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Promoter CpG Methylation of *Hox-a10* and *Hox-a11* in Mouse Uterus Not Altered Upon Neonatal Diethylstilbestrol Exposure

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Mouse *abdominal B*–like *Hoxa* genes are expressed and functionally required in the developing reproductive tracts. Mice lacking either *Hoxa-10* or *Hoxa-11*, two of the *AbdB Hoxa* genes, exhibit abnormal uterine development similar to that induced by in utero diethylstilbestrol (DES) exposure. Indeed, uterine *Hoxa-10* and *Hoxa-11* expression is potently repressed by perinatal DES exposure, providing a potential molecular mechanism for DES-induced reproductive tract malformations. We have shown previously that DES can permanently alter uterine lactoferrin gene expression through modulation of the lactoferrin promoter methylation pattern. Here we ask whether a similar mechanism also functions to deregulate uterine *Hoxa-10* or *Hoxa-11* expression during neonatal DES exposure. We mapped the *Hoxa-10* promoter by cloning a 1.485 kb DNA fragment 5′ of the *Hoxa-10* exon1a. A 5′ rapid amplification of cDNA ends (RACE) experiment revealed a transcription start site for the *a10-1* transcript. Functional analysis of the proximal 200-bp sequences demonstrated significant promoter activity, confirming the location of the *Hoxa-10* promoter. Moreover, methylation assays performed on eight CpGs in *Hoxa-10* and 19 CpGs in *Hoxa-11* proximal promoters demonstrated that all these CpGs were highly unmethylated in both control and DES-dosed mice from postnatal day 5 to day 30. Significant methylation around *Hoxa-10* and *Hoxa-11* promoters was only observed in DES-induced uterine carcinomas in 18-mo-old mice. Our results suggest that DES-induced downregulations of *Hoxa-10* or *Hoxa-11* gene expression are not associated with methylation changes in their proximal promoters and that gene imprinting by developmental DES exposure may be a gene-specific phenomenon. Published 2001 Wiley-Liss, Inc.†

Key words: diethylstilbestrol (DES); methylation; Hoxa-11; Hoxa-10; uterus

INTRODUCTION

Environmental exposures to a variety of compounds play a significant role in the etiology and/or exacerbation of many diseases in women. Among them, estrogenic compounds are implicated as causal agents in many diseases of the uterus and the breast, including cancer. Women exposed to the potent synthetic estrogen diethylstilbestrol (DES) at a critical period of their fetal lives develop a number of reproductive tract abnormalities, including, in some cases, vaginal adenocarcinoma, a rare genital tract lesion [1]. However, the molecular mechanisms underlying developmentally induced cancers in animals and humans remain elusive.

No definitive evidence exists that DES induces genetic changes resulting in later deformities or tumor development of the reproductive organs later in life [2,3]. However, DES causes alteration in expression of genes that are associated with the development of the reproductive organs. DES potently represses expression of *abdominal B Hoxa* genes in the developing reproductive tract in mouse [4,5]. Targeted disruption of *Hoxa-10*, *Hoxa-11*, and *Hoxa-13* results in region-specific developmental defects along the reproductive tract that are similar to those induced by neonatal DES exposure, suggesting that deregulation of *Hoxa* gene expression constitutes a major mechanism for DES teratogenicity [4,6–8]. DES treatment in neonatal mouse also leads to

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Abbreviations: DES, diethylstilbestrol; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; C/EBP, CCAAT enhancer-binding protein; ERE, estrogen response element; PRE, progesterone response element.

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permanent activation of lactoferrin gene expression in the uterus [9,10]. While the function of lactoferrin in the development of reproductive organs and tumorigenesis remains to be elucidated, our studies have shown that one possible mechanism for DES-induced alteration in gene expression may involve modification of the promoter methylation pattern of a target gene, such as the lactoferrin gene [11]. However, it is not clear whether this mechanism represents an isolated scenario or reflects a more general mode of DES function.

Similar to permanent activation of lactoferrin by DES, expression of *Hoxa-10* and *Hoxa-11* gene can be repressed by DES and continues to be repressed in adult uterus by developmental DES exposure [4,5,12]. Alterations in the *Hoxa* gene promoter methylation may be relevant. Here, we tested this hypothesis by examining the effect of DES on the methylation patterns of *Hoxa-10* and *Hoxa-11* promoters. Our data showed that *Hoxa-10* and *Hoxa-11* promoters are generally unmethylated in mouse uterus and neonatal DES exposure does not alter the methylation state of these promoters. Thus, it appears that DES-induced methylation changes may not be a general mechanism for DES action and could very likely be gene specific.

MATERIALS AND METHODS

Cloning and Characterization of the *Hoxa-10* Gene Promoter

The genomic Walker kit was purchased from Clontech (Palo Alto, CA). Based on the known *Hoxa-10* 5′ cDNA (NCBI Accession No. *L08758*) [6], gene-specific primers (GSP1 and GSP2) were designed: GSP1, 5′-GGTTTATAGCGGCGCATTCCAAATAT and GSP2, 5′-TGGACGGGTTTACGACCACATTGT. Following the manufacturer's protocols, amplified PCR products were cloned into the pCR cloning vector (TA cloning kit, Invitrogen, Carlsbad, CA) and sequenced. To identify the 5′ end of the a10-1 transcript of *Hoxa-10*, we designed an oligo PE1, 5′-TTTATAGCGGCGCATTCCAAATATGCAAAT, and performed 5′ rapid amplification of cDNA ends (RACE) using the Marathon cDNA Amplification Kit (Clontech), according to the manufacturer's protocol.

Constructs

Different lengths of the cloned 5′ flanking fragment were inserted into pSEAP-Basic vector (Clontech). Briefly, different sized fragments were amplified by polymerase chain reaction (PCR). For cloning purposes, an XhoI site was added to the 5′ end and a Hin*d*III site to the 3′ end. Then the pSEAP-Basic vector and the amplified fragments were digested by XhoI and Hin*d*III, respectively. After purification, the amplified fragments were ligated with the vector. All deletion constructs were sequence approved and purified for transfection assay.

Cell Transfection and pSEAP Assay

Human endometrial carcinoma cell line Ishikawa were grown in a 60-mm dish in 5 mL of Dulbecco's modified Eagle's medium with F-12 Ham (Sigma, St. Louis, MO) containing 10% fetal bovine serum and antibiotics at 37°C and 5% CO_2 in an incubator. Cell transfection was done using Effectene Transfection Kit (Qiagen, Valencia, CA). When cells were 60–80% confluent, 1 μg of plasmid DNA (final concentration 0.1 μg/μL) was diluted with the DNA-condensation buffer, Buffer EC, to a total volume of 150 μl. Eight microliters of Enhancer reagent was added and mixed by vortexing for 1 s. The mixture was then incubated at room temperature (20–25°C) for 2–5 min. Twenty-five microliters of Effectene Transfection reagent was added to the DNA-Enhancer mixture. Samples were then incubated for 5–10 min at room temperature (20–25°C) to allow complex formation. Meanwhile, growth medium from the plate was gently aspirated and cells washed once with PBS. Four milliliters of fresh growth medium was added to the cells. One milliliter of cell growth medium was added to the reaction tube containing the transfection complexes. Cells were incubated with the complexes at 37°C and 5% CO_2 for 72 h to allow gene expression. Finally, media were collected for pSEAP activity assay according to the manufacturer's instructions.

Animals and DES Treatments

CD-1 mice [Crl:CD-1(ICR)BR] (purchased from Charles River, Raleigh, NC) were injected with 2 μg/pup/day of DES (Sigma) for 5 consecutive days from postnatal day 1–5. The treated mice were killed at days 5, 17, 21, or 30 to obtain uterine tissues. At least 20 uteri were pooled for each experimental group. For long-term uterine tumor development, animals were allowed to age to 18 mo, when they were sacrificed and uterine tissues removed and processed. Mouse genomic DNA was isolated from uterine tissues using the sodium dodecyl sulfate/proteinase K method [13].

Genomic DNA Preparation and Sequencing of the Sodium Bisulfite–Treated *Hoxa-10* and *Hoxa-11* Promoter

Genomic DNA was digested with EcoRI restriction enzyme and denatured by sodium hydroxide. For deamination, alkaline-denatured DNA (10 μg) was incubated with 3.1 M freshly prepared sodium bisulfite, pH 5.0, for 16 h at 50°C and purified using DNA Clean-Up kit (Promega, Madison, WI). The purified DNA was again denatured with 0.3 N sodium hydroxide, neutralized with ammonium acetate, and precipitated with 2 vol of ethanol. The primers 5′ATATATTGAGAAGTATAAGGGTT

and 5′TTTACAACCACATTATCACAACCATCA (from nt −171 to +103) were used to amplify the deaminated *Hoxa-10* promoter region, giving a product of 274 bp containing eight CpG sites, located at nt −97, −87, −73, +26, +29, +33, +37, and +39 (Figure 1, in bold). For amplification of the *Hoxa-11* promoter, primers of 5′TGAAGAAGG-TGTTGAATGTAAGTT and 5′TCCTCAACTTCCTTT-CTTTATAAC were used to amplify a 349-bp fragment (nt −533 to nt −184, NCBI Accession No. *U20371*), containing 19 CpGs located at nt −507, −493, −470, −422, −405, −393, −382, −367, −327, −321, −303, −301, −299, −267, −264, −258, −250, −238, and −231 (Figure 3, in bold).

Statistical Analysis on Promoter CpG Methylation

For each experimental group, 20 *Hoxa-10* and *Hoxa-11* promoter sequences were analyzed for methylation changes. Since there are eight CpGs in *Hoxa-10* and 19 CpGs in *Hoxa-11* proximal promoter regions altogether, we analyzed $20 \times 8 = 160$ CpG sites in the *Hoxa-10* promoter and $20 \times 19 = 380$ CpG sites in the *Hoxa-11* gene promoter in each experimental group. The percentage of unmethylated CpGs was calculated by the number of unmethylated CpGs divided by the total number of CpGs analyzed. Fisher's Exact Test was used to calculate the statistical differences between

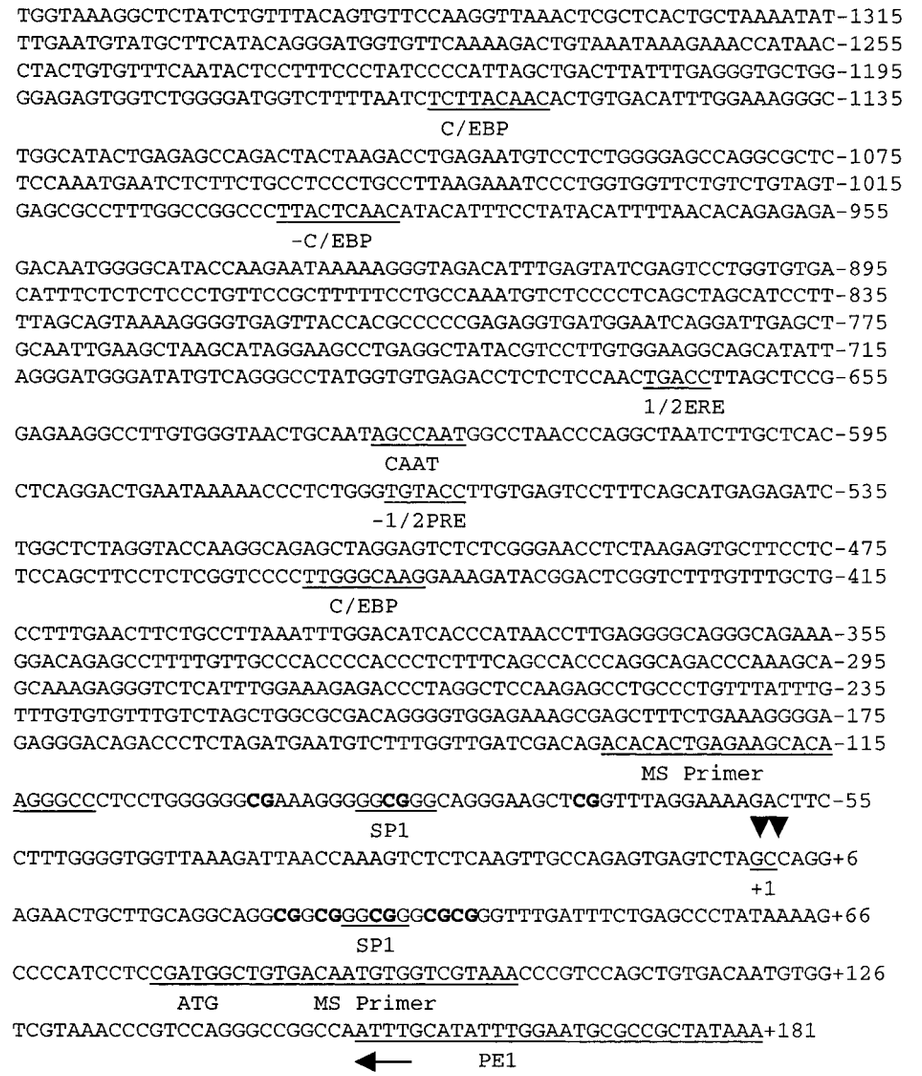

Figure 1. *Hoxa-10* gene 5′ untranslated region. Transcription factor binding motifs, including three C/EPB motifs at nt −1164, −995, and −453, one CAAT site at nt −628; one half ERE at nt −678; one half PRE at nt −567; and two Sp1 sites at −89 and +30 are indicated. The 5′ end of *a10-1* is located at either +1 or +2, indicated by arrows, determined by 5′ RACE experiment using primer PE1. The positions of methylation-specific (MS) primers are underlined. Eight CpG sites within the methylation primers are in bold.

different experimental groups. The level of significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Cloning and Characterization of the *Hoxa-10* Promoter

Using genomic walking, we cloned a 1485-bp DNA fragment containing sequences upstream of exon1a of the *Hoxa-10* gene, including the putative *Hoxa-10* promoter region. The sequence is shown in Figure 1 (NCBI Accession No. *AF246720*). Sequence analysis of this fragment revealed several transcription factor binding motifs, including three CCAAT enhancer-binding protein (C/EPB) motifs at nt − 1164, − 995, and − 453; one CAAT site at nt − 628; one half estrogen response element (ERE) at nt − 678; one half progesterone response element (PRE) at nt − 567; and two SP1 sites at nt − 89 and +30. To map the 5′ end of transcript *a10-1*, we used a primer located in exon1a of the *Hoxa-10* gene to perform 5′ RACE. After linker ligation and PCR amplification, a 150-bp PCR product was obtained, gel-purified, and sequenced. The sequence of the PCR fragment placed the 5′ end of *a10-1* transcript at +2 (Figure 1). A blast search using the 5′ untranslated region of *a10-1* against the mouse expressed sequence tags database revealed one clone (Accession No. *BF784828*) whose sequence ended one base 5′ to our RACE product. Thus, the start of this EST clone represents the 5′-most cDNA for the *a10-1* transcript. We therefore designate this base as +1 for *a10-1* transcription start site (arrow, Figure 1). Sequence analysis of the proximal *Hoxa-10* promoter did not reveal any TATA box; however, a GC-rich region was found around 100 bp upstream of the transcription start site including an Sp1 site at nt − 89, suggesting that transcription of 10-1 transcript of the *Hoxa-10* gene may be under the control of a TATA-less promoter.

To determine whether sequences upstream of the *Hoxa-10* transcription start site have promoter activity, we made deletion constructs by inserting varying length DNA fragments spanning the putative promoter into pSEAP basic vector (Clontech). These constructs were named pSEAP-F1 (nt − 1374 to +78 (1 452 bp)); pSEAP-F2 (nt − 622 to +78 (700 bp)); pSEAP-F3 (nt − 122 to +78 (200 bp)); pSEAP-F4 (nt − 22 to +78 (100 bp)) (Figure 2). The promoter activity was assayed by transfecting deletion constructs into Ishikawa cells. The pSEAP2-Control vector with simian virus 40 promoter, provided by the manufacturer (Clontech), was used as a positive control and the pSEAP basic vector was used as a negative control. As shown in Figure 2, the negative control vector, pSEAP basic vector, showed no promoter activity. In contrast, while pSAEP-F1, F2, and F4 showed weak or no promoter activity, the pSAEP-F3 construct showed significant levels of promoter activity, equal to about 30% of that shown by the positive control vector. Together, the 5′ RACE and the transfection experiments provided strong evidence that the *Hoxa-10* promoter resides within 122 bp upstream of the *a10-1* transcription start site.

DES and the Methylation Patterns of the *Hoxa-10* and *Hoxa-11* Promoters

DNA methylation has been shown to regulate a variety of aspects of cellular physiology, such as growth and differentiation, through alteration in gene expression [14,15]. Our previous results on the

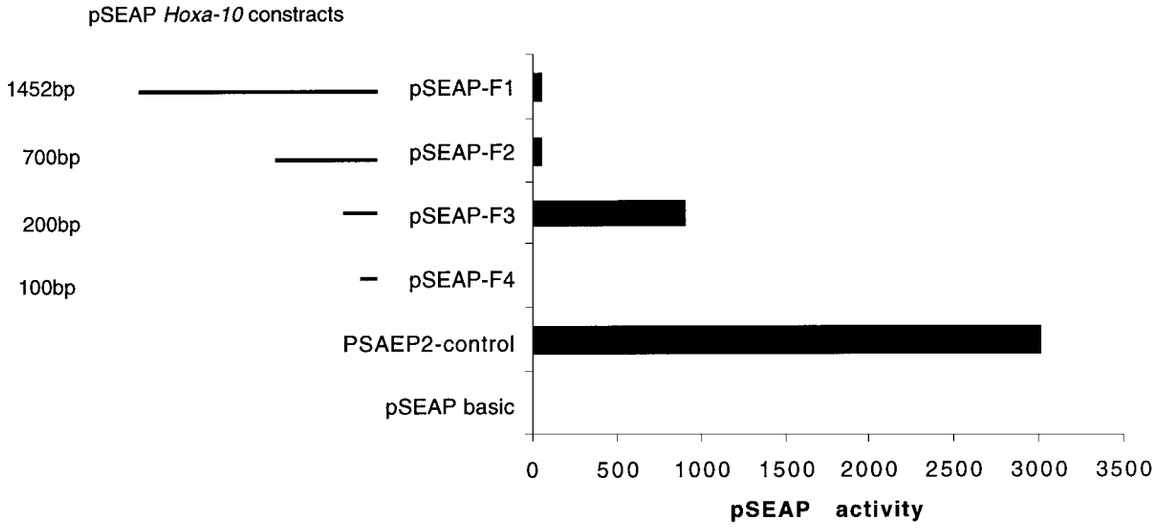

Figure 2. pSEAP activity in deleted fragments of *Hox a-10* 5′ untranslated region. The constructs were made by inserting different lengths of the cloned 5′ flanking fragment into pSEAP-Basic vector, named pSEAP-F1 (1 452 bp); pSEAP-F2 (700 bp); pSEAP-F3 (200 bp) and pSEAP-F4 (100 bp). The promoter activity was assayed by transfecting constructs into Ishikawa cells. The pSEAP2-Control vector with a simian virus 40 promoter was used as a positive control and the pSEAP basic vector was used as a negative control.

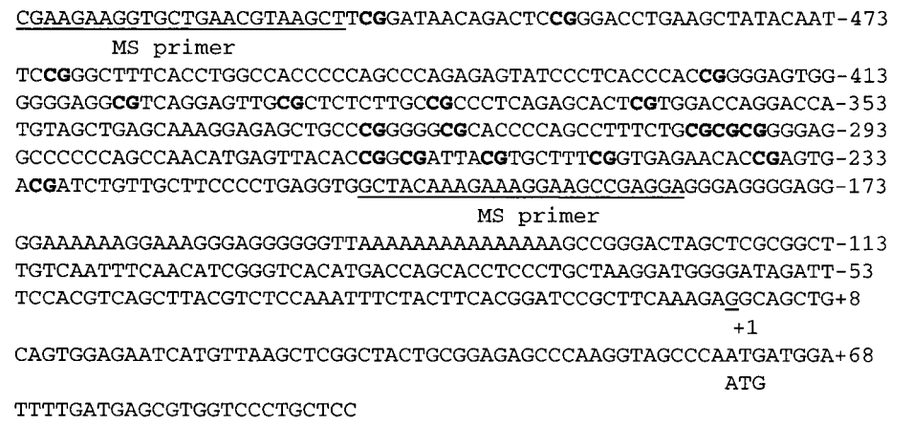

Figure 3. *Hoxa-11* gene 5′ promoter region. The positions of methylation-specific (MS) primers are underlined. The 19 CpG sites within the methylation primers are in bold. The sequences were drawn from GenBank, NCBI Accession No. *U20371*.

regulation of lactoferrin gene expression by DES demonstrated that DES does so by inducing permanent demethylation around the lactoferrin promoter [11]. Thus, in addition to a direct transcriptional regulatory mechanism through estrogen receptors, DES can also alter gene expression via epigenetic pathways, such as changing the promoter methylation status. To determine whether a change in methylation patterns in the *Hoxa-10* and *Hoxa-11* promoters also contributes to deregulation of their uterine expression by DES, we analyzed the methylation status of eight CpG sites in the *Hoxa-10* and 19 CpG sites in the *Hoxa-11* proximal promoters. Twenty sequences in each animal group, a total of 160 CpG sites in *Hoxa-10* and 380 CpG sites in the *Hoxa-11* gene promoter, were analyzed for methylation. Percentages of unmethylated CpGs in both the *Hoxa-10* and *Hoxa-11* promoters were calculated and are shown in Figure 4. Interestingly, almost all the CpGs in the *Hoxa-10* and *Hoxa-11* proximal promoters are unmethylated in control groups.

To assess whether perinatal DES exposure alters the unmethylated state of these CpG sites during uterine development, genomic DNAs were prepared from the uteri of 5-, 17-, 21-, or 30-d-old mice treated neonatally with DES. These CpGs are unmethylated at a high percentage ($>95\%$) in all four experimental groups. Repression of *Hoxa-10* and *Hoxa-11* after developmental DES exposure has been shown by several laboratories [4,5,12]. This repression seemed to be persistent later in adult animals [12]. Hence, it appears that downregulation of *Hoxa-10* and *Hoxa-11* by neonatal DES exposure is not correlated with a change in promoter methylation. We further examined the methylation state of these CpG sites in uterine tumors induced in neonatally DES-treated mice. Neonatal DES treatment in our hands induces neoplastic lesions in 90% of the female mouse uterus at 18 mo, whereas no tumor was observed in control mice [16]. The tumors induced by DES were endometrial carcinomas by histological examination. As shown in Figure 4, 30% of CpGs in *Hoxa-10* and 19% in *Hoxa-11* were methylated in DNAs from uterine tumors ($P < 0.001$). The methylation of the CpG sites in *Hoxa-10* and *Hoxa-11* is random and not site specific.

Alterations in DNA methylation are associated with a variety of processes, including control of gene expression, change in chromatin structure, and gene imprinting. Existing evidence suggests that DNA methylation also plays a major role in carcinogenesis. Whereas hypermethylation of CpG islands is associated with inactivation of tumor suppressor genes [14,15], hypomethylation is linked to aberrant increases in the transcription of oncogene [17]. It was shown that even a single basepair change in a restricted CpG site can have a dramatic effect on gene expression [18]. We have found that DNA methylation of a CpG site in the lactoferrin promoter is specifically altered in response to neonatal exposure to DES [11]. Methylation of a CpG site could interfere with the binding of a transcription factor [18] and, in this case, a transcriptional repressor that normally shuts off gene expression. In the present study, however, we failed to observe any changes in methylation in either the *Hoxa-10* or *Hoxa-11* proximal promoter. A significant increase in methylation of these two promoters was only observed in DES-induced uterine carcinoma in the mouse. These results argue against an involvement of methylation in the deregulation *Hoxa-10* and *Hoxa-11* by DES.

Neonatal DES exposure can provoke permanent changes in gene expression, such as in c-*fos*, lactoferrin, *Hoxa-10*, and *Hoxa-11* in uterus [12,19–21]. At present, the molecular mechanism by which these permanent changes occur remains unclear. Persistently elevated lactoferrin expression in the uteri of neonatally DES-treated mice is

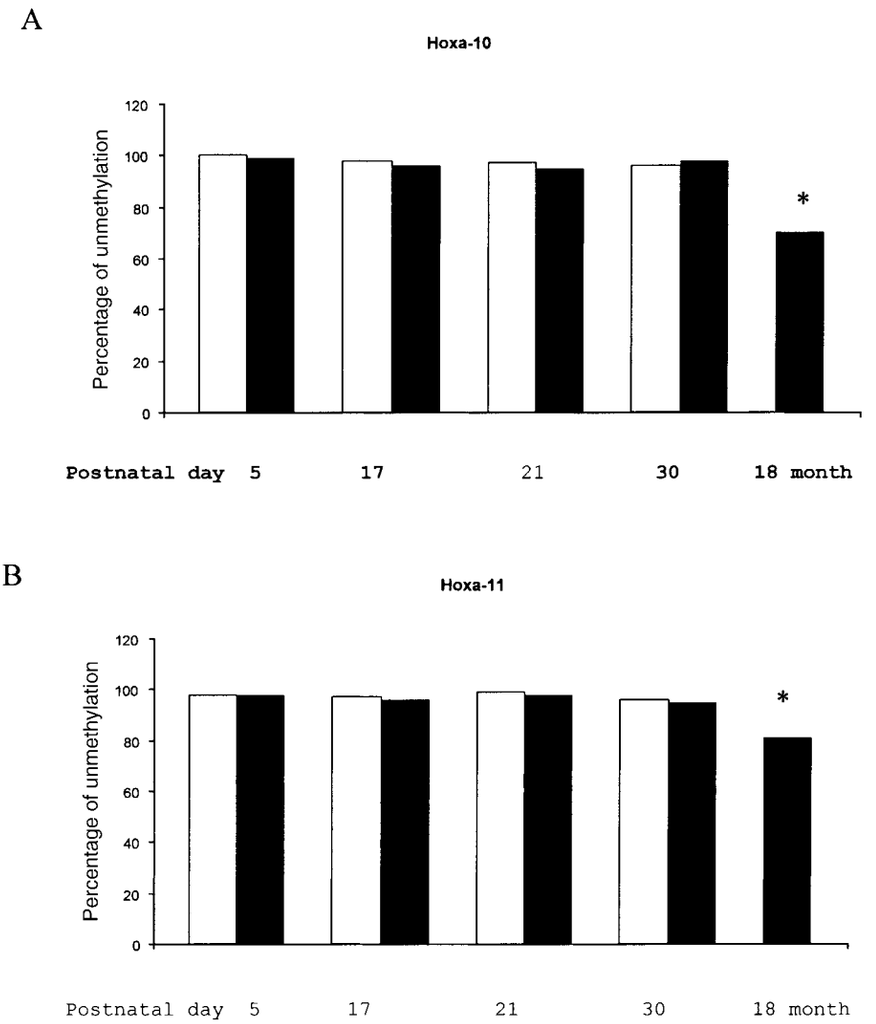

Figure 4. Percentage of unmethylation at all analyzed CpG sites in mouse *Hoxa-10* and *Hoxa-11* gene promoters. Uterine genomic DNAs prepared from the 20 pooled uteri of each neonatal DES-treated (closed bars) or nontreated control (open bars) mice, were treated by sodium bisulfite, amplified, cloned, and sequenced. Percentage of unmethylation is presented as percentage of the number of unmethylated CpGs to the total number of sequences analyzed. A total of 160 CpG sites in *Hoxa-10* gene promoter and 380 CpG sites in *Hoxa-11* gene promoter were analyzed in each group (see Materials and Methods). DNA from uterine carcinoma is designated as the postnatal 18-mo time point. (A) Percentage of unmethylation in *Hoxa-10* promoter region. (B) Percentage of unmethylation in *Hoxa-11* promoter region. $*P < 0.01$.

associated with a demethylation change in its promoter, whereas no change in methylation was detected in *Hoxa-10* and *Hoxa-11* promoters after DES treatment. Therefore, it appears that gene imprinting by developmental DES exposure may not be a general mechanism affecting all genes, but a selective one relevant to some genes. An alternative explanation for our results is that gene imprinting by DES may occur in places other than the proximal promoter regions, such as around ERE, located either further upstream of the promoter or in the introns of a gene [22]. So far, it is not clear whether DES or E2 directly regulates *Hoxa-10* and *Hoxa-11* gene expression. Ma et al. [4] have demonstrated that the 17 β-estradiol repression of *Hoxa-10* during implantation is cycloheximide resistant, suggesting a direct regulation. Moreover, potential EREs exist in the *Hoxa-11/a-10* genomic region which could be functional [4]. Here, we also found a half ERE and a half PRE site in the *Hoxa-10* proximal promoter at positions nt −678 and −567. Couse et al. [12] recently demonstrated that DES repression of *Hoxa-10* and *Hoxa-11* requires a functional estrogen receptor-α, providing additional evidence for a direct regulation. Nonetheless, a demonstration for a direct transcriptional repression by estrogen receptor-α on these two *Hox* genes is still lacking.

In conclusion, cloning of the *Hoxa-10* gene promoter provided a tool for further molecular dissection of the regulation of this gene. Methylation analyses of the *Hoxa-10* and *Hoxa-11* promoters indicated that these promoters are highly unmethy-

lated in the uterus and that developmental DES exposure does not alter this pattern. Gene imprinting by developmental DES exposure may be a gene-specific phenomenon.

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