

# Neonatal Diethylstilbestrol Exposure Induces Persistent Elevation of *c-fos* Expression and Hypomethylation in Its Exon-4 in Mouse Uterus

Shuanfang Li,<sup>1</sup> Roberta Hansman,<sup>1</sup> Retha Newbold,<sup>2</sup> Barbara Davis,<sup>3</sup> John A. McLachlan,<sup>4</sup> and J. Carl Barrett<sup>1\*</sup>

<sup>1</sup>Laboratory of Biosystems and Cancer, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

<sup>2</sup>Laboratory of Molecular Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina

<sup>3</sup>Laboratory of Women's Health, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina

<sup>4</sup>Tulane/Xavier Center for Bioenvironmental Research and Department of Pharmacology, Tulane University Medical Center, New Orleans, Louisiana

Perinatal exposure to diethylstilbestrol (DES) induces reproductive tract cancers later in life in both humans and animals. Because there is no clear evidence that perinatal DES exposure induces gene mutation, we proposed that perinatal DES exposure causes epigenetic methylation changes that result in persistent alterations in gene expression, leading to tumorigenesis. The proto-oncogene *c-fos* is one of the immediately induced genes in uterine epithelium after estrogen simulation and a key player in uterine carcinogenesis. Here, we investigated *c-fos* expression in mice neonatally exposed to DES (2 µg/pup/day on postnatal days 1–5). The mRNA levels of *c-fos* in uteri of neonatal DES-treated mice were persistently 1.4–1.9-fold higher than that in the control mice from day 5 to day 60. Overall, the uterine *c-fos* expression level in the neonatal DES-exposed group was significantly higher than that in the control group. After examination of the methylation status of the *c-fos* gene, we found that the CpGs in promoter and intron-1 regions were completely unmethylated. In exon-4, from day 17 to day 60, the percentage of unmethylated CpGs was higher in neonatal DES-exposed mice uteri than that in control (42%, 51%, 47%, and 42% in DES-exposed mice vs 33%, 34%, 33%, and 21% in control mice at day 17, 21, 30, and 60, respectively). These results suggest that perinatal DES exposure may permanently alter gene expression and methylation, and the methylation modification may occur in either the promoter regions or other regulatory sites in the gene. Published 2003 Wiley-Liss, Inc.†

Key words: DES; *c-fos* gene; mice uteri

## INTRODUCTION

Between the 1940s and 1960s, millions of women were exposed to diethylstilbestrol (DES) when their mothers took the drug during pregnancy to prevent miscarriage. Daughters of mothers exposed to DES during the first 3 mo of pregnancy often exhibited changes in the tissue, or structure, or both, of the uterus, cervix, or vagina. These changes resulted in later infertility problems and also placed them at risk of developing clear cell adenocarcinoma of the vagina or cervix at a young age [1–3]. Perinatal exposure to DES also induces reproductive tract abnormalities in laboratory rodents, such as mouse [4–6], rat [7,8], and hamsters [9,10]. When mice are treated neonatally with 2 µg/DES/pup/day for the first 5 d of life, 90% of animals develop uterine adenocarcinoma at age 18 mo [5]. The underlying mechanism by which developmental DES exposure results in permanent reproductive tract anomalies and cancers remains unclear.

Studies from our laboratory and others show that neonatal DES exposure leads to persistent induction

of certain estrogen-regulated genes, which include lactoferrin, epidermal growth factor (EGF), and proto-oncogenes such as *c-fos*, *c-jun*, and *c-myc* [11–15]. Perinatal DES exposure can also lead to persistent repression of *Hoxa-10* and *Hoxa-11*, which are responsible for structural abnormalities in the reproductive tracts [16–18]. However, there is no clear evidence that perinatal DES exposure induces genetic changes that can be linked to later deformity or tumor development, or both [19,20]. Thus, we have proposed that developmental exposure to DES

\*Correspondence to: Laboratory of Biosystems and Cancer, National Cancer Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892.

Received 11 November 2002; Accepted 23 July 2003

Abbreviations: DES, diethylstilbestrol; PCR, polymerase chain reaction; ERE, estrogen response element; bHLH, basic-helix-loop-helix; TCF, T-cell factor.

DOI 10.1002/mc.10147

may alter gene structure by epigenetic changes, such as altered methylation. To test this hypothesis, we focused on methylation analysis of persistently altered genes after perinatal DES exposure [21,22]. We found that neonatal DES exposure resulted in abnormal demethylation of at least one CpG site of the lactoferrin promoter in the mouse uterus [21]. However, the direct relationship between overexpression of lactoferrin and uterine carcinogenesis is not defined. Moreover, although methylation of CpG islands within the 5' promoter and first exon/intron of genes has been studied extensively, methylation alterations of CpGs located in other regions of a gene have seldom been studied. In some cases, such as in p53 and p16, gene expression changes are associated with methylation changes in downstream exons [23–26]. Hence, to define methylation alterations, methylation assays of the entire genomic region of a gene are required.

The proto-oncogene *c-fos* is one of the “immediate early induced genes” in uterine epithelium after estrogen stimulation, and neonatal DES treatment upregulates *c-fos* expression in mouse uterus [27,28]. Overexpression of *c-fos* plays an important role in uterine epithelial proliferation and in uterine tumorigenesis [29,30]. To verify DES-induced modification of methylation also present in this gene, expression and methylation status of the *c-fos* entire genomic region were studied.

## MATERIALS AND METHODS

### Animals and DES Treatments

CD-1 mice [CrI:CD-1(ICR)BR] obtained from the breeding colony at the National Institute of Environmental Health Sciences (Research Triangle Park, NC) were injected with DES (Sigma Chemical Co., St. Louis, MO; 2 µg/pup/day) dissolved in corn oil, or corn oil alone (control) for 5 consecutive days from postnatal day 1–5. Mice were sacrificed at days 5, 8, 17, 21, 30, and 60, to obtain uterine tissues. At least 16 uteri were collected for each group.

### Real-Time Amplification of *c-fos* mRNA

RNA was isolated from eight pooled uteri from each group with the RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol. Tissues were homogenized by passing through both needles and QIAshredders (Qiagen). RNA was eluted off the RNeasy column with RNase-free water. Reverse transcription was carried out with random hexamers, 10× TaqMan RT Buffer, 25 mM MgCl<sub>2</sub>, deoxy NTP mix, RNase inhibitor, Multiscribe Reverse Transcriptase (all Applied Biosystems, Branchburg, NJ), RNase-free water, and 0.25 µg RNA in a total volume of 10 µL. The reverse transcription reactions were incubated at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. To perform real-time polymerase chain reaction (PCR) amplification of *c-fos* cDNA,

probe/primers were designed with Primer Express™ 1.0 (Applied Biosystems, Branchburg, NJ) and optimized. Probe, 6FAM-CCTTCTCAACGACCCT-GAGCCCA-TAMRA, and primers, 5'-TGGCCTCC-CTGGATTGA and 5'-CCACGTTGCTGATGCTC-TTG, were used. TaqMan Universal PCR Master Mix (Applied Biosystems), and RNase-free water were added to 10 µL cDNA for a total volume of 50 µL. Real-time PCR reactions using TaqMan detection (ABI PRISM 770 Sequence Detector and Sequence Detector v1.7 software) were then run. The results were expressed normalized to both the GAPDH endogenous control and an untreated control sample ( $2^{-\Delta\Delta Ct}$ ), where  $\Delta\Delta Ct = (Ct_{c-fos} - Ct_{GAP}) - (Ct_{c-fos} - Ct_{GAP})_{control}$ .

### Genomic Sequencing of the Sodium Bisulfite-Treated *c-fos* Gene

DNA was isolated from the pooled uteri (>16 for each group) using the QIAmp DNA Mini Kit (Qiagen) as per the manufacturer's protocol. Genomic DNA was digested with EcoRI restriction enzyme and denatured by sodium hydroxide. For deamination, alkaline-denatured DNA (5 µg) was incubated with 3.1 M freshly prepared sodium bisulfite, pH 5.0, for 16 h at 50°C and purified with DNA Clean-Up kit (Promega, Madison, WI). The purified DNA was again denatured with 0.3 N sodium hydroxide, neutralized with ammonium acetate, and cleaned by the QIAquick™ Nucleotide Removal Kit purchased from Qiagen. PCR reactions were then performed to amplify the different regions of the *c-fos* gene. Primers of 5'-GGATGTTTATATTAGGATATTTG and 5'-ATAAATAAACTTCCTACATCACTA were used to amplify a fragment of 284 bp in the promoter region, containing 14 CpGs. Primers of 5'-TGGGTTTTTTTGTAAATATATAG and 5'-ACCTCCCAAACCTAATT AACAAC were used to amplify a fragment of 348 bp in the intron-1, containing 18 CpGs. To amplify the exon-4 region, primers of 5'-TTGGAGTTAGTTAAGAGTATTAG and 5'-CACC-TAAAACATATAAATAAT were used, yielding a 276-bp product, containing 6 CpGs. The amplified products were purified using PCR Prep kit (Promega) and cloned into PCR 3.1 vectors (TA cloning Kit, Invitrogen, Carlsbad, CA) for sequencing.

### Statistical Analysis

Differences between the expression of *c-fos* in neonatal DES and control groups were analyzed by the *t*-test. Percentage of unmethylated CpGs was calculated by the number of unmethylated CpGs divided by the total number of CpGs analyzed. At least 10 clones from each experimental group were sequenced and more than six sequences with complete conversion of C to T, except the CpG sites, were calculated. The chi-square test was used to calculate the statistical differences of unmethylation

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

## RESULTS

### Persistent Elevation of *c-fos* mRNA Level in Neonatal DES Mouse Uterus

Quantitative analyses of *c-fos* mRNA levels were conducted using real-time PCR. The results were expressed normalized to both the GAPDH endogenous control and an untreated control sample as  $2^{-\Delta\Delta Ct}$ , where  $\Delta\Delta Ct = (Ct_{c-fos} - Ct_{GAP}) - (Ct_{c-fos} - Ct_{GAP})_{control}$ . Figure 1 summarizes the expression level of *c-fos* in control and neonatal DES exposed groups from neonatal day 5 to neonatal day 60. The mRNA levels of *c-fos* in the uteri of neonatal DES-treated mice were persistently 1.4–1.9-fold higher than that in the control mice from day 5 to day 60. Overall, the uterine *c-fos* expression level in neonatal DES-exposed group was significantly higher than that in the control group.

### Murine *c-fos* Gene Structure

The murine *c-fos* gene consists of four exons with an identified functional estrogen response element (ERE) sequence, GGTCAnnnCAGCC, at the 3' flanking region, 3 kb downstream of the exon-4 [31–33]. By sequence analysis, we found another ERE with the same sequence at intron-1 (Figure 2). The 5' promoter region we examined contains 14 CpGs, the intron-1 region contains 18 CpGs, and the exon-4 region contains 6 CpGs. The sequences and CpG sites analyzed are shown in Figure 2. Two basic-helix-loop-helix (bHLH) sites and two T-cell factor (TCF) sites were found in the exon-4 region by sequence analysis.

### Hypomethylation on Exon 4 of *c-fos* Gene in Neonatal DES-Exposed Mouse Uterus

Methylation analysis was performed on three regions of the *c-fos* gene containing multiple CpG sites, the 5' promoter, first intron, and fourth exon. The promoter and first intron are the regions where CpG islands overlap, and the fourth exon is the region where a dynamic methylation change occurs during development in mouse organs [34,35]. To determine the methylation status of the CpG sites in the three regions of the *c-fos* gene, DNA from each experimental group was extracted and treated with sodium bisulfite, followed by PCR amplification, cloning, and sequencing. The 14 CpGs sites in the 5' promoter and 18 CpGs sites in intron-1 were found to be completely unmethylated ( $\approx 100\%$ ) in both control and DES-treated mice at all ages (day 5 to day 60) examined.

There are six CpG sites in exon-4 of the *c-fos* gene. The percentage of unmethylated CpGs per total CpGs analyzed in *c-fos* exon-4 is shown in Figure 3. In the control mice uteri, the percentage of unmethylated CpGs varied from 43% at day 5 to 34% at day 60. At day 5, the percentage of unmethylated CpGs in the DES-exposed animals was lower than the control animals (29% vs 43%). At day 8, the percentages in control and DES-exposed mice were similar (21% vs 28%). However, from day 17 to day 60, the percentage of unmethylated CpGs in exon-4 of the *c-fos* gene in neonatal DES-exposed mice uteri was higher than that in control (42%, 51%, 47%, and 42% in DES-exposed mice vs 33%, 34%, 33%, and 21% in control mice at day 17, 21, 30, and 60, respectively). These data suggest that hypomethylation occurs in exon-4 of the *c-fos* gene in uteri of neonatal DES-treated mice

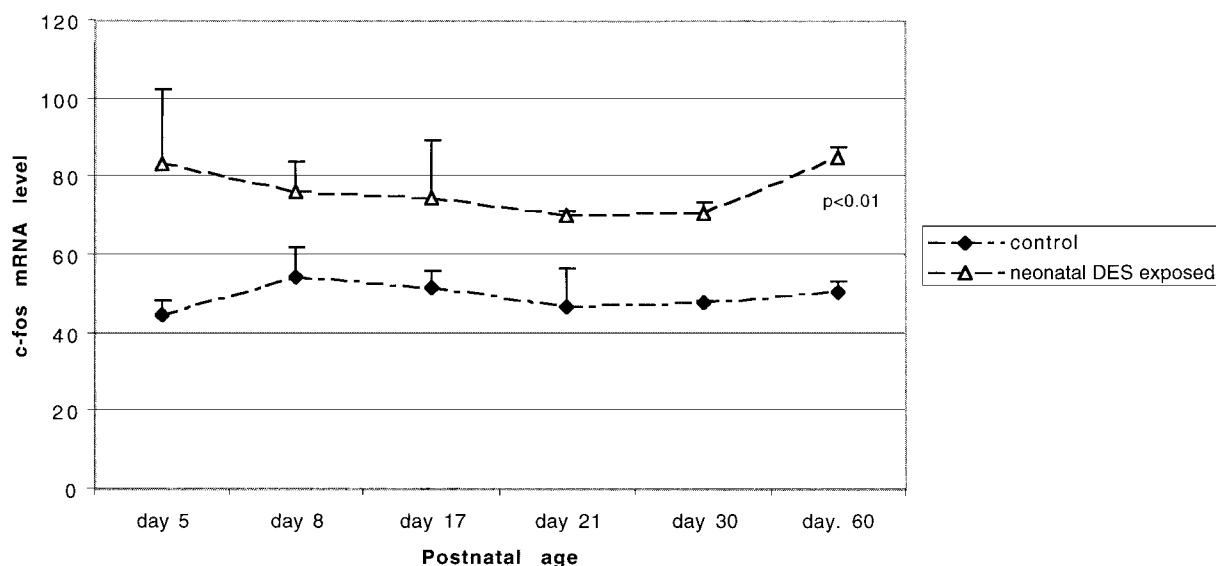


Figure 1. TaqMan real-time PCR results of *c-fos* mRNA in neonatal DES-exposed and control mice. The *c-fos* mRNA level was expressed normalized to both the GAPDH endogenous control and an untreated control sample ( $2^{-\Delta\Delta Ct}$ ), where  $\Delta\Delta Ct = (Ct_{c-fos} - Ct_{GAP}) - (Ct_{c-fos} - Ct_{GAP})_{control}$ . *P*-values represent differences between neonatal DES and control group.

A.

GGATGTCCATATTAGGACATCTGCGTCAGCAGGTTTCCACGGCCGGTCCCTGTTGTTCTGGGGGGGGGA  
CCATCTCCGAAATCCTACACCGCGGAAGGTCTAGGAGACCCCTAAGATCCCAAATGTGAACACTCATAG  
GTGAAAGATGTATGCCAAGACGGGGGTTGAAAGCCTGGGGCGTAGAGTTGACGACAGAGCGCCCGCAGA  
GGCCTTGGGGCGCGCTTCCCCCCCCCTTCCAGTTCCGCCAGTGACGTAGGAAGTCCATCCA (284b)

B.

TGGGCTCTCCTGTCAACACACAGGTGAGTTTGGCTTTGTGTAGCCGCCAGGTCCGCGCTGAGGGTCCGCC  
GTGGAGGAGACACTGGGGTGTGACTCGCAGGGGCGGGGGGGTCTTCCCTTTTTTCGCTCTGGAGGGAGACT  
GGCGCGTTCAGAGCAGCCTTAGCCTGGGAACCCAGGACTTGTCTGAGCGCGTGCACACTTGTTCATAGTA  
 ERE  
AGACTTAGTGACCCCTTCCCGCGCGGCAGGTTTATTCTGAGTGGCCTGCCTGCATTCTTCTCTCGGCCG  
ACTTGTCTGAGATCAGCCGGGGCCAACAAGTCTCGAGCAAAGAGTCGCTAACTAGAGTTTGGGAGGC  
 (348b)

C.

TTGGAGCCAGTCAAGAGCATCAGCAACGTGGAGCTGAAGGCAGAACCCTTTGATGACTTCTTGTTCCG  
 1 2  
GCATCATCTAGGCCAGTGGCTCAGAGACCTCCCGCTCTGTGCCAGATGTGGACCTGTCCGGTTCCTTC  
 3 b-HLH 4  
TATGCAGCAGACTGGGAGCCTCTGCACAGCAATTCCTTGGGGATGGGGCCCATGGTCACAGAGCTGGAG  
 TCF TCF  
CCCCGTGTACTCCCGTGGTCACCTGTACTCCGGGCTGCACTACTTACACGTCTT (276b)  
 5 b-HLH 6

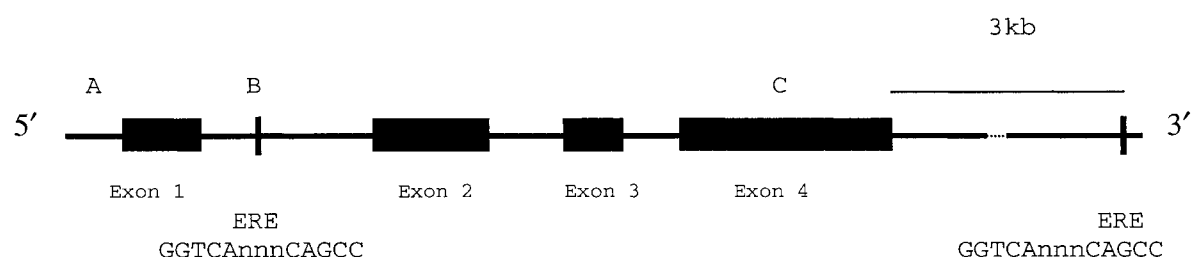


Figure 2. Schematic *c-fos* gene structure based on the published sequences (NCBI access no. V00727 and U62536). Black boxes represent the four exons. Sequences of three regions are given: promoter (A); intron-1 (B); and exon-4 (C). The methylation-specific primer regions are underlined. The CpGs analyzed for methylation status are in bold. The CpGs in exon-4 are numbered 1–6. Precipitated transcription motifs (ERE, bHLH, and TCF) are underlined.

from postnatal day 17. The methylation status of each 6 CpG in exon-4 was also analyzed; the results are shown in Figure 4. No hot spot, nor any specific pattern, was found to be unmethylated on all six CpG sites after neonatal DES exposure (Figure 4).

#### DISCUSSION

In the current study, we found a persistent elevation of *c-fos* mRNA in neonatal DES-exposed mouse uterus by real-time PCR. Through examination of the methylation status of the *c-fos* gene, we found that the CpGs at the *c-fos* promoter and intron-1 regions are completely unmethylated, and neonatal DES exposure does not alter the methylation status in the two regions. However, neonatal DES exposure led to hypomethylation in the exon-4 region.

Although several studies reported the overexpression of *c-fos* after perinatal DES exposure, these reports focused mainly on the hypersensitivity of

*c-fos* to the stimulation by estrogen after neonatal DES treatment [12–14]. Although Yamashita et al. [14] reported that the *c-fos* mRNA level was about 2.2-fold higher in neonatal DES-exposed, ovariectomized mice uteri compared with control at 12 wk of age, Kamiya et al. [12] showed that *c-fos* mRNA level was 6-fold higher in the neonatal DES-exposed, ovariectomized mice uteri compared with control mice at day 50. Our study showed a persistent elevation of *c-fos* mRNA in day 5, 8, 17, 21, 30, and 60 neonatal DES-exposed mice. By real-time PCR analysis, the elevation of *c-fos* mRNA was slightly lower than that in the previous reports, but statistical analysis showed that the elevation was significant. The dose of DES we used in this study was 2 µg/kg/day, which induces uterine epithelial cancer in more than 90% of the mice at age of 18 mo [5]. Yamashita et al. [14] and Kamiya et al. [12] used 4 or 3 µg DES/pup/day from postnatal days 1–5. The relatively higher dose used in their experiments may explain

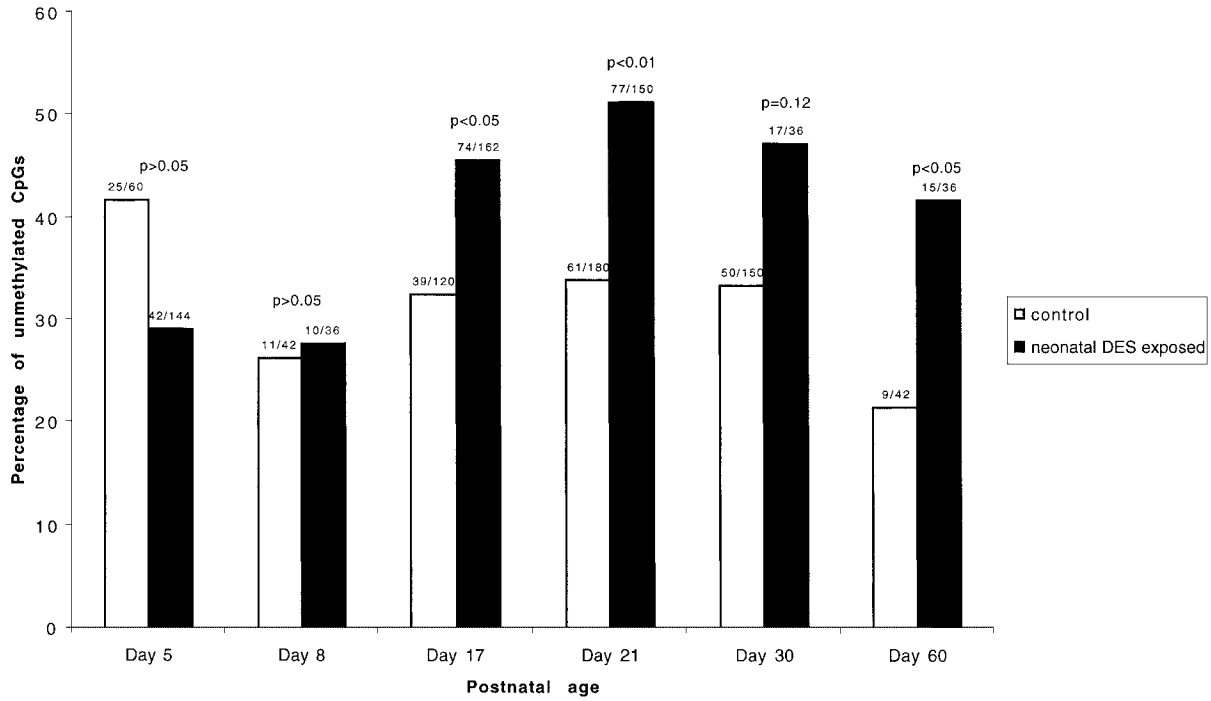


Figure 3. Percentage of unmethylated CpGs in total CpGs analyzed in c-fos exon-4. The numbers on the bars represent the unmethylated CpGs/total analyzed CpGs. P-values represent differences between neonatal DES and control group.

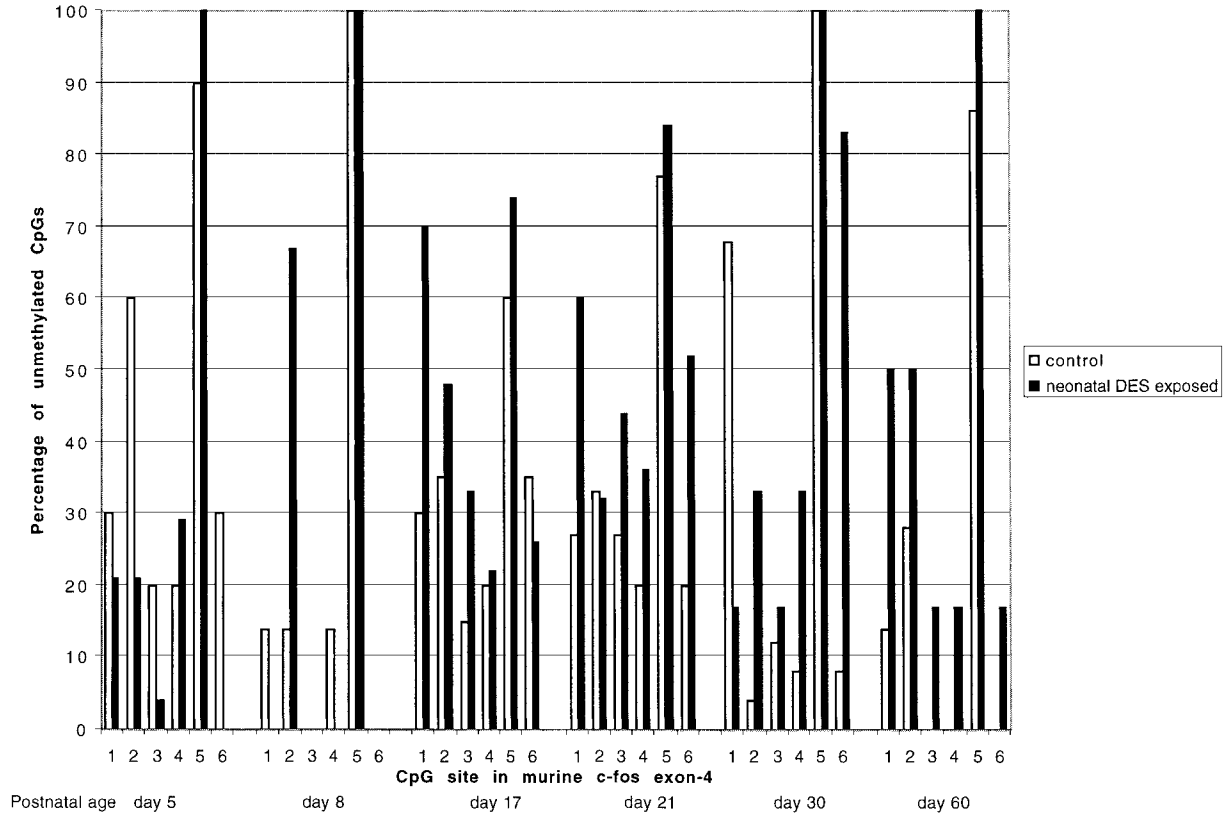


Figure 4. Percentage of unmethylated CpGs in each of CpG analyzed in c-fos exon-4. The six CpGs are numbered 1-6, as shown in Figure 2.

the higher expression of the *c-fos* mRNA level compared with our findings. Thus, our results not only confirmed the elevation of *c-fos* mRNA level in the neonatal DES-exposed mouse uterus but further demonstrate that the elevation is an immediate and persistent phenomenon.

Through examination of the methylation status of the *c-fos* gene, we found that the *c-fos* 5' promoter and intron-1 region were completely unmethylated in the mouse uterus, and that neonatal DES exposure did not alter the methylation status in these regions. By enzyme digestion analysis, Kanduri and Raman reported a stepwise and directional de novo methylation of the *c-fos* gene during murine development [34,35]. Their results showed that the MspI restriction site located before the intron-2 was completely unmethylated in fetal and adult somatic tissues, such as liver, brain, kidney, and spleen. Another study, by Uehara et al. [36], also reported that the CpG sites were completely unmethylated in exon-1 of *c-fos* in mouse liver. Our results show that not only the MspI sites, but also the 14 CpGs in the 5' promoter and the 18 CpGs in intron-1, are completely unmethylated in mouse uterus from postnatal day 5 to day 60, a similar methylation pattern to that reported for the liver. As for the CpGs located in exon-4, our results show that in uteri of 5-d-old control mice, 42% of CpG sites are unmethylated, and about 30% are unmethylated in 8–30-d-old mice. In the uteri of 60-d-old mice, only 21% of CpG sites are unmethylated. This result is also consistent with the study of Dr. Raman's group [34,35], who observed that the MspI restriction sites located downstream of exon-3 are either not methylated, or only partially so, in fetal tissues, but highly or completely methylated in adult mice tissues (liver, brain, kidney, and spleen). To our knowledge, ours is the first report of the methylation status of the *c-fos* gene in the mouse uterus.

Hyder et al. [33] identified an estrogen response element (ERE) at the 3' flanking region of the murine *c-fos* gene that can interact with estrogen receptors in mouse and rat uterus. These investigators suggested that estrogen regulation of *c-fos* gene expression might be through the 3' ERE. Thus, we examined the methylation status of the downstream sequences in the *c-fos* gene and found hypomethylation of exon-4 and elevated expression of *c-fos* in neonatal DES-exposed mice. Hypomethylation of *c-fos* exon-4 may change the binding activity of this region with specific proteins or may change the chromatin structure, affecting the transcription of this gene. In fact, there are two bHLH and two TCF sites in the exon-4 region. However, the direct linkage of *c-fos* exon-4 hypomethylation and the elevation of its mRNA level remain to be elucidated. The neonatal DES-induced *c-fos* exon-4 hypomethylation is observed from postnatal day 17; in contrast, elevation of its mRNA level is observed from postnatal day 5. The later onset of hypomethylation was also found in lactoferrin

promoter [21]. The mechanism for this later onset of demethylation is unknown, but ovarian hormones play a role in neonatal DES-induced methylation changes in the lactoferrin promoter [21], and ovarian hormones may have a similar role in *c-fos* demethylation as well.

The methylation status of each 6 CpG in exon-4 was also analyzed. Hypomethylation of five CpGs in neonatal DES mice appears random. This is different with the demethylation pattern of the lactoferrin promoter, where a CpG site-specific demethylation was found after neonatal DES exposure from day 21. The reason for this difference is unknown but may reflect the diversity of methylation patterns affected by DES among the different genes. Through sequence analysis, we found another ERE that is similar to the reported ERE by Hyder et al. [31–33] located at the intron-1. The physiological role of this ERE remains to be elucidated.

In conclusion, our current results add further support to the hypothesis that developmental DES exposure alters the methylation status of genes in early life as a possible mechanism for tumor development in later life. How and where methylation alterations occur upon developmental exposure to DES may differ with different genes. Methylation changes may occur in CpGs located in 5' promoter and/or other intronic and exonic regions of the gene. Direct linkage between elevated expression of *c-fos* and hypomethylation of its exon-4 in mouse uterus upon neonatal DES exposure remains to be studied further.

#### ACKNOWLEDGMENTS

We thank Ms. Elizabeth Padilla Banks and Glenda Corniffe for technical support, and Drs. Christina Teng and Teddy Devereux for critical review of the paper.

#### REFERENCES

1. Herbst AL, Ulfelder H, Poskanzer DC. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *N Engl J Med* 1971;284:878–881.
2. Herbst AL, Scully RE, Robboy SJ. Prenatal diethylstilbestrol exposure and human genital tract abnormalities. *Natl Cancer Inst Monog* 1979;51:25–35.
3. Herbst AL, Ulfelder H, Poskanzer DC, Longo LD. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. 1971. *Am J Obstet Gynecol* 1999;181:1574–1575.
4. McLachlan JA, Newbold RR, Bullock BC. Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol. *Cancer Res* 1980;40:3988–3999.
5. Newbold RR, Bullock BC, McLachlan JA. Uterine adenocarcinoma in mice following developmental treatment with estrogens: A model for hormonal carcinogenesis. *Cancer Res* 1990;50:7677–7686.
6. Iguchi T, Takasugi N. Postnatal development of uterine abnormalities in mice exposed to DES in utero. *Biol Neonate* 1987;52:97–103.

7. Branham WS, Zehr DR, Chen JJ, Sheehan DM. Uterine abnormalities in rats exposed neonatally to diethylstilbestrol, ethynylestradiol, or clomiphene citrate. *Toxicology* 1988;51: 201–212.
8. Medlock KL, Branham WS, Sheehan DM. Long-term effects of postnatal exposure to diethylstilbestrol on uterine estrogen receptor and growth. *J Steroid Biochem Mol Biol* 1992; 42:23–28.
9. Leavitt WW, Evans RW, Hendry WJ III. Etiology of DES-induced uterine tumors in the Syrian hamster. *Adv Exp Med Biol* 1981;138:63–86.
10. Gilloiseau J, Steggle AW. Endometrium cell surface abnormalities in the Syrian hamster as a result of in utero exposure to diethylstilbestrol. *Scand Electron Microsc* 1985; (Pt 1): 303–309.
11. Nelson KG, Sakai Y, Eitzman B, Steed T, McLachlan J. Exposure to diethylstilbestrol during a critical developmental period of the mouse reproductive tract leads to persistent induction of two estrogen-regulated genes. *Cell Growth Differ* 1994;5:595–606.
12. Kamiya K, Sato T, Nishimura N, Goto Y, Kano K, Iguchi T. Expression of estrogen receptor and proto-oncogene messenger ribonucleic acids in reproductive tracts of neonatally diethylstilbestrol-exposed female mice with or without post-puberal estrogen administration. *Exp Clin Endocrinol Diabetes* 1996;104:111–122.
13. Falck L, Forsberg JG. Immunohistochemical studies on the expression and estrogen dependency of EGF and its receptor and *c-fos* proto-oncogene in the uterus and vagina of normal and neonatally estrogen-treated mice. *Anat Rec* 1996;245: 459–471.
14. Yamashita S, Takayanagi A, Shimizu N. Effects of neonatal diethylstilbestrol exposure on *c-fos* and *c-jun* protooncogene expression in the mouse uterus. *Histol Histopathol* 2001;16: 131–140.
15. Hebert CD, Endo S, Korach KS, et al. Characterization of murine cell lines from diethylstilbestrol-induced uterine endometrial adenocarcinomas. *In Vitro Cell Dev Biol* 1992; 28A:327–336.
16. Ma L, Benson GV, Lim H, Dey SK, Maas RL. Abdominal B (AbdB) Hoxa genes: regulation in adult uterus by estrogen and progesterone and repression in müllerian duct by the synthetic estrogen diethylstilbestrol (DES). *Devel Biol* 1998; 197:141–154.
17. Block K, Kardana A, Igarashi P, Taylor HS. In utero diethylstilbestrol (DES) exposure alters Hox gene expression in the developing müllerian system. *FASEB J* 2000;14:1101–1108.
18. Couse JF, Dixon D, Yates M, et al. Estrogen receptor- $\alpha$  knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract. *Dev Biol* 2001;238:224–238.
19. Barrett JC, Wong A, McLachlan JA. Diethylstilbestrol induces neoplastic transformation without measurable gene mutation at two loci. *Science* 1981;212:1402–1404.
20. Boyd J, Takahashi H, Waggoner SE, et al. Molecular genetic analysis of clear cell adenocarcinomas of the vagina and cervix associated and unassociated with diethylstilbestrol exposure in utero. *Cancer* 1996;77:507–513.
21. Li S, Washburn KA, Moore R, et al. Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. *Cancer Res* 1997;57: 4356–4359.
22. Li S, Ma L, Chiang T, Burow M. Promoter CpG methylation of Hox-a10 and Hox-a11 in mouse uterus not altered upon neonatal diethylstilbestrol (DES) exposure. *Mol Carcinog* 2001;32:213–219.
23. Kim YI, Pogribny IP, Salomon RN, et al. Exon-specific DNA hypomethylation of the p53 gene of rat colon induced by dimethylhydrazine. Modulation by dietary folate. *Am J Pathol* 1996;149:1129–1137.
24. Li B, Goyal J, Dhar S, et al. CpG methylation as a basis for breast tumor-specific loss of NES1/kallikrein 10 expression. *Cancer Res* 2001;61:8014–8021.
25. Nguyen TT, Nguyen CT, Gonzales FA, Nichols PW, Yu MC, Jones PA. Analysis of cyclin-dependent kinase inhibitor expression and methylation patterns in human prostate cancers. *Prostate* 2000;43:233–242.
26. Nguyen C, Liang G, Nguyen TT, et al. Susceptibility of nonpromoter CpG islands to de novo methylation in normal and neoplastic cells. *J Natl Cancer Inst* 2001;93:1465–1472.
27. Loose-Mitchell DS, Chiappetta C, Stancel GM. Estrogen regulation of *c-fos* messenger ribonucleic acid. *Mol Endocrinol* 1988;2:946–951.
28. Jouvenot M, Pellerin I, Alkhalaf M, Marechal G, Royez M, Adessi GL. Effects of 17 beta-estradiol and growth factors on *c-fos* gene expression in endometrial epithelial cells in primary culture. *Mol Cell Endocrinol* 1990;72:149–157.
29. Kennedy AR. Is there a critical target gene for the first step in carcinogenesis? *Environ Health Perspect* 1991;93:199–203.
30. Yoshida A, Newbold RR, Dixon D. Abnormal cell differentiation and p21 expression of endometrial epithelial cells following developmental exposure to diethylstilbestrol (DES). *Toxicol Pathol* 2002;28:237–245.
31. Hyder SM, Stancel GM, Nawaz Z, McDonnell DP, Loose-Mitchell DS. Identification of an estrogen response element in the 3'-flanking region of the murine *c-fos* protooncogene. *J Biol Chem* 1992;267:18047–18054.
32. Hyder SM, Stancel GM. In vitro interaction of uterine estrogen receptor with the estrogen response element present in the 3'-flanking region of the murine *c-fos* protooncogene. *J Steroid Biochem Mol Biol* 1994;48:69–79.
33. Hyder SM, Chiappetta C, Stancel GM. The 3'-flanking region of the mouse *c-fos* gene contains a cluster of GGTC A hormone response-like elements. *Mol Biol Rep* 1998;25: 189–191.
34. Chandrasekhar K, Raman R. De novo methylation of the proto-oncogene, *c-fos*, during development occurs stepwise and directionally in the laboratory mouse. *Mol Reprod Dev* 1997;48:421–432.
35. Kanduri C, Raman R. Characterisation of developmentally regulated chromatin structure in the coding region of the proto-oncogene, *c-fos*, in the male laboratory mouse. *Int J Dev Biol* 1999;43:279–282.
36. Uehara Y, Ono T, Kurishita A, Kokuryu H, Okada S. Age-dependent and tissue-specific changes of DNA methylation within and around the *c-fos* gene in mice. *Oncogene* 1989;4:1023–1028.