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Uterine Responsiveness to Estradiol and DNA Methylation Are Altered by Fetal Exposure to Diethylstilbestrol and Methoxychlor in CD-1 Mice: Effects of Low versus High Doses

L. C. Alworth,*,[1] K. L. Howdeshell,* R. L. Ruhlen,* J. K. Day,†,[2] D. B. Lubahn,† T. H-M. Huang,‡ C. L. Besch-Williford,§ and F. S. vom Saal*

*Division of Biological Sciences, †Departments of Biochemistry, Child Health, and Animal Science and Molecular Biology Program, ‡Department of Pathology and Anatomical Sciences, and §Department of Veterinary Pathobiology, University of Missouri, Columbia, Missouri, 65211

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We examined the effects on female CD-1 mice of fetal exposure to low doses of the drug diethylstilbestrol (DES) (0.1 μg/kg/day) and the insecticide methoxychlor (MXC) (10 μg/kg/day) as well as 1000-fold higher doses: 100 μg/kg/day DES and 10,000 μg/kg/day MXC. Pregnant females were administered these chemicals on gestation days 12–18. At 7–8 months of age, female offspring were ovariectomized and implanted for 7 days with a Silastic capsule containing estradiol. Relative to controls, females exposed to the 0.1 μg DES dose showed significantly heavier uteri, while females exposed to the 100 μg DES dose showed significantly lighter uteri. Females exposed prenatally to the 10 μg/kg dose of MXC had significantly heavier uteri relative to females exposed to the 10,000 μg/kg dose of MXC, but neither group differed significantly from controls. Liver weight for females exposed to both doses of DES was significantly greater than controls. Using a microarray approach to analyze DNA methylation, an increase in ribosomal DNA (rDNA) methylation was observed. Sequence data and Southern analysis indicate an increase in 18S rDNA and 45S pre-rDNA methylation in uterine samples exposed prenatally to low and high doses of DES. We thus found opposite effects of fetal exposure to a low and a high dose of DES on the uterine response to estradiol (inverted-U dose–response relationship). In contrast, there was a monotonic dose–response relationship found for prenatal DES exposure on both liver weight and ribosomal DNA hypermethylation. © 2002 Elsevier Science (USA)

Key Words: DES; methylation; DNA imprinting; inverted-U; dose-response; low dose; liver; kidney.

During fetal development, classical hormones and other signaling molecules involved in differentiation permanently turn off or on specific genes and also determine subsequent rates of gene expression (now referred to as imprinting) in cells possessing receptors for the hormones (Holliday, 1987; Lyn-Cook *et al.*, 1995; Li *et al.*, 1997). There are dozens of chemicals used in household products that can mimic these hormones or otherwise interfere with endocrine function, and these are referred to as endocrine disrupting chemicals (EDCs) (Colborn *et al.*, 1993). Exposure of the fetus to EDCs can interfere with the normal balance of fetal hormones important to development, resulting in permanent alterations in DNA imprinting and in subsequent organ function. Some of these alterations, such as changes in the functioning of reproductive organs, may only become apparent subsequent to sexual maturation, while other effects may be detected in the fetus upon detailed examination (Johnson *et al.*, 1979; Driscoll and Taylor, 1980; vom Saal *et al.*, 1997). Endocrine disruption can occur via many different mechanisms (Colborn *et al.*, 1998). However, the most thoroughly characterized class of EDCs are chemicals that can bind to estrogen receptors and exert estrogenic effects in tissues (NRC, 1999).

There has been considerable controversy among toxicologists concerning the issue of low-dose effects that are opposite to those seen at high doses (vom Saal and Sheehan, 1998; Welshons *et al.*, 1999). The U.S. National Institutes of Health recently held a meeting (at the request of the US-EPA) devoted to the "low-dose" issue (NTP, 2001). At this meeting low dose was defined as doses below the range typically used in toxicological studies, where the dose range seldom extends more than 50-fold below the maximum tolerated dose (Calabrese and Baldwin, 1997; vom Saal and Sheehan, 1998). At the Low Dose NIH meeting the consensus of the review panel was that "low-dose effects were clearly demonstrated for estradiol and several other estrogenic compounds" (NTP, 2001).

Of considerable interest is the mechanism(s) by which hormones and chemicals that mimic hormones permanently alters cellular functions when exposure occurs during organogenesis in fetal life. One mechanism that is generating considerable interest is the epigenetic modification of DNA by the addition

[1] Current address: Division of Animal Resources, University of Illinois, Urbana–Champaign, IL.

[2] Current address: Epigenomics Inc., Seattle, WA.

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of methyl groups to specific bases, particularly those located in the promoter region of genes. DNA methylation is the covalent addition of methyl groups to the 5-position of cytosines that are 5-prime to guanine nucleotides in the DNA sequence. Throughout the genome CpG dinucleotides are found at approximately 10% of their expected frequency, except in regions known as CpG islands, where they are at or above predicted values (Gardiner-Garden and Frommer, 1987; Antequera and Bird, 1993). CpG islands are normally protected from DNA methylation; exceptions include various imprinted genes, X-chromosome inactivation, and cancer, where this protective effect is lost (Lyn-Cook *et al.*, 1995; Yan *et al.*, 2000). Alterations in methylation profiles have been linked to changes in chromatin structure and subsequent gene expression. Many of the differentially methylated genes produce critical factors in cell cycle progression, growth regulation, and tumor suppression. Hypermethylation of such genes acts in a manner analogous to a classical genetic mutation and results in the lack of a functional protein product. The result can be a subsequent breakdown in homeostasis (Jost and Saluz, 1993; Larid and Jaenisch, 1994).

There are numerous hypotheses as to the factors that regulate DNA methylation patterns. Estrogenic compounds are beginning to receive attention as regulators of methylation, since during critical periods of organogenesis in fetal life they are reported to result in tissue-specific patterns of DNA methylation (Lyn-Cook *et al.*, 1995; Li *et al.*, 1997). A possible example of an epigenetic modification of genes is that there appears to be a multigenerational consequence to exposure of fetuses to diethylstilbestrol (DES), such that an increased probability of reproductive tract tumors present in fetuses directly exposed to DES is "inherited" by future generations, even though these generations were not themselves exposed to DES; in contrast, there is an absence of findings that DES is a classical mutagen (Herbst, 1981; Newbold *et al.*, 1990, 1998; Turusov *et al.*, 1992; Walker and Kurth, 1995). This suggests the intriguing possibility of transgenerational "memory" of at least some "epigenetic imprinting" effects of DES and thus other estrogenic chemicals.

We examined the uterine response to estradiol (uterine weight and methylation pattern of CpG islands) in adult female offspring of mothers administered low versus high doses of DES (0.1 and 100 μg/kg/day). We also examined the effect of administration of a low and high dose of methoxychlor (MXC) (10 and 10,000 μg/kg/day) during pregnancy on uterine weight in female offspring. Methoxychlor is a currently used insecticide sprayed on livestock, pets, and plants to control pests. Methoxychlor is a particularly interesting chemical, since it has been shown to interact with multiple receptors, including as yet uncharacterized receptors (Ghosh *et al.*, 1999). The *in vivo* hydroxy and bis-hydroxy metabolites of methoxychlor have been shown to have estrogenic activity (ATSDR, 1994). Since our focus was on the effects of methoxychlor on the uterus, we did not control for the more recently discovered antiandrogenic action of methoxychlor in this study (Gray *et al.*, 1999).

We report here opposite effects (low-dose stimulation and high-dose inhibition) on the uterine growth response to estradiol in adult females as a result of fetal exposure to a low versus a high dose of both DES and methoxychlor. These findings are thus similar to previously reported opposite effects on the prostate of fetal exposure to low versus high doses of both estradiol and DES (vom Saal *et al.*, 1997; Welshons *et al.*, 1999; Gupta, 2000a). We also report that both low and high doses of DES resulted in an increase in methylation of ribosomal DNA (rDNA), which is a potential mechanism by which permanent effects on reproductive organs might occur due to fetal exposure to estrogenic chemicals.

GENERAL METHODS

Animals, housing, diet, and timed mating. These experiments were approved by the University of Missouri Animal Care and Use Committee. For our animal model, we used the CD-1 house mouse (*Mus musculus domesticus*). The CD-1 mouse has proven to be a valuable predictor of developmental effects of DES in humans (Newbold, 1995). CD-1 mice were initially purchased from Charles River Laboratories. Animals were maintained in an outbred colony and housed in standard (11.5 × 7.5 × 5 in) polypropylene mouse cages on corncob bedding (The Andersons, Maumee, OH). Pregnant and lactating females were fed Purina 5008 (soy-based) breeder chow and, after weaning, animals were fed Purina 5001 (soy-based) chow. Water was provided *ad libitum* in glass bottles and purified by ion exchange followed by a series of carbon filters. Rooms were maintained at 25 ± 2°C under a 12:12 L:D cycle, with lights on at 10:00 h. To produce pregnant mice, adult females (3–5 months old) were placed daily with stud males for 4 h until a vaginal plug was observed (gestation day 0). Parturition typically occurs prior to the onset of the light phase of the L:D cycle on gestation day 19.

Maternal administration of chemicals. Pregnant females were randomly assigned to one of six treatment groups and housed 3 per cage. Diethylstilbestrol (Sigma, St. Louis, MO) was administered by sc injection to 10 mice at 0.1 μg/kg/day (low dose) and 100 μg/kg/day (high dose). Technical grade methoxychlor (bis-*p*-methoxy *o,p'*DDT; 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane (Kincaid Enterprises, Nitro, WV) was administered orally as described previously (vom Saal *et al.*, 1995) at 10 μg/kg/day (low dose) and 10,000 μg/kg/day (high dose). We chose to administer DES by injection to replicate the DES administration procedure used by Newbold and colleagues (Newbold *et al.*, 1998, 1999). Methoxychlor was administered orally, since the parent compound lacks hormonal activity, and bioactivation by demethylation in the liver after oral administration is required to observe effects (ATSDR, 1994). Mice readily consume corn oil, and feeding the chemical provides an accurate and less stressful method of oral delivery relative to gavage. DES and methoxychlor were administered dissolved in tocopherol stripped corn oil (Fisher, Cat. No. 901415, ICN, Aurora, OH). The doses were based on the average body weight of the mice between gestation days 12 and 18 (~50 g). All animals were treated once daily on days 12 through 18 of gestation. The gonads begin differentiating on gestation day 12, and the accessory reproductive organs begin differentiating on gestation day 15 in mice (Brody and Cunha, 1989a,b; vom Saal *et al.*, 1992).

There were two control groups: oil fed and oil injected. Oral administration consisted of approximately 30 μl of solution administered into the mouth using a micropipetter. Injected mice (DES and oil-injected controls) were also treated once daily on days 12 through 18 of gestation. Approximately 40 μl of solution was injected subcutaneously in the back. The mice were singly housed after the final treatment on day 18 of gestation and monitored for parturition. At

weaning on postnatal day 24, pups were weighed and housed in groups of five or fewer same-sex siblings per cage.

Differential methylation hybridization (DMH). Here we employed a DNA array-based approach recently developed by Huang and colleagues (Huang *et al.*, 1997) known as differential methylation hybridization analysis. This technique allows the analysis of genome-wide changes in methylation patterns, because it uses arrays that are generated from CpG islands representing regions of the mouse genome that may potentially be altered in their methylation patterns due to exposure to estrogenic chemicals during fetal life.

High-molecular-weight DNA was isolated from DES-treated and control uterine tissue using the DNAeasy tissue kit (Qiagen, Inc., Valencia, CA). Amplicons were generated by digesting 1 μg DNA with 10 U of *Mse*I following the conditions recommended by the supplier (New England Biolabs, Beverly, MA). DNA was ligated to linker-oligonucleotides (H-24: 5′-AGG CAA CTG TGC TAT CCG AGG GAT-3′ and H12: 5′-TAA TCC CTC GGA-3′) that were combined and cooled from 65°C to 25°C. DNA was then digested with either *Hpa*II or *Bst*UI, which are methylation-sensitive endonucleases, or the non-methylation-sensitive isoschizomer *Msp*I. After restriction, DNA was amplified by linker-PCR in the presence of Deep Vent (exo-) DNA polymerase, 0.4 μM H-24 primer, 200 μM deoxynucleotide triphosphates in a buffer provided by the supplier. PCR conditions have been described previously (Huang *et al.*, 1999). The amplicons were purified and ^{32}P-labeled using the Megaprime labeling system (Amersham–Pharmacia Biotech, Piscataway, NY) for array hybridization.

The mouse CpG island microarrays were prepared from a genomic library, CpGIL (Human Genome Mapping Project, Cambridge, UK), specifically enriched to contain GpC-rich genomic fragments. A total of 300 CpG island clones were cultured in 96-well plates. DNA templates were used to generate CpG island inserts by PCR (Huang *et al.*, 1999). Amplified products were denatured and dotted in duplicate on nylon membranes using a Multi-Print replicator (V & P Scientific). The arrays were then hybridized with ^{32}P-labeled amplicons overnight at 65°C using the High Efficiency Hybridization system (HEHS, Molecular Research, Piscataway, NY). Membrane washing was performed three times, each for 25 min in 0.01% SDS–0.2× SSC at 65°C. Autoradiographic exposure varied depending on the intensity of hybridization probes.

Methylation analysis by Southern hybridization. Genomic DNA (2.5 μg) was digested to completion with 20 U of restriction endonuclease *Msp*I or *Hpa*II (New England Biolabs). The restriction products were separated on 1.2% agarose gels and transferred to nylon membranes. The membranes were hybridized with ^{32}P-labeled 3MC2-A4 (a 45S ribosomal sequence) or pHsrDNA5.1 (human 18S ribosomal sequence) in the HEHS hybridization buffer overnight at 65°C. Posthybridization washing was performed as described above. Methylation intensities were analyzed using Scion Imaging software (Scion Corp., Frederick, MD). Increases in rDNA methylation were defined by the shift in intensity from unmethylated bands (un-bands) to the larger molecular weight methylated band (Me-band, Figs. 3 and 4). The fold increase in rDNA methylation was measured as the ratio of methylated band intensity to unmethylated band intensity compared to control samples.

Statistical procedures. Data were analyzed by analysis of covariance (ANCOVA) or analysis of variance (ANOVA) as indicated using the Statistical Analysis System (SAS), General Linear Model procedure. Planned comparisons of differences between group means were made using the LS means test in SAS. All organ weight data were analyzed by ANCOVA to determine whether organ weight measures needed to be corrected for body weight. If body weight accounted for a significant component of the variance, then group means were adjusted for body weight. The correlation between organ weight and body weight was also determined using Pearson's correlation analysis. If body weight did not account for a significant component of the variance for a specific measure, then the data were reanalyzed by ANOVA, and group means were not adjusted for body weight. We note that the direction of the correlation between organ weight and body weight (positive or negative) was not a factor in our decision to use ANCOVA when the above criterion was met.

In these experiments low and high doses of chemicals were administered. The objective was to test the hypothesis that low and high doses of endocrine disrupting chemicals would result in opposite effects. We thus conducted planned comparisons on results from the low- and high-dose groups and also compared each experimental group with controls. Planned comparisons were only conducted if the overall ANOVA or ANCOVA was statistically significant.

EXPERIMENTAL METHODS AND RESULTS

Controls

In the experiments here and in prior studies, chemicals have been administered to pregnant mice by feeding and by injection. In this study we included controls for these different routes of administration: oil fed via a micropipetter (not gavage) and sc oil injection. A separate analysis conducted just on these control groups revealed that they were not significantly different for any of the variables measured in either Experiment 2 or Experiment 3. A pooled control group referred to as "controls" was thus used in all of the subsequent analyses for females examined at 7 and 8 months of age.

To control for variability in estradiol levels and estrogen-responsive tissues throughout the estrous cycle, we ovariectomized the female offspring of treated mothers. The ovariectomized females were treated with estradiol, thus allowing us to compare the uterine response to estradiol in females from different prenatal treatment groups.

Experiment 1: Determination of Estradiol Doses That Stimulate the Uterus

The objective of this study was to determine a dose of estradiol administered sc via Silastic capsule that resulted in an increase in uterine weight that was below the maximum uterine weight in response to estradiol and within the linear region of the dose–response curve for use in Experiment 2. We also sought to determine the range of doses to be used in Experiment 3 in order to conduct a more detailed dose–response assessment and to compare the slopes of the dose–response curves to estradiol based on prenatal treatment. One cannot study the effects of prenatal treatments on estrogen-responsive organs in gonadally intact, cycling adult females without controlling for stage of estrus or stabilizing estrogen levels (EDSTAC, 1998). We chose to control estradiol levels via Silastic capsules, since attempting to control for the stage of estrus in intact females is complicated by the fact that the characteristics of estrous cycles vary considerably between individuals (vom Saal, 1981, 1989).

Adult 7- to 8-month-old previously untreated female mice were ovariectomized using a mixture of ketamine, xylazine, and acepromazine for anesthesia, and all females were implanted with a Silastic capsule (0.62 in ID, 1.25 in OD) that was 10 mm long between the capped ends. The capsules contained 0 (controls), 0.25, 0.5, 1.0, 1.25, and 1.5 μg 17β-estradiol dissolved in 0.02 ml tocopherol stripped corn oil (Fisher). Prior to implantation, the capsules were preincubated in physiological saline for 24 h to stabilize the release of

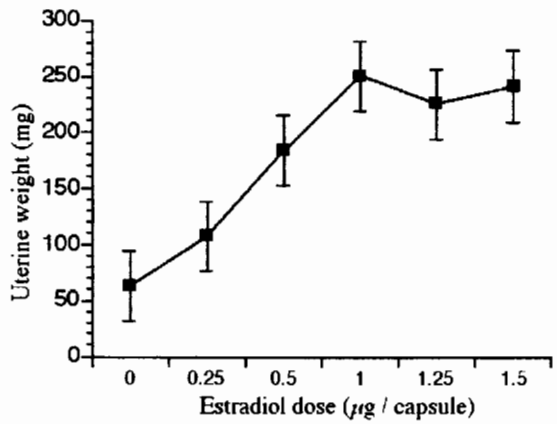

FIG. 1. Mean (±SEM) uterine weight in 8-month-old control CD-1 female mice in Experiment 1. Females were ovariectomized and implanted with a Silastic capsule containing different doses of estradiol, and organs were collected 7 days later.

estradiol. Seven days after the capsules were implanted, the females were killed, body weights were recorded, and uteri were collected and weighed. The data shown in Fig. 1 reveal that the maximum response occurred with the 1.0 μg capsule and that the 0.5 μg capsule dose resulted in a response that was about 70% of maximum and within the linear portion of the dose–response curve. In Experiment 2 we thus used the 0.5 μg estradiol capsule, and in Experiment 3 we used capsules containing 0 to 1.0 μg estradiol.

Experiment 2: Uterine Response to a Single Dose of Estradiol: Organ Weights and Methylation of CpG Sites

In this study we examined organ weights in females that were ovariectomized and implanted with a Silastic capsule containing one dose (0.5 μg) of estradiol determined in the prior study. The objective of this study was to determine whether females exposed to a low versus a high dose of DES or methoxychlor during fetal life would show a different response to the same submaximal dose of estradiol. We thus sought to examine differences between control females and females in each treatment group, as well as differences due to prenatal dose (low versus high) for each chemical.

At 7 months of age, one female was randomly selected from each litter for use in this experiment. The data were thus not adjusted for litter effects. Each female was ovariectomized and implanted with a Silastic capsule containing 0.5 μg 17β-estradiol as described in Experiment 1. Seven days later the females were killed, body weights were recorded, and organs were collected, weighed, and stored for subsequent analyses.

Body weight. The 100 μg/kg/day prenatal dose of DES significantly increased body weight by 19% relative to control females and females exposed to the low (0.1 μg/kg) dose of DES) ($P < 0.001$). Females exposed to methoxychlor or the low dose of DES did not differ significantly in body weight relative to controls. All treated females had also been weighed when they were 24 days old, and body weight of the DES 100 females (16.5 ± 0.5 g) was also significantly ($P < 0.05$) elevated relative to controls (14.3 ± 0.3 g), while none of the other groups differed significantly from controls.

Uterine weight. Body weight accounted for a significant portion of the variance in uterine weight ($P < 0.001$), and the data were analyzed by ANCOVA. Overall, there was a significant effect of prenatal treatment on uterine weight ($P < 0.001$). Relative to controls, females exposed to the 0.1 μg DES dose had significantly heavier uteri (by 27%), while females exposed to the 100 μg DES dose had significantly lighter uteri (by 49%). The uteri of females exposed to methoxychlor did not differ significantly from controls. For both DES ($P < 0.001$) and methoxychlor ($P < 0.05$), females exposed prenatally to the low dose of the chemical had significantly heavier uteri relative to females exposed to the high dose of the same chemical (Table 1).

Liver weight. Body weight accounted for a significant component of the variance in liver weight ($P < 0.001$), and the data were analyzed by ANCOVA. There was a significant effect of prenatal treatment on liver weight ($P < 0.01$). Females exposed to both the low and the high doses of DES

TABLE 1
Mean (±SEM) Body Weight and Organ Weights for Control Females and Females Exposed Prenatally to Diethylstilbestrol (DES) or Methoxychlor (MXC)

	N	Body weight (g)	Uterine weight (mg)	Liver weight (g)	Spleen weight (mg)	Kidney weight (mg)
Controls	20	33 ± 0.8	150 ± 14	1.70 ± 0.04	133 ± 10	249 ± 7
DES 0.1	20	35 ± 0.8††	191 ± 12*††	1.87 ± 0.03**	147 ± 8	258 ± 6
DES 100	13	41 ± 1.0**	77 ± 23**	1.93 ± 0.07**	161 ± 16	268 ± 12
MXC 10	23	34 ± 0.7	162 ± 11†	1.79 ± 0.03	132 ± 8	252 ± 6
MXC 10,000	20	35 ± 0.8	129 ± 11	1.77 ± 0.03	141 ± 8	241 ± 6

Note. When females were 7 months old they were ovariectomized and implanted sc with a Silastic capsule containing 0.5 μg estradiol. Significant difference from controls is indicated by * (*$P < 0.05$, **$P < 0.01$); Significant difference between low and high doses of the same chemical is indicated by † (†$P < 0.05$, ††$P < 0.01$).

showed a significant enlargement of the liver relative to controls ($P < 0.005$). Females exposed to the low dose of methoxychlor tended to show an increase in liver weight ($P = 0.06$) relative to controls (Table 1).

Spleen and kidney weight. Neither the spleen nor the kidney differed significantly from controls for females exposed to either chemical (Table 1).

Differential methylation hybridization. An array-based DMH technique was used to screen changes in DNA methylation of uteri from adult female mice exposed during fetal life to DES or no chemical (control). Candidate CpG island sequences were arrayed on nylon membranes and screened with amplicons generated from uterine DNA that had been exposed prenatally to 0, 0.1, or 100 μg/kg/day DES. PCR amplified differences inherent to each amplicon are a direct result of the inability of methylation-sensitive restriction enzymes to cut methylated sites. The treatment-specific differences are then able to subsequently anneal CpG island arrays. The methylation sensitive enzymes *Bst*UI and *Hpa*II were used to characterize treatment-specific differences in DNA methylation patterns. *Msp*I, an isoschizomer of *Hpa*II, was used to determine whether differences in array hybridization patterns were due to changes in DNA methylation or based upon genotypic polymorphisms between mice.

Of the 300 CpG island loci screened by DMH, a high degree of DNA methylation variation was identified in DES-exposed mice relative to the control group (Fig. 2). This altered DNA methylation identified in the DES-treated group showed detectable hybridization signals in CpG island tags probed with the DES-treated amplicons, but not in the same tags probed with the control amplicons. This is because methylated *Hpa*II sites in the treated DNA were protected from restriction within CpG island sequences, which were then amplified by linker-PCR and hybridized to the corresponding tags. The same sites, however, were unmethylated in control DNA and were restricted by the isoschizomer *Hpa*II; therefore, no hybridization signals were detected in the arrays. Hypomethylated sequences can also be detected in a similar fashion, except that hybridization signals would be present in the "control" arrays, but not in the corresponding positions of the "treated" arrays.

Sequencing analysis of five positive loci for hypermethylation revealed that they were part of ribosomal DNA sequences, which are abundant in the mouse genome. Their transcribed domain, including the enhancers, spacers, and 45S pre-rDNA, shares many characteristics of single-copy CpG islands. Our findings show that they were subjected to alteration in their methylation contents in the animals prenatally exposed to DES.

Southern analysis was conducted to confirm this finding. When hybridized to a probe in the rDNA spacer region (3MC2-A4), unmethylated DNA fragments (<0.56 kb) were detected, showing that the patterns of methylation-sensitive *Hpa*II restriction were largely the same as that of methylation-insensitive *Msp*I restriction in all DNA lanes (Fig. 3). Also present in *Hpa*II lanes was a larger fragment (~8 kb). This DNA fragment could be a result of resistance to enzymatic restriction or due to DES-induced methylation in some of the rDNA spacers. However, the larger DNA fragment was more intense in the lanes of the DES-treated animals. Uteri from mice treated with the low dose of DES showed a 1.4- to 2.8-fold increase in intensity of this band, while the high-dose DES uteri showed a 1.8- to 3.4-fold increase in methylation compared to the control. Results of the 45S pre-rDNA hybridization also showed similar methylation patterns (Fig. 4).

Experiment 3: Uterine Response to a Range of Estradiol Doses

In this experiment we examined organ weights using the remaining females from each prenatal treatment group. However, this experiment differed from the previous experiment in that the females were ovariectomized and implanted with Silastic capsules containing a range of doses of estradiol determined in Experiment 1 (Fig. 1). The objective was to determine whether the dose–response curve for stimulation by estradiol of uterine weight gain in adult females differed significantly based on prenatal treatment. Specifically, at 8 months of age females were ovariectomized and implanted with Silastic capsules as described above, except that in this experiment the capsules contained one of four doses of estradiol: 0, 0.25, 0.5, and 1.0 μg per capsule.

Statistics. Whereas in the prior experiment only one female per litter was used, the remaining females in each litter were assigned to different estradiol dose groups in this experiment. Thus, in addition to correcting for body weight, we also corrected for maternal effects. To control for maternal (litter) effects, litter was entered as a main effect, and the F value for treatment was divided by the F value for litter; all F values presented below were adjusted for litter effects. If the overall ANOVA or ANCOVA was statistically significant, planned comparisons were made using the LS means test; the means from these analyses were all adjusted for litter effects, and the significance levels also were adjusted for litter. We also conducted a regression analysis (using SAS) in order to compare the slope of the estradiol dose–response curve for each prenatal treatment group relative to controls.

Body weight. Adult treatment with estradiol did not influence body weight, and comparisons of prenatal treatment groups were made without regard to the dose of estradiol administered (Table 2). Regardless of the adult estradiol dose, prenatal treatment with the 100 μg dose of DES significantly increased body weight by approximately 20% relative to control females ($P < 0.001$) or females exposed to the low dose of DES ($P < 0.001$), thus replicating the findings of Experiment 1. Body weight did not differ from controls for females exposed prenatally to methoxychlor.

Uterine weight. Body weight accounted for a significant component of the variance in uterine weight ($P < 0.001$), and

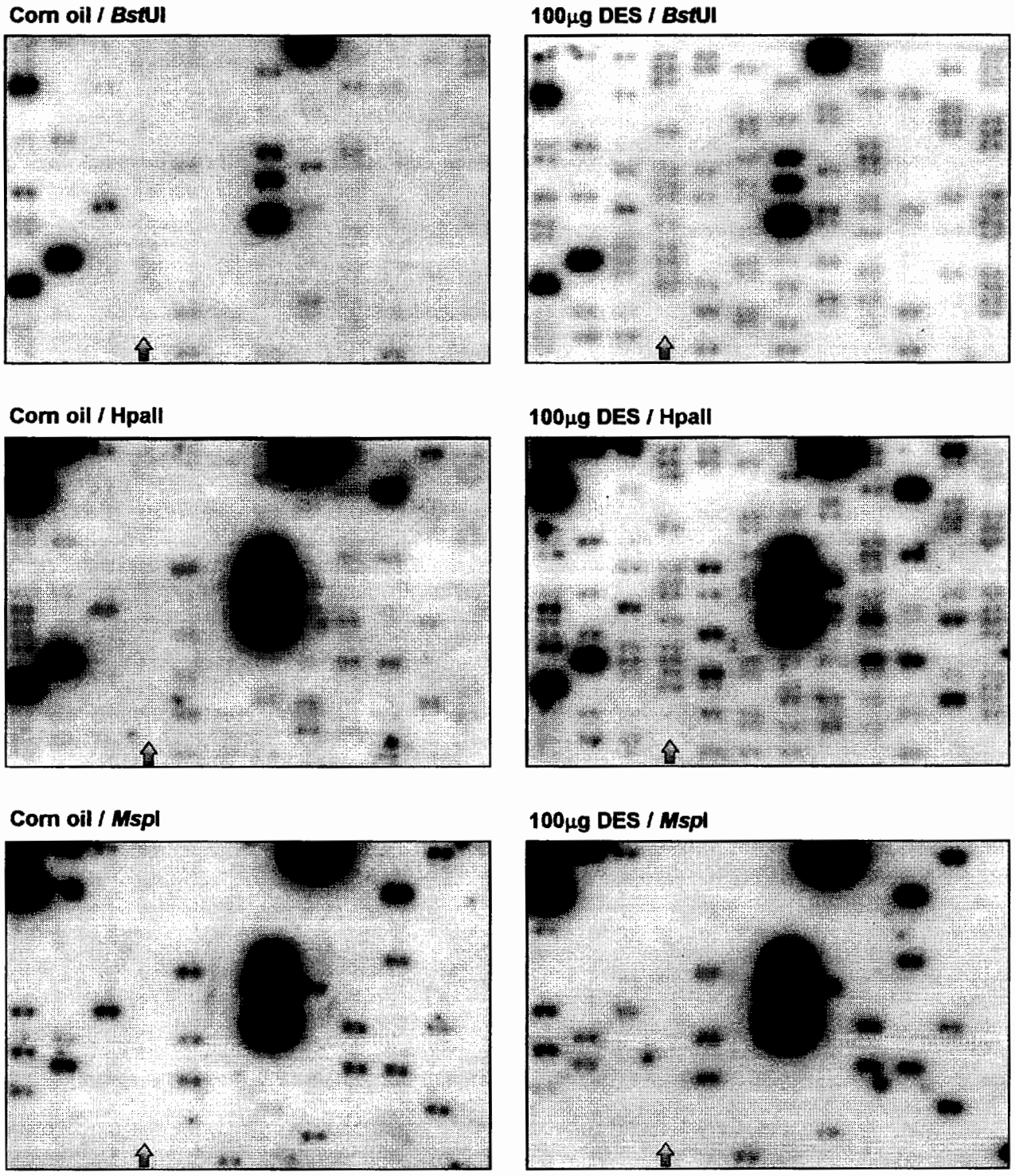

FIG. 2. Differential methylation hybridization arrays generated using the methylation-sensitive restriction enzymes *Bst*UI and *Hpa*II. *Msp*I was included as a control to show consistency between DNA samples. Arrows indicate candidate genes which were differentially methylated after DES exposure. Pregnant CD-1 mice were injected with corn oil (control), 0.1 μg/kg/day DES (low dose), or 100 μg/kg/day DES (high dose) on days 12–18 of gestation. Uteri used were from Experiment 2.

the data were analyzed by ANCOVA. For prenatal control females, uterine weight increased by 362%, from 56.6 to 261.3 mg between the 0 and 1.0 μg doses of estradiol per Silastic capsule. In sharp contrast, for the females exposed to the 100 μg dose of DES, there was only a 79% increase in uterine weight (from 52.2 to 93.5 mg) in response to increasing doses

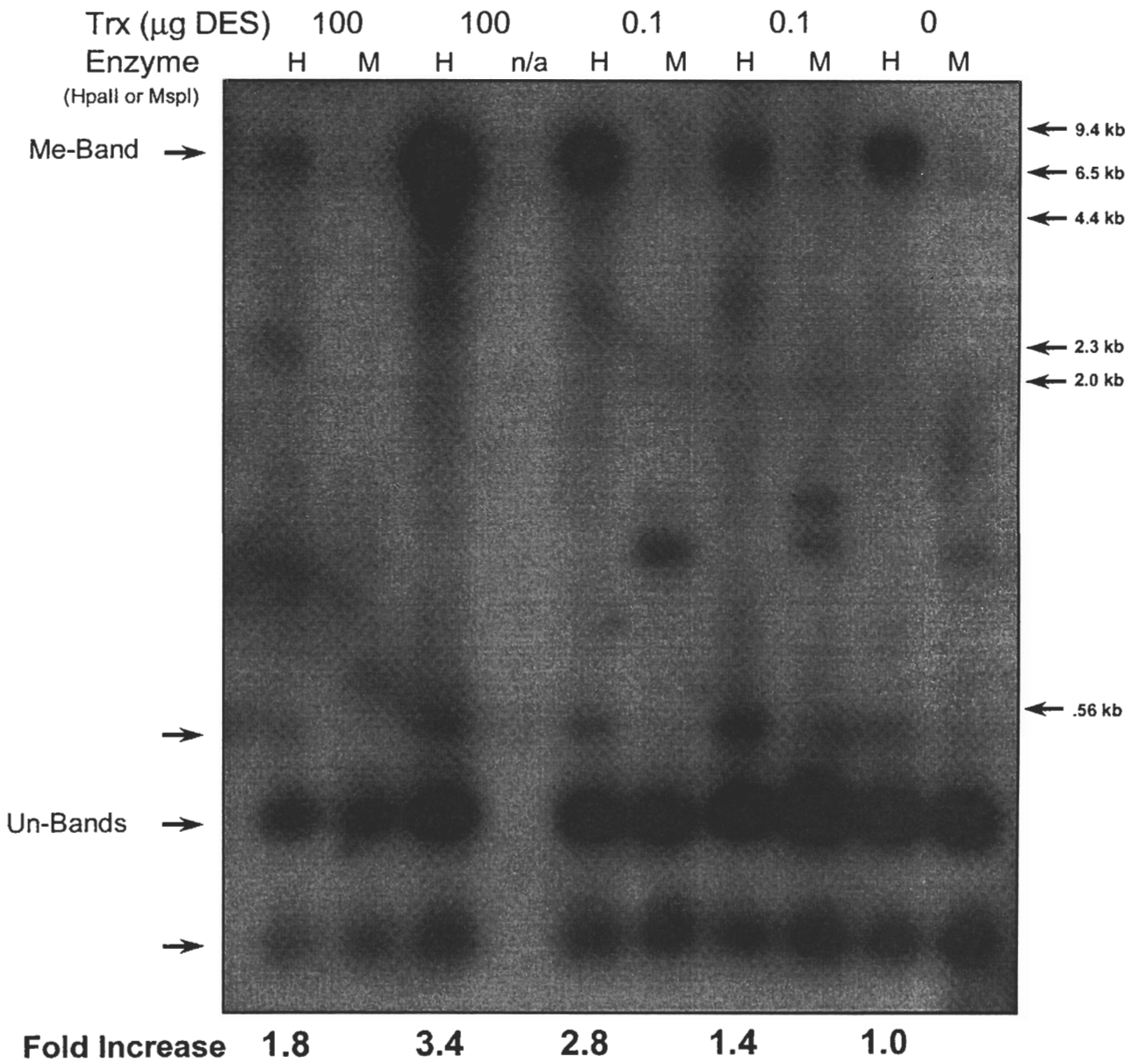

FIG. 3. An increase in ribosomal DNA (rDNA) methylation after prenatal exposure to diethylstilbestrol. Southerns were run using 3MC2-A4, a candidate CpGIL gene with 99% homology to mouse pre-45S rDNA. The Me-band represents methylated bands and Un-bands represent unmethylated fragments. For enzyme designations, M is *Msp*I and H is *Hpa*II. The samples were not digested with *Mse*I prior to digestion with *Msp*I and *Hpa*II. Pregnant CD-1 mice were injected with corn oil (control), 0.1 μg/kg/day DES (low dose), or 100 μg/kg/day DES (high dose) on days 12–18 of gestation. Uteri used were from Experiment 2.

of estradiol. Thus, while there was a significant increase in uterine weight in response to estradiol in the females exposed prenatally to 100 μg DES, the response was markedly attenuated relative to prenatal control females (Fig. 5).

The 0.1 μg/kg dose of DES resulted in a significant increase in uterine weight relative to prenatal control females, ignoring doses of estradiol in the Silastic capsules. We note that one of the females in the 0.1 DES group exposed in adulthood to 1.0 μg estradiol had a uterine weight that was about two times the mean for the group. However, even if the data from this animal were excluded from the analysis, the females exposed *in utero* to 0.1 μg DES still showed significantly enlarged uteri relative to prenatal controls ($P < 0.05$). There was also a dramatic difference ($P < 0.001$) between the low and high DES groups in the uterine response to estradiol based on ANCOVA (ignoring dose of estradiol in the Silastic capsules). This difference was confirmed by a significant difference between the slopes of the dose–response curves to estradiol based on the regression analysis.

Females exposed prenatally to the 10 μg dose of methoxychlor tended ($P = 0.06$) to have heavier uterine weight relative to controls based on a comparison of just the females exposed to the 1.0 mg dose of estradiol. In contrast, females

TABLE 2

Mean (±SEM) Body Weight and Organ Weights for Control Females and Females Exposed Prenatally to Diethylstilbestrol (DES) or Methoxychlor (MXC)

	N	Body weight (g)	Uterine weight (mg)	Liver weight (g)	Spleen weight (mg)	Kidney weight (mg)
Controls	50	36 ± 0.6	161 ± 13	1.81 ± 0.03	144 ± 7	255 ± 5
DES 0.1	30	35 ± 0.8††	215 ± 17*††	1.91 ± 0.03*	154 ± 9	253 ± 6†
DES 100	30	42 ± 0.8**	72 ± 25**	1.98 ± 0.05*	188 ± 14*	277 ± 9*
MXC 10	28	36 ± 0.6	183 ± 12†	1.75 ± 0.02	134 ± 6	244 ± 4
MXC 10,000	35	35 ± 0.7	140 ± 15	1.80 ± 0.03	144 ± 8	254 ± 5

Note. When females were 8 months old they were ovariectomized and implanted sc with a Silastic capsule containing 0, 0.25, 0.5, and 1.0 μg estradiol. The data presented here are collapsed across adult estradiol treatment groups, since only uterine weight differed as a function of adult estradiol dose (see Figs. 5 and 6). Significant difference from controls is indicated by * (*$P < 0.05$, **$P < 0.01$); Significant difference between low and high doses of the same chemical is indicated by † (†$P < 0.05$, ††$P < 0.01$).

Kidney weight. Body weight accounted for a significant component of the variance in kidney weight ($P < 0.001$), and the data were analyzed by ANCOVA. There was no significant effect of estradiol dose on kidney weight. Ignoring the dose of estradiol administered in adulthood, there was a significant effect of prenatal treatment on kidney weight ($P < 0.05$), due to the 100 μg dose of DES significantly increasing adult kidney weight relative to controls. Methoxychlor did not significantly influence kidney weight (Table 2).

Spleen weight. Body weight accounted for a significant component of the variance in spleen weight ($P < 0.001$), and the data were analyzed by ANCOVA. Adult treatment with estradiol did not influence spleen weight, but there was a significant effect of prenatal treatment ($P < 0.01$). Ignoring adult treatment with estradiol, the 100 μg dose of DES significantly increased adult spleen weight relative to controls ($P < 0.01$). No other comparison was statistically significant (Table 2).

Histopathology. There were no visible differences between treated and control livers seen on light microscopy, even though the DES-treated livers were enlarged. Histopathologic examination of spleens (one representative from each prenatal exposure and adult estradiol treatment combination) revealed hematopoieses and follicular lymphoid hyperplasia. However, these changes were observed in both enlarged and nonenlarged spleens, and no lesions were observed.

DISCUSSION

The major finding reported here is that the response of the adult uterus to estradiol is altered by exposure to estrogenic chemicals during fetal life, with the direction of the effect (enhancement versus inhibition of response) being dependent on the dose to which the females were exposed as fetuses. Specifically, when female mice were ovariectomized and administered estradiol in a Silastic capsule, we found that prenatal exposure to a low dose of DES (0.1 μg/kg/day) resulted in an enhanced uterine response to estradiol, while a dose of DES 1000 times higher (100 μg/kg/day) produced the opposite

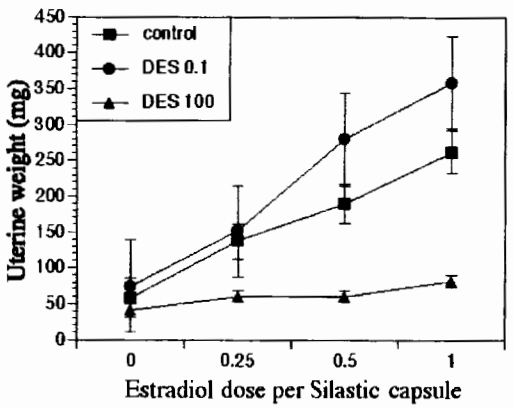

FIG. 5. Mean (±SEM) uterine weight in 8-month-old CD-1 female mice exposed to vehicle (controls), 0.1 μg/kg/day DES (low dose), or 100 μg/kg/day DES (high dose) from gestation days 12–18 via maternal administration in Experiment 3. Females were ovariectomized and implanted with a Silastic capsule containing different doses of estradiol, and organs were collected 7 days later.

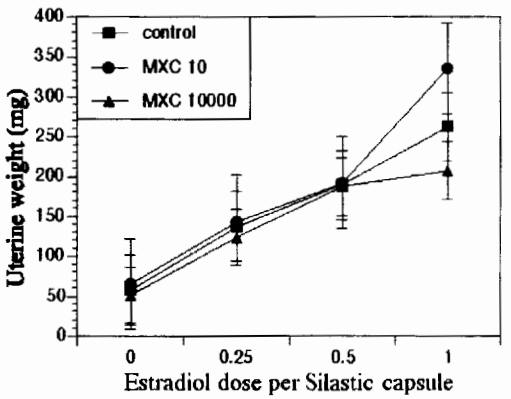

FIG. 6. Mean (±SEM) uterine weight in 8-month-old CD-1 female mice exposed to vehicle (controls), 10 μg/kg/day (low dose), or 10,000 μg/kg/day (high dose) of methoxychlor (MXC) from gestation days 12–18 via maternal administration in Experiment 3. Females were ovariectomized and implanted with a Silastic capsule containing different doses of estradiol, and organs were collected 7 days later.

outcome, an inhibition of response to estradiol, in each case relative to controls. A significant difference in uterine response to estradiol was also observed between females exposed to low (10 μg/kg/day) versus high (10,000 μg/kg/day) doses of methoxychlor. However, while the low- and high-dose groups differed from each other, neither of the methoxychlor groups differed significantly from controls in uterine weight. Our findings suggest that a similar low-dose stimulation and high-dose inhibition occurred for methoxychlor and DES, but the magnitude of the difference between the low and high doses of methoxychlor was less than that observed for DES.

There is actually considerable evidence from both *in vitro* and *in vivo* studies that hormones and EDCs can exert stimulatory effects at low doses and inhibitory effects at high doses (Amara and Dannies, 1983; Bigazzi *et al.*, 1992; Shelby *et al.*, 1996; Somjen *et al.*, 1998; Welshons *et al.*, 1999). For example, Shelby *et al.* (1996) found inverted-U dose–response curves for DES, estradiol, and other estrogenic chemicals in the mouse prepubertal uterotrophic assay. Inverted-U dose–response curves can thus occur due to fetal programming of subsequent responsiveness of tissues to hormonal stimulation (for example, vom Saal *et al.*, 1995, 1997) or due to opposite effects of low and high doses of natural hormones or EDCs on the functioning of tissues after development (for example, Shelby *et al.*, 1996).

Low-dose stimulation and high-dose inhibition of responses by hormones and hormone-mimicking EDCs, such as those reported by Shelby *et al.* (1996), can be explained by findings that, as dose increases from zero receptor occupancy, response increases as a function of increased receptor occupancy until a maximum response is reached. As dose continues to increase and more receptors are occupied, the response can then begin to decrease, associated with receptor down-regulation and/or activation of competing responses mediated by other response systems (NRC, 1999). There is direct evidence that the enhanced responsiveness of the uterus to estradiol that we observed here due to developmental exposure to a low dose of estrogenic chemicals may be caused by a permanent up-regulation of uterine estrogen receptors. In contrast, the decreased responsiveness of the uterus caused by developmental exposure to much higher doses of estrogen chemicals may be caused by a permanent down-regulation of estrogen receptors. For example, different doses of estradiol administered to adult female rats via Silastic implants lead to up-regulation of estrogen receptors at a low dose and down-regulation at a higher dose (Medlock *et al.*, 1991). In adult females these changes in estrogen receptors are typically reversible, whereas changes in estrogen receptors induced by exposure to estrogenic chemicals during uterine development are permanent and irreversible.

During uterine differentiation in rodents, mesenchyme induces epithelial cytodifferentiation, but there is also a reciprocal interaction between mesenchyme and epithelium, with epithelium inducing myometrial differentiation from mesenchyme (Cunha *et al.*, 1989). While estrogen receptors are expressed in mesenchyme as early as gestation day 13, they first appear in uterine epithelium during the first week after birth (Korach *et al.*, 1988). Changes in uterine epithelial as well as myometrial morphology and function following treatment with estrogenic chemicals during prenatal (Wordinger *et al.*, 1991) and/or early neonatal (Brody and Cunha, 1989a,b) life in rodents are assumed to be mediated by binding to estrogen receptors in mesenchyme. Studies on a mouse with a mutant α estrogen receptor show that ERα mediates uterine growth in response to estrogen in the mouse (Lubahn *et al.*, 1993). Extensive growth and differentiation of the female reproductive tract occur postnatally in rodents, and at birth the stage of development in rodents is similar to that in human fetuses at about gestational week 14 (Robboy *et al.*, 1982).

Our prior studies with methoxychlor involved administration of a wide range of doses to pregnant mice, including the reference dose currently considered "safe" for daily human intake (20 μg/kg/day). Prenatal exposure to this very low dose of methoxychlor increased both territorial marking behavior (vom Saal *et al.*, 1995) and prostate size (Welshons *et al.*, 1999) in male CF-1 mouse offspring. In male mice fetal exposure to other estrogenic chemicals (for example, DES, ethinyl estradiol, and bisphenol A) at low doses also led to a permanent increase in prostate size and prostate androgen receptors. In contrast, fetal exposure to much higher doses of estrogenic chemicals leads to a permanent decrease in androgen receptors, associated with a marked reduction in prostate size (vom Saal *et al.*, 1997; Gupta, 2000a,b; Thayer *et al.*, 2001). Thus, in the developing prostate there is a molecular pathway linking the estrogen-response system and the androgen-response system, with the result that estrogen modulates the response to androgen in the developing prostate. Importantly, the direction of the effect of estrogenic chemicals on androgen receptors is dependent on dose.

There are also other possible mechanisms which must be considered regarding the increased estrogen responsiveness of the uteri from females exposed to a low DES dose in contrast to the marked decrease in responsiveness with a high DES dose. For example, the liver is known to possess estrogen receptors (Forsberg, 1984; von Schoultz *et al.*, 1989). Alterations in the number of hepatic estrogen receptors due to DES exposure could also affect hepatic metabolism of estrogen. Exogenous estrogens are known to affect the metabolic activity of the liver (von Schoultz *et al.*, 1989). We reported elsewhere (Ganjam *et al.*, 2001) that while liver weight of the female mice used in the present study was increased by both the low and the high doses of DES, the activity of the glucocorticoid-regulating enzyme, 11β-hydroxysteroid dehydrogenase (11β-HSD) Type I, showed an inverted-U dose–response relationship for prenatal DES treatment; this also occurred for kidney 11β-HSD Type II activity.

We also conducted a comparison of methylation patterns of 300 candidate CpG island sequences in uteri from control and DES-treated females. A number of ribosomal genes showed an

increase in methylation in response to both the low and the high doses of DES relative to the control. Our prediction had been that permanent, inhibitory effects of high doses of DES on the uterine response to estradiol would be associated with hypermethylation of regulatory genes, while the low-dose stimulatory effects of DES would be associated with hypomethylation. Hypermethylation of CpG islands in the promoter region of genes is associated with gene silencing, and ribosomal DNA is typically protected from methylation, as are CpG islands (Bird and Taggart, 1980; Reilly *et al.*, 1982), supporting the hypothesis that these genes are constitutively active. In breast cancer, rDNA has been shown to become hypermethylated (Yan *et al.*, 2000). While our finding that both low and high doses of DES increased methylation of CpG island sites in ribosomal genes does not shed light on the basis for the opposite effects of low and high doses of DES on the uterus, our identification of aberrant increases in ribosomal DNA methylation is important because of the role they play as templates for ribosomal subunits and subsequent changes in protein expression. These findings also support the hypothesis that early exposure to DES may result in a breakdown of boundaries that generally protect these regions from DNA methylation. Changes in ribosomal subunit expression could thus alter translation and subsequently alter normal cellular homeostasis.

DES is one of the most studied estrogenic chemicals, since it was administered to millions of women during the 1950s and 1960s prior to being banned in 1972. There is thus an extensive literature concerning the long-term effects of exposure to high, nonphysiological doses of DES during fetal life in humans and during fetal/neonatal life in animals (Takasugi and Bern, 1988; Newbold, 1995; Swan and vom Saal, 2001). The supraphysiological doses used in most animal studies are similar to doses administered to pregnant women and thus have clinical relevance. Developmental exposure to high doses of DES has been shown to cause uterine malformations, cancer, and infertility or subfertility in humans and rodents (Brody and Cunha, 1989a,b; Mittendorf, 1995; Newbold, 1995). High doses of DES induce uterine hyperplasia acutely in neonatal rats, followed by hypoplasia (Medlock *et al.*, 1988, 1992), and uterine hypoplasia is also observed in adult women exposed as fetuses to DES (Mittendorf, 1995). A high dose of DES also inhibits uterine gland differentiation in rats (Medlock *et al.*, 1988, 1992).

DES is now commonly used as a positive control in studies of environmental chemicals, such as methoxychlor, that have estrogenic activity. The discussion of DES here is thus based on the importance of this chemical as a positive control in studies involving the effects of estrogenic endocrine disrupting chemicals. Only a few studies besides ours (vom Saal *et al.*, 1995, 1997; Welshons *et al.*, 1999) have examined the effects of administration of DES at doses below 0.1 μg/kg/day administered to pregnant mice. This is important since it is within this dose range that DES would be an appropriate positive control for a number of estrogenic EDCs (Welshons *et al.*, 1999). Specifically, McLachlan *et al.*, (1982) reported that at a low maternal dose of 0.01 μg/kg/day, DES decreased the number of ova shed in response to gonadotropin challenge and had a small adverse effect on fertility in female offspring. At this same low DES dose administered to newborn mice, Newbold *et al.* (1999) reported an enhanced uterine response to estrogen challenge, similar to our findings here with a 0.1 μg/kg/day dose of DES.

An experiment conducted by Ashby (1999) also examined the effects of prenatal exposure to 0.2 μg/kg/day DES on the size of the uterus in adult female CF-1 mice. The conclusion drawn from this study was that administration of a 0.2 μg/kg/day dose of DES to pregnant mice did not have any effect on uterine weight in female offspring. This finding would thus appear to contradict our finding here. However, in the Ashby study females were not ovariectomized and administered estradiol, and no attempt was made to control for stage of the estrous cycle at the time the female mice were killed when they were 11 months old. An EPA panel charged with designing tests for endocrine disrupting chemicals deemed it inappropriate to study the effects of estrogenic chemicals on the uterus without controlling for adult estrogen levels (EDSTAC, 1998, pp. 5–26).

After conducting the above study in which DES failed to show a positive effect, Ashby (Ashby, 1999) proposed that low doses of DES (within the range of 0.01–0.1 μg/kg/day) should not be expected to produce the effects we observed here or those that were reported by Newbold and colleagues (1999). This argument was made without acknowledging that in the Ashby (1999) study, the 0.2 μg/kg/day dose of DES was initially reported as being the positive control (SPI, 1998). In commenting on "a study in which the positive control chemical does not produce the expected positive response," the NIH Low Dose Review panel stated that "the prudent course of action may be in such cases to declare the study inadequate and repeat it" (NTP, 2001, p. 89). In our study here, the positive findings with the positive control chemical DES are thus critical with regard to interpreting which of the positive findings with methoxychlor are likely to reflect estrogenic effects and which negative findings can legitimately be interpreted as representing the absence of such effects seen with a positive control. Toxicological studies should include positive controls that yield positive results, since without positive controls, one cannot interpret negative results.

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