1:50 for 10 min. Sections were washed 2 × 5 min in TBS before being subjected to pressure cooking for 5 min on full heat. Tap water was used to cool the slides at the end of cooking. Sections were blocked in 20% NDS/TBS/5% BSA for 30 min at room temperature prior to overnight incubation at 4°C with 3μg/ml of Active Motif Chip-IT Express kit (Cat no. 053009). Glycine stop fixation solution was used to stop the fixation reactions. The fixed testes were lysed and their chromatin fragmented using a Bioruptor (Diagenode).

The quality of the chromatin fragmentation procedure was checked using 1% agarose gel electrophoresis. The fragmented DNA size was shown to be between 100 and 500 bp when assessed against DNA molecular weight markers in the gel (data not shown).

Four different antibodies were used for ChIP. The antibodies used for the ChIP reactions were raised against the following proteins: RNA polIII (Active Motif-39097), SF1 (Upstate Millipore 07-618), CBP (Santa Cruz Biotechnology Ltd no. SC-369), and PPARα (Sigma P0669-100UG).

Eight testes from different litters from vehicle- and DBP-treated dams were sampled at GD15 and GD19, corresponding to four biological replicates per time point or treatment, and analyzed using ChIP microarrays. Fragmented DNA samples were divided into four 90 μl aliquots of immunoprecipitated (IP) DNA samples and four 20 μl aliquots of input DNA samples. IP DNA samples were immunoprecipitated with the antibodies using the Active Motif ChIP-IT Express kit (Cat no. 053009) according to the kit method. Input samples were stored at −70°C prior to DNA purification. The IP DNA samples were reverse crosslinked and treated with proteinase K. The genomic (input) DNA was also treated with proteinase K, and both IP and input samples were purified using a DNeasy blood and tissue cleanup kit (Qiagen). The samples were eluted in a volume of 20 μl, and 5 μl of this sample was amplified using the Sigma WGA4 kit (described subsequently).

Whole-genome amplification of the IP and input DNA. Chromatin IP and input DNA samples were amplified before labeling and microarray hybridization. A whole-genome amplification kit (WGA4, Sigma) was used to amplify the IP and input DNA to produce a quantity of DNA suitable for labeling and hybridization on the microarrays, according to the kit method.

Expression microarray data analysis. Expression microarray data derived from a previously published study (Plummer et al., 2007) were reanalyzed using Rosetta Resolver 6 software to define a list of significantly altered genes (the “Signature List”). Resolver software calculates a p value based on an error-weighted mean of the data for the different replicates. The Rosetta error model (Weng et al., 2006) calculates both the propagated (technical) and scattered (biological) error and combines these two errors to make a more reliable error estimation. The p value allows an estimation of the confidence that a given feature/gene is not significantly different (the null hypothesis) relative to the reference. A p value < 0.01 was considered significant. Rosetta Resolver trends analysis was used to identify genes displaying similar patterns of alteration across time.

Ingenuity Pathways Analysis (IPA) Network analysis and Functional Pathways representation analysis were used to examine bias in lists of genes significantly altered by the treatment toward particular biological functions or pathways.

ChIP, purification, and quality control. ChIP was performed as the first step in a process designed to test whether DNA sequences in the promoter regions of DITMD “target” genes were differentially bound by the transcription factors (SF1 and PPARα) and coactivators/transcription machinery proteins (CBP and RNA polII) in response to the treatment. These antibodies have been previously validated for ChIP (Ferraz-de-Souza et al., 2011, Saramaki et al., 2009; Active Motif instructions for product no. 39097). Briefly, eight testes from each litter were pooled and used to prepare samples for chromatin fragmentation for each treatment (control and DBP treated) and each time point (GD-15 and GD-19) in quadruplicate (four biological replicates). Frozen testis samples were fixed or crosslinked with formaldehyde solution according to the Active Motif Chip-IT Express kit (Cat no. 053009) method. Glycine stop fixation solution was used to stop the fixation reactions. The fixed testes were lysed and their chromatin fragmented using a Bioruptor (Diagenode).

The quality of the chromatin fragmentation procedure was checked using 1% agarose gel electrophoresis. The fragmented DNA size was shown to be between 100 and 500 bp when assessed against DNA molecular weight markers in the gel (data not shown).

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ChIP microarray design. Agilent custom Rat ChIP-on-chip microarray slides 8 × 15k (G4499A, AMADID-023386) designed using Agilent eArray software were used for the study. Briefly, the UCSC Genome browser was used to retrieve transcription start (TS) sites for DITMD “hypothesis” genes. Excel
software was used to generate list of chromosome coordinates 5.5kb upstream (promoters) and 2kb downstream (coding regions) of the TS site. Agilent Array software was used to perform a probe search within the chromosome coordinates and to generate a probe list from the Agilent database at 100bp spacing. The UCSC genome browser was used to confirm binding of probes in the promoter regions of target genes.

**DNA labeling and ChIP microarray analysis.** ChIP microarray analysis involved two color labeling of DNA (Cy5-labeled IP DNA and Cy3-labeled input DNA) derived from immunoprecipitations with four different antibodies of four control (corn oil) and four DBP-treated litters, each at two time points (4 x 2 x 4 x 2 = 64 arrays).

Amplified IP and input DNA of 1.3 µg (13 µl) were fluorescently labeled using a Genomic DNA Labeling kit plus (Agilent no. 5188-5309). The amplified IP DNA samples were labeled using Cy5-labeled nucleotides (red channel) and the input (genomic) DNA samples were labeled with Cy3-labeled nucleotides (green channel). The labeled DNA samples were purified using “Microcon filters.” Agilent 8 x 15 K Rat custom Chip-on chip arrays (Amadid no. 023386) were hybridized, washed, and scanned using an “Agilent Microarray Scanner.” All methods used followed the “Agilent Chip-on-chip microarray analysis.”

**ChIP microarray data processing and bioinformatics.** Images from the scanner were processed using Agilent Feature Extraction Software v 9.5 to generate raw data (.txt files). These Cy5/Cy3 ratio data were used for subsequent statistical analysis. Peaks (DNA-protein binding events) constituting collections of a minimum of two probes with significantly raised Cy5/Cy3 ratios were screened for and detected using the Whitehead per-array neighborhood peak detection algorithm by Agilent ChIP analytics software. The algorithm scores peaks by performing a random sampling of the data and determining the likelihood/probability that a putative peak found in the data could have been identified by chance. A binding event was considered significant if the mean p values of the peak probe and its two neighbors pbar were less than 0.05 (pbar < 0.05). Perr is calculated from the mean of the ChIP analytics error model–derived p value of the central probe and its two neighbors. Peaks were reviewed or visualized according to their location in the promoters of target genes in order to assess to which region(s) of the DBP-induced testicular maldevelopment promoter(s) the gene regulatory protein was bound. In order to assess the effects of DBP on DNA-protein binding, we used ChIP analytics software to generate a mean of the data from each of the four replicates for each of the two treatments (control and DBP). Student’s t-test was used to assess whether there were significant differences between peak-probe Cy5/Cy3 ratios between control and DBP-treated samples. Probes on the ChIP array that were contained in peaks were also tested for homology with the SFI and PPARG “consensus” binding sequences, AAGGTCA and AGGTCAANNAGGTCA, respectively, using the EMBOSS alignment tool from the European Bioinformatics Lab (EBL) website. The microarray data for this study are held publically in the NCBI Geo database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE39225.

**Expression microarray data processing and bioinformatics.** A Lowess curve was fitted to the log-intensity versus log-ratio plot, and 20.0% of the data were used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control channel was lower than 10, then 10 was used instead.

**RESULTS**

**Expression Array Analysis**

Previously derived expression microarray data (Plummer et al., 2007) were reanalyzed using Rosetta Resolver and GeneSpring software and IPA software. Our previous analysis of the data using Rosetta luminator software (Plummer et al., 2007) had indicated that steroidogenic genes CYP11a and CYP17a were not significantly regulated at GD15; however, the Resolver reanalysis showed that these genes were significantly (p < 0.01) downregulated (~1.5-fold) at GD15 (Supplementary table 1). The IPA of GeneSpring multiple test–corrected differentially expressed gene lists showed that the functional pathway of lipid metabolism was the top-ranked overrepresented one at GD15 and GD19 (Fig. 1; Supplementary data).

Examination of the genes in the lipid metabolism functional pathway showed that the majority were induced at GD15 and repressed at GD19 (Supplementary table 1). Closer review of the lipid metabolism genes repressed at GD19 highlighted that many, including genes involved in steroidogenesis, were regulated by SFI. However, at GD15, several of the lipid metabolism genes were involved in mitochondrial β oxidation and lipid transport pathways and regulated by PPARG.

Trends analysis of steroidogenic and lipid metabolism genes across time showed that the dynamics of changes to genes in these pathways was strikingly similar indicating the possibility that they may be coregulated (Fig. 2).

**ChIP microarray analysis (GD15 and GD19)**

**SFI.** To investigate a possible relationship between the antiandrogenic effects of DBP and the binding of SFI to steroidogenic gene promoters, binding events (peaks) of SFI protein to SFI-regulated gene promoters in GD 19 fetal testes were assessed using a custom ChIP microarray designed...
FIG. 1. IPA representation analysis histogram showing the top 10 functional pathways overrepresented in the signature lists (a) GD15 and (b) GD19.
against genes altered by DBP in the lipid metabolism/steroidogenesis pathways (Methods section). Peaks for SF1 binding were found in three genes in the steroidogenic cascade as follows: cytochrome P450, family 11, and subfamily A (CYP11a), \( p_{\text{qbar}} < 0.01 \), in the promoter and first intron (Figs. 3a and 3b), respectively; steroidogenic acute regulator (STAR), \( p_{\text{qbar}} < 0.05 \) (Fig. 4), cytochrome P450, family 17, and subfamily A (CYP17a) (Table 2). Probes on the ChIP array that were contained in the peaks were tested for homology with the SF1 “consensus” binding sequence (AAGGTCGA) (Chou et al., 1996) using the EMBOSS alignment tool (Table 2). Peak probes in CYP11a, STAR, and CYP17a contained sequences that “matched” the SF1 consensus sequence with 70–85% homology consistent with the idea that they are SF1-binding sites (Table 2). DBP-treatment caused a significant \( (p < 0.05) \) reduction in peak probe intensity ratios (Cy5/Cy3 ratios) for SF1 binding compared with control for CYP11a, STAR, and CYP17a (Figs. 3a, 3b, and 4 and Table 2).

Follicle-stimulating hormone receptor (FSHR) previously shown to be regulated by SF1 (Heckert, 2001) was found to contain SF1-binding peaks. However, its expression was unaffected by DBP treatment in our expression microarray data.

Consistent with its lack of effect on FSHR expression, DBP also did not cause any significant changes to the height of the SF1-binding peaks in the promoter of this gene (Fig. 5 and Table 2).

No SF1 peaks were detected in the promoters of the inhibit alpha (INHA), insulin like 3 (INSL3), scavenger receptor, class B, member 1 (SCARB1), and hydroxymethylglutaryl coenzyme a synthetase/reductase (HMGC/S/R) genes at GD19 (data not shown). We checked the effects of DBP treatment on probes that correspond to a previously published SF1 regulatory binding site in the INHA, INSL3, SCARB1, and HMGC/S genes (Ito et al., 2000; Lopez et al., 1999; Mascaro et al., 2000; Zimmermann et al., 1998). Analysis of INHA showed that DBP caused a reduction in the log-ratio value of a probe in the main SF1 regulatory binding site (~83 bp for TS site), which approached significance \( (p < 0.06) \) (Table 2). Although no peak was detected (NB: requires two or more probes to be significantly \( [p_{\text{qbar}} < 0.05] \) above background), this indicates that DBP likely inhibits SF1 binding at this key site in the INHA gene. We also checked probe coverage for literature-reported SF1 regulatory binding sites in the INSL3, SCARB1, and HMGC/S promoters. The SF1 regulatory binding sites for these genes were found not to be covered by our ChIP microarray probes; hence, no conclusion could be drawn from these data on the potential impact of DBP exposure on SF1 binding for these genes.

In GD15 fetal testes, there were no SF1 protein–binding peaks in STAR (data not shown), suggesting that SF1 activation of this gene is not optimal at this time point. This is consistent with a minimal repressive effect of DBP on expression of this gene at this time point compared with later time points (Supplementary table 1).

PPAR\( \alpha \) We initially tested the PPAR\( \alpha \) antibody for its ability to immunoprecipitate PPAR\( \alpha \)/DNA chromatin complexes in the context of our ChIP-on-chip array using rat hepatocytes exposed to Wyeth 14,643 (Wy) as a positive control. In this validation experiment, we examined the binding of PPAR\( \alpha \) to the promoter region of the canonical PPAR\( \alpha \)-regulated gene acyl CoA oxidase 1 (ACOX1). We found that treatment of rat hepatocytes with a concentration of Wy (10\( \mu \)M), previously reported to induce fatty acid coenzyme oxidase in rat hepatocytes (Tamasi et al., 2008), induced ACOX1 mRNA expression (13-fold) and caused a significant PPAR\( \alpha \)-binding peak in the ACOX1 promoter to a site ~600 bp from the ACOX1 TS site (Supplementary fig. 1) previously identified by Tugwood et al. (1992). Hence, the corroboration of a previously identified PPAR\( \alpha \)-binding site in rat hepatocytes with the PPAR\( \alpha \) antibody (Sigma PO869) indicates that this antibody can detect PPAR\( \alpha \) protein/DNA binding in the context of our ChIP-on-chip array assay and is thus fit to use for this purpose.

Fetal testes contained significant \( (p_{\text{qbar}} < 0.01) \) PPAR\( \alpha \) protein–binding peaks in Acs11 at GD15 (Fig. 6a), CYP11a at GD15 (not shown) and GD19 (Fig. 6b), and STAR at GD15 (Table 2). Homology matching in the PPAR\( \alpha \) peak probes showed they contained sequences ~70% homologous to the PPAR\( \alpha \) “consensus” binding sequence (AGGTTCAGTCA) (Ilpenberg et al., 1997; Table 2). A search for PPAR\( \alpha \) consensus binding sites in the Acs11 gene identified a cluster of PPAR\( \alpha \) consensus binding sites in the boundary between the first exon and first intron, i.e., in the same region where the Acs11 DBP-induced PPAR\( \alpha \) peak was detected (Fig. 7).

In contrast to its effect on SF1, DBP treatment caused significant \( (p < 0.05) \) increases in the height (Cy5/Cy3 ratio) of these peaks compared with control (Table 2). We also detected PPAR\( \alpha \)-binding peaks for CYP17a, INHA, SCARB1, and HMGC/S (data not shown). As there was no detection of SF1 regulatory binding peaks for SCARB1 and HMGC/S in control
FIG. 3. SF1 binding to the (a) CYP11a promoter and (b) first intron in control (green lines) and in utero DBP-exposed (red lines) GD19 fetal rat testes. The top panel of (a) shows visualization of probe Cy5/Cy3 intensity ratios for each of four replicates of control and DBP-treated samples in the region of the CYP11a promoter. The bottom panel of (a) shows mean data from the four replicates for each treatment. SF1 protein–binding peaks, detected using the Whitehead algorithm (Methods section), are highlighted in orange. The x-axis represents the genomic position of probes in the CYP11a promoter or gene body. The y-axis is the Cy5/Cy3 fluorescence log intensity ratio (binding signal). The green and red lines represent log ratios of probes in the control and DBP-treated samples, respectively. Probes in the peaks were searched for homology to a consensus binding sequence for SF1. A probe in the promoter peak contained a “consensus” SF1-binding sequence matched at ~70% homology (red), which supports the contention that SF1 binds to this DNA region.
FIG. 4. Binding of SF1 to the StAR promoter in control (green line) and in utero DBP-exposed (red line) GD19 fetal rat testes. Orange boxes show significant peaks detected using the Whitehead peak detection algorithm. The x-axis represents the genomic position of probes (crosses). The y-axis shows the Cy5/Cy3 fluorescence log intensity ratio. DBP treatment inhibits binding of SF1 to the StAR promoter. Results are the mean of data for the four replicate treatments.

TABLE 2
Intensity Ratio (Cy5/Cy3) Data for Probes Contained in Binding Peaks for SF1 and PPARα in SF1-Transactivated Gene Promoters
Printed on the Agilent ChIP-on-Chip Microarray

<table>
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<tr>
<th>ChIP Ab/Gene name</th>
<th>Probe name</th>
<th>Consensus sequence similarity</th>
<th>Location</th>
<th>Cy5/Cy3 ratio mean</th>
<th>SD</th>
<th>Cy5/Cy3 ratio mean</th>
<th>SD</th>
<th>p value</th>
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<tr>
<td>SF1</td>
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<td>0.20</td>
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<td>0.76</td>
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<td>0.3</td>
<td>0.04</td>
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Note: The table shows fluorescence intensity signals for probes contained in SF1, PPARα, or RNA polII–protein binding peaks in DITMD “target” genes. The data for each probe are represented as ratios between the Cy5 and Cy3 mean signals (Cy5/Cy3). The Cy5 and Cy3 signals represent the intensity of hybridization of IP and input DNA for each treatment sample on a probe on the array. Peaks were identified in Agilent DNA analytics software using the Whitehead peak detection algorithm (Methods section). The sequence of each probe contained in SF1 and PPARα peaks was matched with a “consensus” DNA-binding sequence of these two transcription factors (Chou et al., 1996; Ijpenberg et al., 1997; Neve et al., 2000), respectively, to generate a % homology value. The distance of the probe upstream from the TS site is shown in column 4 (location). Results are means ± SD of four biological replicate data points per treatment. A Student’s t-test was used to calculate a p value for each probe. P values < 0.05, representing lower than 5% probability that a difference between the two treatments occurred by chance, were considered significant.
samples, it was not possible to assess whether this transcription factor was affected by DBP treatment. We did not detect any differences in PPARα binding in the promoter region of FSHR (Fig. 8) consistent with a lack of effect of DBP on this SF1-regulated gene. Hence, increased binding of PPARα to SF1-regulated gene promoters in steroidogenesis genes repressed by DBP treatment (CYP11a, StAR, CYP17a, and INHA) correlates with inhibition of binding of SF1 in these regions.

CBP. No CBP-binding peaks were detected by probes contained in the SF1-binding peaks for CYP11a, StAR, or CYP17a in either control or DBP-treated samples. By contrast, peak detection analysis of the CYP11a and Acsl1 genes showed CBP peaks in the same location as that for PPARα binding at GD19 and GD15 (not shown), respectively (Fig. 9 and Supplementary table 2).

RNA polII. RNA polII is an essential part of the eukaryotic transcriptional machinery, and transcriptional rate of genes is correlated to the number of RNA polII molecules associated with gene coding regions in DNA. Hence, we assessed RNA polII binding in the coding regions of DITMD “target” genes in order to assess whether the treatment affected their transcriptional rate.

We detected increased RNA polII binding downstream of the TS site in the CYP11a gene in control GD19 fetal testes. This binding was inhibited by DBP treatment (Table 2). Increased RNA polII binding was also detected in StAR; however, this occurred upstream of the TS site (Table 2). We did not detect RNA polII binding up- or downstream of the TS site for CYP17a at GD19. As it seems likely that CYP17a is actively transcribed by RNA polII at this time because CYP17a expression is required for fetal testis steroidogenesis, we conclude that this is not reflected by our analysis of RNA polII-DNA binding at this time (data not shown). The reason for the discrepancy is not clear but may reflect heterogeneity in the kinetics of steroidogenesis gene transactivation in the testes over this period.

No RNA polII peaks were detected for INHA, SCARB1, and INSL3. There were RNA polII peaks for HMGCS downstream of the TS site that had higher mean log-ratio values in control compared with DBP-treated samples. However, this difference was not reflected by significant differences in the log ratios of probes in these peaks (data not shown).

PPARα immunostaining. PPARα was expressed in the interstitial region of the GD15 testis, with little or no detectable expression in seminiferous cords (Fig. 10a). We also detected PPARα expression in the interstitial region at GD19 (data not shown). The expression of PPARα was dispersed throughout the interstitial region, but some areas displayed higher levels of expression than others. There was some colocalization of PPARα staining and 3βHSD staining (Leydig cell marker; Fisher et al., 2003) in cells in the interstitial region. A small number of Leydig cells showed only 3βHSD staining; however, the majority of interstitial cells stained weakly for 3βHSD (Fig. 10b).

Higher magnification images showed that PPARα protein in interstitial regions of the testes was partially colocalized as shown by dual staining for 3βHSD and PPARα (Fig. 10b). As there were a large number of interstitial cells that showed only PPARα staining, this indicates that PPARα expression was not confined to differentiated fetal Leydig cells.

DISCUSSION

Expression microarray pathway analysis and ChIP microarray analysis were employed to investigate the potential effects of DBP on binding of selected transcription factors to genes
postulated to be involved in the mechanism(s) of DBP-induced testicular maldevelopment. This analysis facilitated examination of DNA binding to genes that we had previously found to be altered in fetal rat testis tissue (Plummer et al., 2007). Inhibition by DBP of transcription factor SF1-regulated gene expression correlated with inhibition of SF1-DNA binding to the promoters of these genes, suggesting that the effects of DBP could be mediated by SF1 transrepression. These data suggest that the effects of DBP on steroidogenesis gene expression are partly mediated by inhibition of SF1 DNA binding in the promoter regions of CYP11a, StAR, CYP17a, and INHA. This correlates with existing data that showed that DBP inhibited SF1 binding to DNA regions from the CYP11a, StAR, and CYP17a promoters (Kuhl et al., 2007). These investigators postulated that because “inactive” phthalate compounds (e.g., diethylphthalate) also caused reductions in SF1 binding to these genes, this transcription factor could not be solely responsible for the repressive effects of “active” phthalates, e.g., DBP.

**FIG. 6.** PPARα binding to (a) Acs1l (first intron) and (b) CYP11a (promoter) in control (green line) and in utero DBP exposed (red line) in GD15 and GD19 fetal rat testes, respectively. The orange boxes show significant peaks detected using the Whitehead peak detection algorithm. The x-axis represents the genomic position of probes. The y-axis shows the Cy5/Cy3 fluorescence log intensity ratio (binding signal). The green and red lines represent log ratios of probes in the control and DBP-treated samples, respectively. PPARα binding is increased by DBP treatment relative to control. Results are the mean of data for four replicates per treatment.
on steroidogenesis genes. However, the present data support the contention that SF1 is a “target” for DBP-mediated repression of certain steroidogenesis genes. These data have shown that DBP inhibits SF1 binding in the promoters and introns of SF1-transactivated genes that are downregulated by DBP at the mRNA level (CYP11a, StAR, and CYP17a). Previous studies have shown that nuclear hormone receptor (NHR)-regulated genes show binding of these transcription factors to intronic regions (Dong et al., 2009), and intronic regulatory sites appear to be common for several NHRs.

The binding of SF1 to the SF1-transactivated gene promoter of FSHR, which was unaffected by DBP at the mRNA level (Plummer et al., 2007), was not inhibited by DBP in this study. Hence, the effect of DBP on SF1-regulated steroidogenesis genes in fetal testes correlates with inhibition of SF1 binding to their promoters. This supports the hypothesis that the effects of DBP on SF1 are indirect as not all SF1 regulated genes are affected by DBP exposure. These data also support the contention that the repressive effects of DBP on SF1-regulated steroidogenic genes are mediated, in part, via inhibition of SF1-DNA binding to steroidogenic promoters. This contention is contradictory to the recent work of Kuhl et al. (2007) who postulated that c/ebp rather than SF1 is a target for the antiandrogenic effects of DBP (Kuhl et al., 2007). These investigators also suggested that SF1 does not play an important role in steroidogenesis in the rat, a suggestion that is incompatible with data indicating that SF1 is required for the expression of StAR (Sandhoff et al., 1998), a protein that is rate limiting for steroidogenesis in rats (Manna and Stocco, 2005).

Data from investigations of SF1 conditional knockout mice indicate that SF1 is required for fetal testis steroidogenesis and StAR expression in mice (Jeyasuria et al., 2004). In this regard, it is interesting that fetal mice are refractory to the antiandrogenic effects of certain phthalates (Gaido et al., 2007). Because the effects of DBP appear indirect (Plummer et al., 2007), it seems possible that mice, unlike rats, lack a “target” that mediates the antiandrogenic effects of DBP in fetal testes. It is not known whether fetal mice or humans express PPARα in testes although adult human testes do express PPARα (Schultz et al., 1999). In this regard, it is interesting that diethylhexylphthalate (DEHP) has recently been shown to inhibit steroidogenesis in adult human testis explants (Desdoits-Lethimonier et al., 2012).

FIG. 7. Homology mapping of PPARα “consensus” binding sites in a region of the Acsl1 gene 5.5 kb upstream of the TS site to 2 kb downstream of the TS site. The red circle shows a cluster of PPARα consensus binding sites in the same region where a DBP-induced PPARα-binding peak was detected (Fig. 6a).

FIG. 8. Lack of binding peaks of PPARα on the FSHR promoter in control (green line) and in utero DBP-exposed (red line) GD15 fetal rat testes. Results represent the mean of four biological replicates per treatment. The x-axis represents the genomic position of probes (crosses). The y-axis shows the Cy5/Cy3 fluorescence log intensity ratio. The binding of PPARα to the FSHR promoter was not significantly altered by DBP treatment. Results are the mean of data for the four replicate treatments.
The profile of DBP-mediated changes to SF1-regulated steroidogenic genes across time was similar to the profiles of change taking place in PPARα-regulated lipid metabolism genes. The inhibitory effects of DBP on the binding of SF1 to the SF1-regulated steroidogenesis genes correlated with changes in the binding of PPARα, a nuclear receptor known to be activated by DBP (Hurst and Waxman, 2003). There are in vitro data suggesting that PPARα is involved in the antiandrogenic effects of phthalates on Leydig cells (Gazouli et al., 2002), and it has been suggested that PPARα could also be involved in the in vivo effects of these compounds on steroidogenesis in testes (Corton and Lapinskas, 2005). In this study, SF1-transactivated steroidogenesis genes inhibited by DBP treatment showed DBP-mediated increases in PPARα binding. There were also coincident DBP-mediated increases in CBP binding to some of the PPARα-DNA-binding sites.

The effects of DBP on PPARα in fetal testes and the effects of DBP on SF1 gene expression and SF1 binding in DITMD promoters are related in that increased PPARα binding correlated with inhibition of SF1 binding. DBP-mediated increases in PPARα binding also correlated with DBP-mediated SF1-regulated steroidogenic gene repression. We did not detect the binding of PPARα in the promoter of FSHR. These results are consistent with a lack of DBP-treatment-mediated repression of FSHR at the mRNA level (Plummer et al., 2007) and support the contention that PPARα binding could be involved in SF1-transregulated gene repression in certain DBP-induced testicular maldevelopment “target” genes.

DBP-mediated increases in binding of PPARα to steroidogenic genes occur at binding sites different from those of SF1, indicating that PPARα may be an indirect repressor of SF1 binding. This suggests that PPARα is an indirect transrepressor of these SF1-regulated genes. It is possible that PPARα could act via sequestration of the shared coactivator CBP. CBP-binding peaks were detected in CYP11a and Acs1 coincident to PPARα-binding sites consistent with the fact that CBP forms part of a transcriptional complex with PPARα and is required for PPARα transactivation (Misra et al., 2002). This indicates that CBP is associated with PPARα and that sequestration of this coactivator could partly explain the repressive effects of DBP on SF1. Another possible explanation for the PPARα effects could be the involvement of a direct SF1 transrepressor. Possible candidates for this are DAX (Yu et al., 1996) and COUP-TF2 (Martin and Tremblay, 2010), a direct repressor of SF1 (Yu et al., 1996). COUP-TF2 also transrepresses PPARα by directly binding to a PPRE in the malic enzyme and fatty acyl-CoA dehydrogenase promoters (Baes et al., 1995, Miyata et al., 1993). The COUP-TF2 promoter contains an HRE which, in the presence of COUP-TF2, inhibits its own transactivation (Soosaar et al., 1996). As COUP-TF2 transrepresses PPARα by binding to a PPRE, it is plausible that the converse would also be true as both transcription factors bind to PPRE/HRE sites. Hence, it is possible that PPARα could increase the expression of COUP-TF2 through inhibition of this negative feedback loop. It has recently been shown that in utero DBP (500mg/kg) treatment increases the expression of COUP-TF2.
SF1-binding peaks were not detected in the SCARB1 and HMGCS promoters. There are data suggesting that SF1 transcriptionally activates these genes in testes (Val et al., 2003). Lack of SF1 peak detection in the promoters of these genes in this study could be due to insufficient density of probes on the ChIP microarray that contain binding sites for these proteins.

DBP is metabolized in rodents to MBP which is thought to be the “active” metabolite of DBP with regard to its effects on testicular development (Corton and Lapinskas, 2005; Williams and Blanchfield, 1975). Other phthalates such as DEHP and its active metabolite monoethylhexylphthalate (MEHP) have also been found to cause testicular maldevelopment in rats (Albro and Lavenhar, 1989; Gray et al., 2000). However, some phthalates, e.g., diethylphthalate (DEP), are inactive in this regard (Gray et al., 2000). MBP, MEHP, and monoethylphthalate (MEP) have been found to gain access to the fetal testes during a critical “window,” GDs 16–19 (Carruthers and Foster, 2005; Clewell et al., 2008, 2009). Hence, it has been suggested that the ability of these compounds to cause testicular maldevelopment is determined mainly via differences in their potency to inhibit testosterone production rather than differences in their ability to gain access to the fetal testes. MBP and MEHP are both agonists for PPARα (Hurst and Waxman, 2003). However, MEP, unlike MEHP and MBP, does not activate PPARα (Bility et al., 2004; Corton and Lapinskas, 2005). Hence, the PPARα agonist activity of these phthalate metabolites correlates with their abilities to induce TMD in rats. It is not known whether nonphthalate PPARα agonist compounds induce testicular maldevelopment in rats. Recently, Hannas et al. (2012) found in utero treatment with the PPARα agonist Wy did not cause testicular maldevelopment or inhibit fetal testis testosterone synthesis or gene expression and suggested that this indicated that PPARα could not be involved in the antiandrogenic effects of phthalates (Hannas et al., 2012). However, as Wy undergoes extensive first pass metabolism in rats (NTP Technical report on the toxicity studies of Wy; National Toxicology Program, 2007) and also may not cross the placenta to gain access to the fetal testes, it is possible that the lack of effects of Wy in this model may be due to lack of fetal testis bioavailability. The inhibitory effect of fenofibrate, a potent PPARα agonist, on StAR gene expression in mouse ovary was reported to be directly mediated via PPARα in studies involving PPARα knockout mice (Toda et al., 2003). Hence, there is evidence that nonphthalate PPARα agonist compounds can also repress SF1-transactivated genes in a PPARα-dependent manner in vivo. DBP does not cause antiandrogenic effects in mice (Gaido et al., 2007), so it is not possible to assess whether there is concurrence in PPARα-mediated repression of SF1-regulated steroidogenic genes and inhibition of testosterone synthesis in wild-type and PPARα knockout mouse strains. It will be necessary to assess the expression of PPARα in fetal mouse testes to determine whether the lack of induction of testicular maldevelopment by DBP in mice could be caused by the lack of expression of PPARα in mouse fetal Leydig cells. Lack of expression of PPARα in mouse fetal testes could explain why
fetal mouse testes are resistant to the antiandrogenic effects of in utero DBP treatment. Recent studies have shown that human fetal testis explants (Heger et al., 2012; Mitchell et al., 2012) are also refractory to the antiandrogenic effects of DBP and a similar explanation could also account for this observation. A recent study by Desdoits-Lethimonier et al. (2012) has found that DEHP and its metabolite MEHP inhibit testosterone production in adult human testes. In this regard, it is intriguing that PPARα has been shown to be expressed in Leydig cells of adult human testes (Schultz et al., 1999). As the chromosomal context of steroidogenic genes in human testes undoubtedly is different from that of the rat, it will be interesting to assess whether PPARα binds to the regulatory regions of these genes in human testes.

To our knowledge, this study is the first one to apply ChIP microarray analysis (ChIP on chip) in fetal testes. This has facilitated coordinated analysis of transcription factor binding to a battery of genes involved in DBP-induced testicular maldevelopment. The binding of SF1 to several testicular maldevelopment genes involved in steroidogenesis and testis development was found to be inhibited by DBP treatment, and this correlates with increased binding of PPARα and CBP at sites peripheral to SF1-binding sites. Hence, these data support the conclusion that PPARα could inhibit SF1 by indirectly disrupting the binding and activity of this transcription factor in fetal rat testes. A schematic summarizing this hypothesis is shown in Figure 11. We have shown for the first time that PPARα binds to the promoters of certain steroidogenic genes and that increased PPARα binding correlates with DBP-mediated inhibition of SF1 binding and steroidogenic gene expression.

Our immunostaining studies have confirmed that PPARα is expressed in the fetal rat testes at GD15; hence, the ontogeny of its expression is consistent with a postulated role in mediating antiandrogenic effects of DBP at this time in development. The results showed that the majority of interstitial cells, staining weakly for 3βHSD, also contained PPARα staining, indicating that these cells could be differentiating Leydig cells. Studies of fetal testes using electron microscopy have shown that at GD17 the interstitial tissue contains a significant number of differentiating fetal Leydig cells (Kerr and Knell, 1988). Hence, it seems likely that the ability of PPARα to interfere with SF1 binding/transactivation caused by exposure to DBP could precede functional maturity of Leydig cells, affecting their ability to produce testosterone. PPARα expression was not observed in tubular regions including Sertoli cells. This is consistent with the lack of effect of DBP on FSHR that is expressed primarily in Sertoli cells (Salhanick and Wiebe, 1980).

To conclude, expression microarray pathways clustering analysis and ChIP-on-chip microarray analysis of SF1 and PPARα binding in in utero DBP-exposed fetal rat testes have shown inhibition of testosterone synthesis gene expression correlates with inhibition of binding of SF1 to “target” gene promoters. This effect also correlated with increased binding of PPARα, a receptor known to be activated by DBP, to the promoters of these genes. The SF1-regulated gene FSHR, unaffected by DBP at the RNA level, showed no DBP-mediated alteration to SF1 or PPARα binding. Hence, the effects of DBP on SF1 binding and expression of steroidogenic genes correlate with increased binding of PPARα, which is consistent with its postulated role as an indirect transrepressor of steroidogenic (SF1-regulated) genes in this model.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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