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Developmental Exposure to Diethylstilbestrol Elicits Demethylation of Estrogen-responsive Lactoferrin Gene in Mouse Uterus

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ABSTRACT

Alteration of DNA demethylation in five CpG sites (−547, −533, −475, −464, and −454) immediately upstream from the estrogen response element of lactoferrin promoter was determined in the uteri of immature (17-day-old) and mature (21- and 30-day-old) mice treated neonatally with DES. Only the CpG/−464 was found to be abnormally demethylated by diethylstilbestrol (DES) treatment in the mature uteri. This abnormal demethylation occurred in specific response to DES in neonatal mice, because DES injected into the 30-day-old mature mice did not demethylate CpG/−464. This site, however, remained methylated in the neonatally DES-treated/ovariectomized mice, indicating that this DES-elicited demethylation is under hormonal control. Thus, neonatal DES treatment appeared to imprint an abnormal, site-specific demethylation of CpG/−464, which requires ovarian hormones to occur in adult mice. Moreover, the demethylation was maintained in uterine tumors of the neonatally DES-treated mice. This mode of demethylation is reminiscent of uterine tumor formation, which also depends on both neonatal DES exposure and ovarian hormone stimulation in adulthood. Thus, neonatal DES treatment may induce tumor formation as well as demethylation through a common cellular process.

INTRODUCTION

Women exposed at a critical period of their fetal life to the potent synthetic estrogen DES[2] develop a number of reproductive tract abnormalities and, in some cases, a rare genital tract lesion, vaginal adenocarcinoma (1). Prenatal exposure to DES during critical stages of development has also resulted in a low incidence of vaginal adenocarcinoma in mice (2). Moreover, when mice were treated with DES for the first 5 days of neonatal life, a high incidence of uterine adenocarcinoma was seen (3). Although DES has been shown to neoplastically transform cells in culture (4, 5), the molecular events underlying developmentally induced cancers in animals and humans are unresolved. Earlier studies have shown that tumors derived from developmentally DES-exposed mice or women do not express detectable mutations in the common oncogene *ras* or the tumor suppressor gene *p53*. There were, however, indications of genomic instability in the form of nucleotide repeat sequences in these tumors (6).

We have chosen the lactoferrin gene to examine a persistent alteration ("programming" or "imprinting") of genes caused by estrogen early in the uterine development and tumorigenic process. Lactoferrin is a member of an iron-binding glycoprotein family, which is expressed in various tissues, including the mammary gland and reproductive organs (7, 8). The expression of lactoferrin gene is regulated by estrogens in the uterine epithelial cells of adult mice (9–12). In the neonatally DES-treated mice, however, this gene is expressed abnormally following neonatal treatment in epithelial cells in the immature mice (7). Moreover, this abnormal expression appears to persist in the neonatally DES-treated adult mice ovariectomized at 39 days of age, which indicates that it is no longer under regulation by estrogen in these mice (8).

The abnormal overexpression of lactoferrin in the uteri of mice treated neonatally with DES suggests a developmentally induced structural alteration in its gene. Normal as well as abnormal cell growth and differentiation are often under the control of DNA methylation patterns (13–16). Moreover, abnormal alteration of DNA methylation is suggested as an important factor that generally plays a critical role in tumor development (17–23). Most directly, for example, the intestinal neoplasia caused by the adenomatosis polyposis coli (*Apc*) gene is suppressed by DNA hypomethylation (19). A CpG island in the estrogen receptor gene is hypermethylated in human breast cancer cells (20), and the hypermethylation of the CpG island is also reported to link with the earliest disposition to sporadic colorectal tumorigenesis in humans (21).

In an effort to find additional molecular markers for DES-dependent alteration in uterine tissue, we have examined DNA methylation of the lactoferrin promoter to determine whether methylation of CpG sites patterns is specifically altered in response to neonatal exposure to DES. Our results suggest that changes in methylation status may be an early event in, or indicative of, the tumorigenic process induced by DES.

MATERIALS AND METHODS

Animals and DES Treatments. For the neonatal DES treatment, newborn CD-1 mice [Crl:CD-1(ICR)BR; purchased from Charles River Breeding Laboratories, Raleigh, NC) were injected with 2 μg/mouse/day of DES (Sigma Chemical Co., St. Louis, MO) for 5 consecutive days from postnatal day 1 through day 5. The treated mice were sacrificed at days 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 months, when they were sacrificed, and three uteri with endometrial cystic hyperplasia were removed and processed. To determine the effect caused by DES injected in mature animals, DES (2 μg/kg of body weight/day) was injected daily from days 30–34 and sacrificed at day 35. For controls, mice were injected with sesame seed oil. Mouse genomic DNA was isolated from the uterine tissues using the SDS-proteinase K method (24). To verify the effect of ovarian hormones on the lactoferrin gene, groups of mice, control or DES-treated, were ovariectomized at day 15 and sacrificed at day 21 to prepare genomic DNA.

Preparation of Genomic DNA and Sequencing of the Sodium Bisulfite-treated Lactoferrin Promoter. Genomic DNA was digested with *Eco*RI and denatured by sodium hydroxide. For deamination (25), alkaline-denatured DNA (10 μg) was incubated with 3.1 M freshly prepared sodium bisulfite (pH 5.0) for 48 h at 50°C and purified using the DNA Clean-Up kit (Promega, Madison, WI). The purified DNA was again denatured with 0.3 N sodium hydroxide, neutralized with ammonium acetate, and then precipitated by 2 volumes of ethanol. The following oligonuclotide primers were used to amplify a region of the deaminated sequence (from −592 to −418) of the lactoferrin promoter (10): 5′-GGTTAATTTTGGGTGGTATTT and 5′-AAACAACTACTCAAACCTAACCAC for the 5′ and 3′ primers, respectively.

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[2] The abbreviation used is: DES, diethylstilbestrol.

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The amplified DNAs were purified using the PCR Prep kit (Promega, Madison, WI) and cloned into M13mp19 vectors for sequencing.

Genomic Southern Hybridization. Genomic DNAs (10 μg) were digested first with *Hinc*II and *Hph*I, followed by further digestion with either *Msp*I or *Hpa*II. These DNA digests were resolved on 1.5% agarose gel, transferred onto a Hybond-N$^+$ nylon filter (Amersham Corp., Arlington Heights, IL), and hybridized with the ^{32}P-labeled *Hinc*II-*Hph*I fragment (−590/−47) of the lactoferrin gene using Quik-Hyb hybridization solution (Stratagene, La Jolla, CA).

RESULTS

CpG Sites in Lactoferrin Promoter. There are five CpG sites within the region from −590 to −330 of the lactoferrin promoter at positions −547, −533, −475, −464, and −454 that reside immediately upstream from the estrogen response element (Fig. 1). To determine the methylation status of these cytosines, a 172-bp DNA fragment including the five CpG sites was deaminated *in vitro* by sodium bisulfite, amplified, cloned and sequenced. Fig. 2 depicts sequences of normal (Fig. 2*A*) and deaminated (Fig. 2*B*) DNAs from the uterine tissues of 5-day-old mice. All other cytosines except those in the five CpG sites were converted into thymidines by the bisulfite deamination treatment. The results, therefore, indicate that the five CpG sites are methylated completely in the uteri of 5-day-old mice.

Neonatal DES Exposure and Demethylation. To examine whether exposure to DES altered the demethylation of these CpG sites during uterine development, genomic DNAs were prepared from the uteri of 17-, 21-, or 30-day-old mice treated neonatally with DES (Fig. 3*A*). The upstream three CpG sites at positions −547, −533, and −475 were found to be demethylated at a high level (>74%) in the 17-day-old immature uteri of both the control and DES-treated mice (Fig. 4*A*). As expected, these CpG sites were also demethylated in uteri of 21- and 30-day-old mature mice. Thus, demethylation at these three CpG sites of the lactoferrin gene is apparently a normal developmental process in the mouse uterus, and neonatal DES exposure did not alter the demethylation. The two downstream CpG sites at positions −464 and −454 were heavily methylated in the immature as well as the mature uteri of the control mice. CpG/−464, however, was demethylated in the mature uteri of neonatally DES-treated mice (Fig. 4*A*). The degree of this demethylation at −464 was more than 90% in the 30-day-old mice, which is increased 9-fold compared to the less than 10% demethylation in the 21-day control mice. This large increase suggested that the demethylation occurred not only in the uterine epithelial cells but also in the other types of uterine cells. On the contrary, CpG/−454 remained methylated in the mature uteri regardless of the neonatal DES treatment (approximately 20% demethylation). These methylations of CpG/−454 were also confirmed by an additional experiment using the digestion of genomic DNA by the methylation-sensitive and -insensitive restriction enzymes *Hpa*II and *Msp*I, respectively (data not shown). Fig. 5 compares the normal methylation patterns of the lactoferrin promoter at the five CpG sites with the DES-altered pattern. These results indicate that neonatal DES exposure specifically demethylates CpG/−464 in mature uterine tissues.

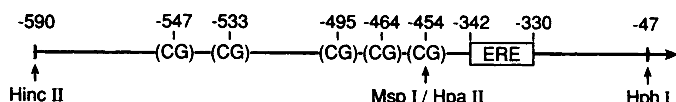

Fig. 1. Map of CpG sites in the lactoferrin promoter. The CpG sites, estrogen response element (*ERE*), and restriction endonuclease-digestion sites are shown, based on data obtained in earlier articles (10, 28).

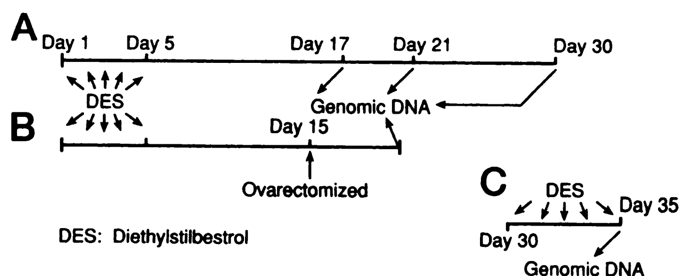

Fig. 3. Protocols for DES treatment.

Effect of Endogenous Hormones on the Neonatal DES-dependent Demethylation. To examine whether the abnormal demethylation of CpG/−464 required endogenous hormones during the uterine development, neonatally DES-treated mice were ovariectomized on day 15 and sacrificed on day 21 (Fig. 3*B*). CpG demethylation of the lactoferrin promoter was analyzed (Fig. 3*B*). The five CpG sites in the ovariectomized/control mice remained methylated, which indicates that the normal developmental demethylation at CpG sites −547, −535, and −475 in the lactoferrin promoter is under the control of ovarian hormones (Fig. 4*B*). Surprisingly, CpG/−464 also remained methylated in the uteri of the ovariectomized/DES-treated mice. The demethylation induced by neonatal DES exposure of CpG/−464 appears to be governed by ovarian hormones in adulthood.

DES Exposure in Adult Mice. To determine whether the demethylation of CpG/−464 is specific to the neonatal DES treatment, 30-day-old mice were treated daily with DES for 5 consecutive days and then sacrificed to prepare uterine DNA on day 35 (Fig. 3*C*). CpG/−464 and CpG/−454 were found to be methylated in these mice, whereas CpG sites at positions −541, −533, and −475 were demethylated (Fig. 4*C*). Consequently, the methylation/demethylation patterns of these five CpG sites were identical to that observed in the

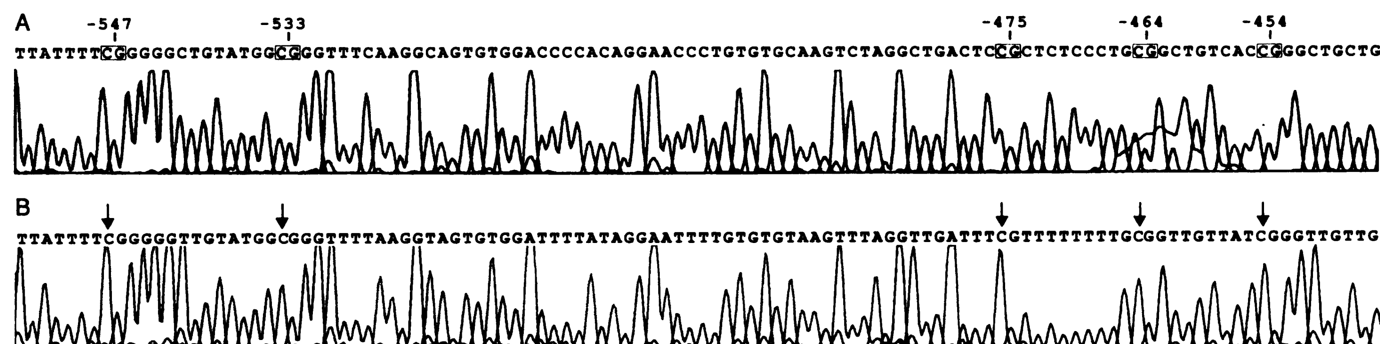

Fig. 2. DNA sequences of normal and deaminated lactoferrin promoter. Genomic DNA was prepared from uterine tissues of 5-day-old mice. Normal (*A*) and deaminated (*B*) DNA fragments were amplified, cloned, and sequenced as described in "Materials and Methods." *Boxes*, the five CpG sites; *number above each box*, position in the lactoferrin promoter; *arrows*, methylated cytosines.

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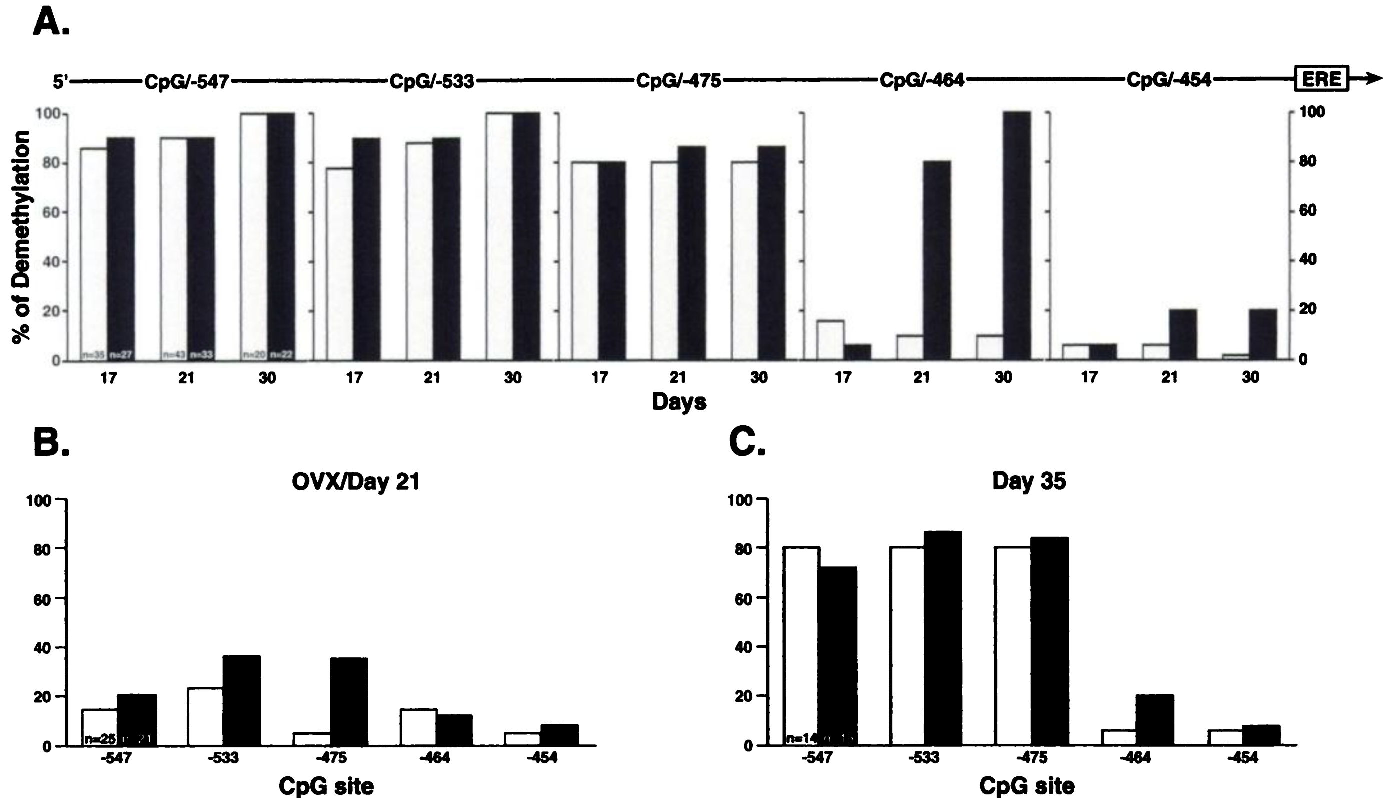

Fig. 4. Degree of demethylation at each CpG site in response to DES. Uterine DNAs prepared from the pooled uteri of all DES-treated (■) or nontreated control (□) mice were treated by sodium bisulfite, amplified, cloned, and sequenced. Degree of demethylation at each site is presented as percentage of the number of demethylated sequences to the total number (*n*) of sequences analyzed. These numbers are indicated only for the CpG/−547 site and apply to all other sites shown in each figure part. *A*, demethylation in neonatally DES-treated and control mice. Mice were treated by DES, as shown in Fig. 3*A*. *ERE*, estrogen response element. *B*, demethylation in the DES-treated/ovariectomized mice (see Fig. 3*B* for animal treatment). *C*, demethylation in mice treated by DES in adulthood (see Fig. 3*C* for animal treatment).

normal mature mice. CpG/−464, which was demethylated by neonatal DES treatment, remained methylated in the 35-day-old mice. The results indicate, therefore, that mature mice exposed to DES under these conditions do not alter the demethylation of the lactoferrin promoter.

Demethylation in Uterine Tumors. We examined whether the abnormal demethylation of CpG/−464 was maintained in uterine tumors of the neonatally DES-treated mice. As shown in Table 1, CpG/−464 of the lactoferrin gene in the tumor tissues was found to be completely demethylated, similarly to the uterine tissues of the neonatally DES-treated 21-day-old mice. These results indicate, therefore, that the demethylation is maintained in the tumors. At the other CpG sites, CpG/−475 in the tumors was less methylated, whereas CpG/−454 became more demethylated compared with those in the uterine tissues of the 21-day-old mice. The demethylation at CpG/−475 and methylation at CpG/−454 were indicative of a tumor-specific alteration of DNA methylation and also served as an excellent internal control for the persistent demethylation of CpG/−464 in the tumors.

Table 1 *Degree of demethylation in uterine tumors in DES-exposed mice*[a]

CpG sites	−547	−533	−475	−464	−454
Demethylation (%)	88	75	50	81	43

[a] Uteri with endometrial cystic hyperplasia caused by neonatal DES treatment were taken from three mice at the age of 18 months. Genomic DNA was isolated from a pool of those mice and chemically deaminated, cloned, and sequenced as described in "Materials and Methods." Degree of demethylation at each CpG site is the number of demethylated CpG expressed as percentage of the total number of the 17 sequences.

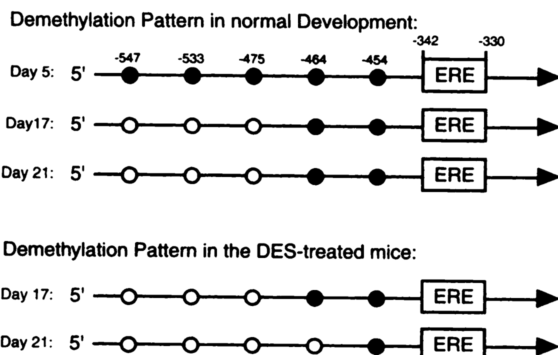

Fig. 5. Schematic representation of normal and abnormal demethylations of lactoferrin promoter. The demethylation patterns are based on the results shown in Fig. 4*A*. *ERE*, estrogen response element.

DISCUSSION

The abnormal demethylation of CpG/−464 in the lactoferrin promoter occurs specifically in response to neonatal DES exposure and adult ovarian hormones. Moreover, the demethylation is observed continuously in the uterine tumors of the neonatally DES-treated mice. This finding is the first example that estrogenic xenobiotics can abnormally demethylate DNA sequence during organ development.

Neonatal DES treatment is known to cause the development of uterine tumors in adult mice, and this tumor development also depends on continuous exposure to ovarian hormones (3, 26), which is reminiscent of the demethylation process of CpG/−464 in lactoferrin promoter: both require exposure to DES in the neonates and ovarian hormones in adulthood. This apparent correlation suggests that a

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common cellular process that is altered in response to DES may be involved in both tumor formation and DNA demethylation in mouse uteri. CpG/−464 demethylation seems to be organ specific to the uterus. It does not occur, at least, in the livers of the DES-treated mice (data not shown). DES-dependent tumors are also induced preferentially in the mouse uterus, providing additional evidence to support the hypothesis that a common factor may regulate both demethylation and tumorigenesis. It remains to be seen, however, whether this type of abnormal alteration of DNA methylation by a xenobiotic chemical can occur in many other genes, including those involved directly in neoplastic transformation, such as proto-oncogenes. It is noteworthy that neonatal exposure to phytoestrogens was associated with the hypermethylation of c-H-*ras* gene in the rat pancreas (22). Abnormal DNA methylation such as in the case of the CpG/−464 of the lactoferrin promoter, therefore, may occur more frequently than first thought. The postnatal development of the uterus in rodents is approximately equivalent to that in the human fetus at the end of the first trimester of pregnancy. Because transplacental DES exposure during the first trimester is known to cause genital tract abnormalities (1–3), future research may also provide direct evidence that the abnormal alteration of DNA methylation can be a critical factor in the abnormalities, including cancers caused by xenobiotics in humans.

As discussed above, the abnormal demethylation of the lactoferrin gene requires ovarian hormones in adulthood. Nelson *et al.* (8) reported that the expression of lactoferrin persisted in the neonatally DES-treated adult mice ovariectomized at 39 days of age. Although the methylation of lactoferrin gene does not necessarily correlate with the expression of the gene, an apparent discrepancy in the results obtained from these studies may be due to the different ages when ovariectomy was performed. For our present methylation study, ovariectomy was done at the 15th day of age (prepuberty), whereas it was done at the 39th day of age (puberty) in the study by Nelson *et al.* (8). In an original tumor study, ovariectomy was performed at the 17th day of age (prepuberty), and it was found that the presence of ovarian hormones is essential for development of uterine tumor in neonatally DES-treated mice (3). With respect to the hormone requirement, this may be the reason why the demethylation is correlated with the tumor development but not with the persistent expression of lactoferrin gene. It remains to be investigated whether the demethylation persists in the mice ovariectomized at the 39th day of age.

DNA methylation is known to regulate cellular physiology by altering gene expression and is programmed in the growth and differentiation processes (13–16). Given the caveat that gene regulation of lactoferrin expression has not been studied with respect to methylation, the demethylation of CpG/−464 appears not to be essential for the lactoferrin gene to be expressed and regulated by ovarian hormones, because this CpG site remains methylated in the lactoferrin-expressing uteri of normal adult mice. The role of the methylated CpG/−464 for constitutive or abnormal expression of the lactoferrin gene in the mouse uterus as well as other tissues needs to be investigated in future studies. For example, Walmer *et al.* (27) have reported recently that in human endometrial tumors, expression of lactoferrin is abnormally high. Interestingly, a CpG site is present within the estrogen-responsive element of the human lactoferrin gene, which may provide a better model to study the DES regulation mechanism of the methylation and expression.

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