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# Effect of Prenatal Exposure to Diethylstilbestrol on Müllerian Duct Development in Fetal Male Mice*

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## ABSTRACT

The clinical use of diethylstilbestrol (DES) by pregnant women has resulted in an increased incidence of genital carcinoma in the daughters born from these pregnancies. Also, in the so-called DES-sons abnormalities were found, mainly, the presence of Müllerian duct remnants, which indicates that fetal exposure to DES may have an effect on male sex differentiation. Fetal regression of the Müllerian ducts is under testicular control through anti-Müllerian hormone (AMH). In male mice, treated *in utero* with DES, the Müllerian ducts do not regress completely, although DES-exposed testes do produce AMH. We hypothesized that incomplete regression in DES-exposed males is caused by a diminished sensitivity of the Müllerian ducts to AMH. Therefore, the effect of DES on temporal aspects of Müllerian duct regression and AMH type II receptor (AMHRII) messenger RNA (mRNA) expression in male mouse fetuses was studied.

It was observed that Müllerian duct regression was incomplete at E19 (19 days *post coitum*), upon DES administration during pregnancy from E9 through E16. Furthermore, analysis of earlier time points of fetal development revealed that the DES treatment had clearly delayed the onset of Müllerian duct formation by approximately 2 days; in untreated fetuses, Müllerian duct formation was complete by E13, whereas fully formed Müllerian ducts were not observed in DES-treated male fetuses until E15.

Using *in situ* hybridization, no change in the localization of AMH and AMHRII mRNA expression was observed in DES-exposed male fetuses. The mRNA expression was quantified using ribonuclease protection assay, showing an increased expression level of AMH and AMHRII mRNAs at E13 in DES-exposed male fetuses. Furthermore, the mRNA expression levels of Hoxa 11 and steroidogenic factor-1 (SF-1) were determined as a marker for fetal development. Prenatal DES exposure had no effect on Hoxa 11 mRNA expression, indicating that DES did not exert an overall effect on the rate of fetal development. In DES-exposed male fetuses, SF-1 showed a similar increase in mRNA expression as AMH, in agreement with the observations that the *AMH* gene promoter requires an intact SF-1 DNA binding site for time- and cell-specific expression, although an effect of DES on SF-1 expression in other tissues, such as the adrenal and pituitary gland, cannot be excluded. However, the increased expression levels of AMH and AMHRII mRNAs do not directly explain the decreased sensitivity of the Müllerian ducts to AMH. Therefore, it is concluded that prenatal DES exposure of male mice delays the onset of Müllerian duct development, which may result in an asynchrony in the timing of Müllerian duct formation, with respect to the critical period of Müllerian duct regression, leading to persistence of Müllerian duct remnants in male mice. (*Endocrinology* **139:** 4244–4251, 1998)

ANTI-MÜLLERIAN hormone (AMH), a member of the transforming growth factor β (TGFβ) superfamily of peptide growth and differentiation factors, is the earliest protein known to be secreted by the fetal Sertoli cells (1, 2). In contrast to other family members, which have a broad range of functions, AMH has a very specific role during sex differentiation. AMH, which is produced only by the fetal testes and not by the ovaries during fetal development, might play a role in gonadal differentiation, as indicated by the formation of ovotestes in female mice overexpressing AMH (3). Most importantly, in the male, AMH induces the regression of the Müllerian ducts, which form the anlagen of the uterus, oviducts, and upper part of the vagina. It has been shown that the timing of AMH action on the Müllerian ducts is very critical. In the rat, exposure of female fetuses to AMH after E16 (16 days *post coitum*) does not result in Müllerian duct regression (4, 5).

The cellular and molecular mechanisms by which AMH induces Müllerian duct regression are poorly understood. However, the identification and cloning of the AMH type II receptor (AMHRII) has contributed to the elucidation of this question (6, 7). AMHRII messenger RNA (mRNA) is expressed in the fetal gonads and in the mesenchymal cells located adjacent to the Müllerian duct epithelium, which corresponds to the sites of action of AMH (6, 7). Recent results have shown that AMH elicits its effect on the Müllerian duct epithelium via the surrounding mesenchymal cells, a process which may also involve induction of programmed cell death (4, 8).

AMHRII is a member of the transmembrane serine/threonine kinase receptor family, to which also the TGFβ and activin receptors belong (9). Members of the TGFβ superfamily exert their action through a heteromeric signaling complex consisting of a type I and a type II receptor (10). Failure in AMH action, as a result of a gene mutation leading to either inactive AMH or AMHRII, causes inhibition of Müllerian duct regression, resulting in a rare form of pseudohermaphroditism in man known as persistent Müllerian duct syndrome (11, 12). Gene knockout experiments in mice have confirmed that, in the absence of AMH or AMHRII, Müllerian ducts do not regress (13, 14).

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In chickens, unilateral regression of Müllerian ducts occurs in the female. The left Müllerian duct is retained, whereas the right Müllerian duct regresses, because of the fact that, in contrast to mammalian species, AMH is also expressed by the fetal ovary (15). It has been suggested that estrogens protect the left duct from regression. This is supported by the observation that the concentration of estrogen receptor in the left duct is higher than that in the right duct (16). Furthermore, inhibition of estrogen production in female chick fetuses, by treatment with an aromatase inhibitor during egg incubation, resulted in regression of both ducts (17). Exposure to estrogen during egg incubation prevents Müllerian duct regression in both male and female chick fetuses (18, 19).

Although it is a large step from chicken to human, it is of interest to compare the data from the experiments with chickens with clinical data. In humans, intrauterine exposure to diethylstilbestrol (DES), a potent synthetic estrogen that has been administrated during pregnancy to prevent miscarriages, has led to an increased incidence of reproductive tract abnormalities. The effects of prenatal DES exposure in so-called DES-daughters, such as an increased risk of genital carcinoma, have been well documented (20). However, also the sons born from DES-controlled pregnancies have an increased incidence of genital tract abnormalities, including epididymal cysts, cryptorchidism, and the presence of Müllerian duct remnants (21, 22). This indicates that DES has an effect on male sex differentiation. To study the prenatal effects of DES on the developing genital tract in an animal model, McLachlan *et al*. (23) injected DES daily into pregnant mice during the phase of growth and differentiation of the fetal reproductive tract. Observations on the male offspring of these DES-treated mice indicated that the developing reproductive tract of the fetus is sensitive to DES exposure. Hypoplastic testes and Müllerian duct remnants were found (23, 24). It is, however, not clear how DES mediates its inhibitory effect on reproductive tract differentiation.

In a mouse organ culture system, after *in vivo* DES treatment, Newbold *et al.* (25) studied whether the inhibitory effect of DES on Müllerian duct regression results from suppression of fetal testicular AMH production or a change in responsiveness of the Müllerian ducts to AMH. Control Müllerian ducts regressed normally when cultured in the presence of control testes, whereas DES-exposed Müllerian ducts in the presence of DES-exposed testes did not regress. Combination of control Müllerian ducts and DES-exposed testes resulted in normal regression. However, in the reciprocal combination, DES-exposed ducts and control testes, only partial regression of the Müllerian ducts was observed. These results indicate that DES-exposed testes still produce bioactive AMH and that the effect of DES is caused mainly by a decrease in AMH responsiveness of the Müllerian ducts.

We hypothesized that the change in sensitivity of the Müllerian ducts to AMH may result from an effect of DES on the expression of AMHRII. In this paper, we describe the effects of DES exposure of mouse male fetuses on the Müllerian ducts; in particular, AMH and AMHRII mRNA expression during the period of reproductive tract differentiation. As a control for possible effects of DES exposure on general fetal development (26, 27), the expression of Hoxa 11 mRNA was measured. The mRNA expression level for steroidogenic factor-1 (SF-1) mRNA, an orphan nuclear receptor essential for the development of steroidogenic tissues (28), was measured as a control for possible effects of DES exposure on urogenital ridge development. The results of this study may contribute to our knowledge about the possible involvement of exposure to exogenous estrogenic compounds in the postulated increased incidence of reproductive tract disorders in wild-life and perhaps also in humans (29, 30).

## Materials and Methods

### *Animals and treatment*

FVB mice were kept under standard animal housing conditions in accordance with NIH Guidelines for the Care and Use of Experimental Animals. Vaginal plug detection was considered day 0 (E0) of pregnancy. Pregnant mice were given daily sc injections with DES (100 μg/kg BW; Janssen Chimica, Beerse, Belgium) dissolved in olive oil, or olive oil alone, on days E9–E16 of gestation. Pregnant mice were killed by cervical dislocation at E13, E14, E15, E17, or E19 of gestation. Fetuses were isolated and snap-frozen in liquid nitrogen and stored at −80 C. Total RNA was isolated using the LiCl/urea method (31). In addition, fetuses from the same litter were also fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned transversally at 7 μm. PCR reactions, using placental genomic DNA (32), were performed as described by Mitchell *et al.* (33) using primers for the mouse genes *Sbx* and *Sby* (34) to determine the sex of the fetuses.

### *In situ hybridization*

A *Pst*I fragment containing bp 1243–1640 of the rat AMHRII complementary DNA (cDNA) and an *Nhe*I fragment containing bp 38–400 of the rat AMH cDNA were subcloned in pBluescript KS (Stratagene, Westburg, Leusden, The Netherlands) and used to generate sense and antisense $[^{35}S]$-uridine 5′-triphosphate (UTP)-labeled (Amersham, ’s Hertogenbosch, The Netherlands) transcripts *in vitro*. *In situ* hybridization was performed as described by Zeller and Rogers (35), with some modifications (6). Sections were mounted on slides that were coated with 3-aminopropyl-ethoxysaline. After deparaffinization, sections were treated with 0.2 M HCl (20 min), treated with proteinase K (1 μg/ml in 0.2 M Tris (pH 7.5), 2 mM $CaCl_2$; incubation for 15 min at 37 C), and postfixed in 4% paraformaldehyde in 0.1 M PBS. After treatment with dithiothreitol and blocking of nonspecific binding with 0.1 M triethanolamine, followed by 0.1 M triethanolamine and acetic anhydride, sections were incubated with $[^{35}S]$-UTP-labeled antisense and sense AMH and AMHRII RNA probes at a final concentration of $5 \times 10^5$ cpm/μl. Hybridization was carried out as described previously (6). Sections were exposed at 4 C for 1 week, developed, counterstained with hematoxylin, and mounted.

### *Ribonuclease (RNase) protection assay*

A mouse AMHRII DNA template for *in vitro* transcription was generated by RT-PCR. The RT-PCR reaction was carried out on 100–200 ng total RNA, extracted from 25-day-old mouse testis, using random hexamers. A sample of the RT reaction product was used in the PCR reaction using the primers 5′GCTCCGGAGCTCTTGGACAAG3′ (forward primer) and 5′CAGGCGCTGCTGCACACACTC3′ (reverse primer) corresponding to kinase subdomains VIII, IX, and X of the *AMHRII* gene transcript. A 350-bp PCR product was subcloned in pBluescript KS and used to generate $[^{32}P]$-UTP-labeled antisense probe. The AMH RNA probe was obtained using a 430-bp *Pst*I fragment, containing exon 1, of mouse genomic DNA. The SF-1 RNA probe was obtained using a 252-bp *Hin*dIII-*Eco*RI fragment of mouse SF-1 cDNA (36). The Hoxa 11 RNA probe was obtained using a 300-bp *Bam*HI-*Bgl*II fragment of the mouse Hoxa 11 cDNA (26). The control glyceraldehyde 3-phosphate dehydrogenase (GAPD) RNA probe was synthesized using a construct containing a 163-bp *Acc*I-*Sau*3AI fragment of the rat GAPD cDNA. RNase protection assays of 50 μg total fetal RNA with these probes were performed as described by Baarends *et al.* (6). GAPD was used as a control for RNA loading. The relative amount of protected mRNA band was quantified through exposure of the gels to a phosphor screen (Molecular Dynamics, B and L Systems, Zoetermeer, The Netherlands), followed by a calculation of the relative density of the obtained bands using a phospho-imager and Image Quant (Molecular Dynamics) as computer analysis software. The arbitrary units are expressed as the rations after division by the corresponding GAPD values.

## Results

### *Effect of DES exposure on Müllerian duct formation*

The development of the Müllerian duct was studied in male fetuses at E13, E15, and E19, at three positions along the axis of the Müllerian ducts (Figs. 1, 2, and 3). Position I indicates the most cranial part of the ducts, at the level of the fetal testes. Position II is at the level where the Müllerian and Wolffian ducts cross each other. Position III indicates the caudal part of the Müllerian ducts, near the urogenital sinus.

In control fetuses at E13, no morphological signs of regression could be detected along the axis of the Müllerian ducts (Fig. 1, A–D). In DES-exposed E13 fetuses, on the other hand, Müllerian ducts were only found at position I (Fig. 1F). Caudally, at positions II and III, the Müllerian ducts were not formed, indicating a delay in their formation caused by the DES treatment (Fig. 1I, position III).

At E15, differences in Müllerian duct regression between control and DES-exposed fetuses were observed. In E15 control fetuses, regression of the Müllerian ducts had started but was not complete (Fig. 2, A–D). The regression of the Müllerian ducts was initiated cranially at position I, and concomitantly, we observed the characteristic presence of a whorl of mesenchymal cells surrounding the Müllerian ducts (Fig. 2A). No signs of Müllerian duct regression could be detected at positions II and III at E15, indicating that degeneration of the Müllerian ducts is initiated cranially and then progresses caudally (Fig. 2D). In contrast, regression of the Müllerian ducts in the DES-exposed E15 fetuses was not initiated at all three positions, as indicated by the absence of the typical whorl of mesenchymal cells (Fig. 2, F–I). The appearance of the Müllerian ducts in DES-exposed fetuses at E15 corresponds to that of the Müllerian ducts in control fetuses at E13, implicating that the onset of Müllerian duct regression is delayed by approximately 2 days.

It was observed that regression of the Müllerian ducts in control male fetuses resulted in their complete absence at E19 (Fig. 3A). In the DES-exposed male fetuses at E19, regression of the cranial part of the Müllerian ducts was complete at positions I and II, because no Müllerian structures could be detected (results not shown). However, more caudally, at position III, the Müllerian ducts were still present (Fig. 3B). The epithelial and mesenchymal cells of the Müllerian duct remnants, in DES-exposed male fetuses at E19 (Fig. 3B), were differentiated and had an appearance comparable with that found in control female fetuses of the same developmental stage (results not shown).

These results are schematically summarized in the *top panels* of Figs. 1, 2, and 3.

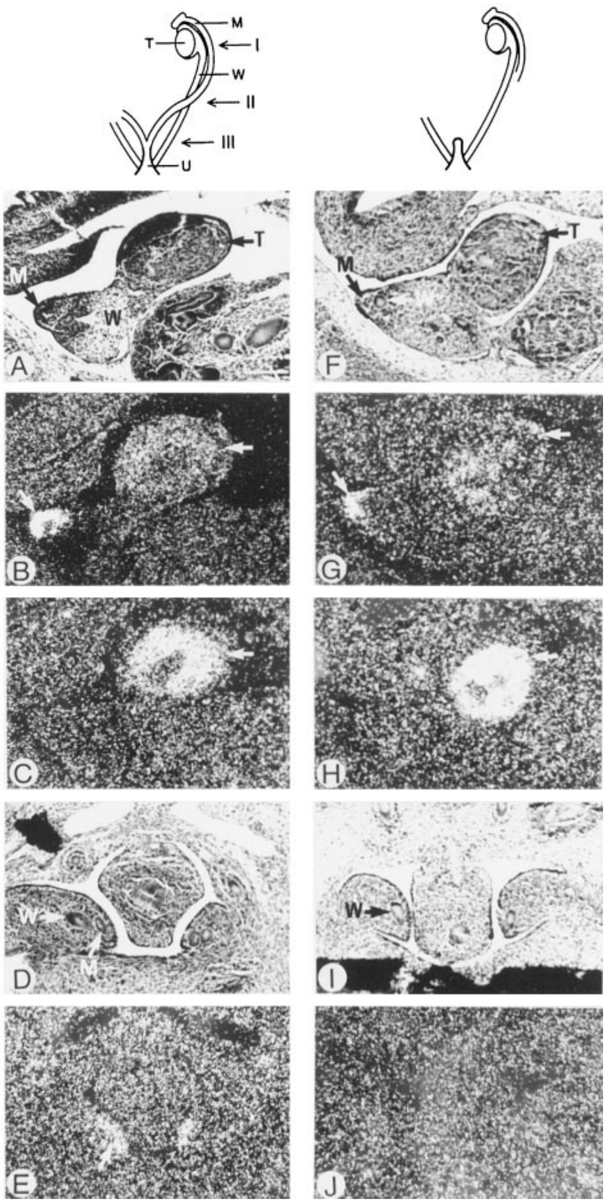

FIG. 1. Histology of Müllerian ducts and expression of AMH and AMHRII mRNAs in control and DES-exposed male mouse fetuses at E13. The formation of the Müllerian ducts is represented schematically in the *top panel*. The Roman numerals indicate positions I, II, and III, at which sections were taken for morphology study and *in situ* hybridization. The *left figures* (position I: A, B, C; position II: D, E) and the *right figures* (position I: F, G, H; position II: I, J) are sections from control and DES-exposed fetuses, respectively. At position I, the Müllerian ducts are present in control fetuses (A) and in DES-exposed fetuses (F), although less differentiated. At position II, the Müllerian ducts are found in control (D) but not in DES-exposed fetuses (I). Expression of AMH and AMHRII mRNAs was determined using *in situ* hybridization. Darkfield views of AMHRII (control: B, E; DES-exposed: G, J) and AMH (control: C; DES-exposed: G) mRNAs are shown in adjacent sections. No difference between control and DES-exposed fetuses is found in the localization of AMH and AMHRII mRNAs expression; AMH mRNA expression is found in the testes, and AMHRII mRNA expression is found in the testes and the mesenchymal cells surrounding the Müllerian ducts. *Arrows*, Expression sites; T, testis; W, Wolffian duct; M, Müllerian duct. The *scale bar* represents 100 $\mu$m.

### *Expression of AMH and AMHRII mRNAs*

The expression of AMH and AMHRII mRNAs was studied by *in situ* hybridization. AMH mRNA expression was localized in the gonads of DES-exposed male fetuses, similar to

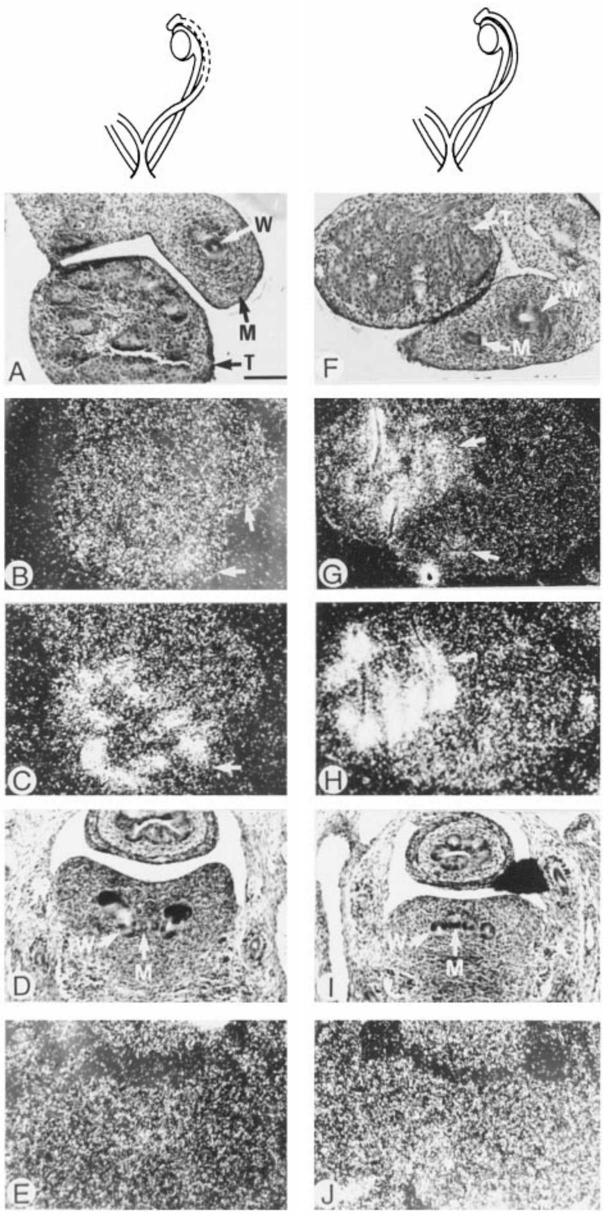

FIG. 2. Histology of Müllerian ducts and expression of AMH and AMHRII mRNAs in control and DES-exposed male mouse fetuses at E15. In the *top panel*, the regression of the Müllerian ducts is represented schematically. The *stippled lines* indicate the regressed Müllerian ducts. The *left figures* (position I: A, B, C; position III: D, E) and the *right figures* (position I: F, G, H; position III: I, J) are sections from control and DES-exposed fetuses, respectively. Regression has initiated in control fetuses at position I (A), but not at positions II and III (D). In DES-exposed fetuses, the Müllerian ducts are completely present (F, I), although no signs of regression are found at position I (F). Note the presence of a whorl of mesenchymal cells surrounding the Müllerian duct in control fetuses (A). Expression of AMH and AMHRII mRNAs was determined using *in situ* hybridization. Darkfield views of AMHRII (control: B, E; DES-exposed: G, J) and AMH (control: C; DES-exposed: H) mRNAs are shown in adjacent sections. AMH and AMHRII mRNA expression is increased in fetal testes of DES fetuses. At position I, mesenchymal cells of control fetuses do not express AMHRII mRNA, whereas in DES-exposed fetuses, a low expression is found. At position III, no expression of AMHRII was found. *Arrows*, Expression sites; T, testis; W, Wolffian duct; M, Müllerian duct. The *scale bar* represents 100 $\mu$m.

control fetuses, although differences in the quantitative level of expression were detected. The testes of DES-exposed fetuses at E13 showed a marked increase in AMH mRNA expression, compared with control testes (Fig. 1, C–H). This increase in AMH mRNA expression was also present on E15 (Fig. 2, C–H), whereas testicular expression of AMH mRNA could hardly be detected in both control and DES-exposed E19 fetuses (results not shown).

AMHRII mRNA expression was also studied at the three positions indicated in Fig. 1. Expression of AMHRII mRNA in DES-exposed fetuses was found in the same tissues as in control fetuses, the fetal gonads and the mesenchymal cells surrounding the Müllerian ducts (Fig. 1, B–G). It is important to note that, although the formation of the Müllerian ducts was not complete by E13 in DES-exposed fetuses, AMHRII mRNA was already expressed. More caudally, at positions II and III, the Müllerian ducts were absent in DES-exposed fetuses; hence, expression of AMHRII mRNA could not be detected at these sites (Fig. 1J). In control fetuses, AMHRII mRNA was expressed along the whole axis of the Müllerian ducts, although expression decreased caudally (Fig. 1B/E).

In control fetuses at E15, expression of AMHRII mRNA could no longer be detected in the mesenchymal cells surrounding the Müllerian ducts, at all three positions studied (Fig. 2B/E). The mesenchymal cells of the cranial Müllerian ducts in DES-exposed E15 fetuses did still express AMHRII mRNA (Fig. 2G), although the expression was lower, compared with that in E13 DES-exposed fetuses. Caudally, at position III, expression could not be detected (Fig. 2J). In the testes of control fetuses, AMHRII mRNA was only weakly expressed, whereas the testes of DES-exposed fetuses at E15 still showed a clear AMHRII mRNA expression (Fig. 2B/G). An increase in testicular AMH mRNA expression in DES-exposed fetuses, compared with control fetuses, was still observed at E15 (Fig. 2C/H).

At E19, testicular AMHRII mRNA expression was equally low in both control and DES-exposed fetuses. AMHRII mRNA expression in the mesenchymal cells of the Müllerian ducts could not be detected in control and DES-exposed fetuses, although the Müllerian ducts were still present in

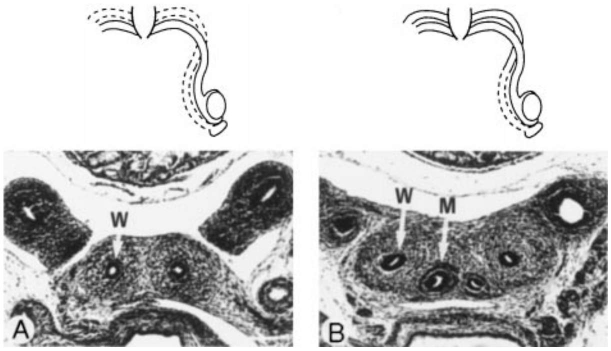

FIG. 3. Histology of Müllerian ducts in control and DES-exposed male mouse fetuses at E19. In the *top panel*, the regression of the Müllerian ducts is represented schematically. At position III, the Müllerian ducts have completely regressed in control fetuses (A), whereas in DES-exposed fetuses, remnants of Müllerian ducts are clearly visible (B). The Müllerian duct remnants show differentiation of epithelial and mesenchymal cells. W, Wolffian duct; M, Müllerian duct. The *scale bar* represents 100 $\mu$m.

DES-exposed male fetuses near the urogenital sinus (results not shown).

Expression levels of AMH and AMHRII mRNAs were quantified more precisely using an RNase protection assay (Fig. 4A). Furthermore, the expression of SF-1 mRNA was included as a marker for urogenital ridge development. The results of the RNase protection showed that the expression patterns of AMH and AMHRII mRNAs mimic the expression pattern of SF-1 mRNA (Fig. 4B). In DES-exposed male fetuses at E13 the expression of SF-1 mRNA is strongly increased, compared with control fetuses. A similar increase in AMH mRNA expression was measured in DES-exposed male fetuses, as was observed with *in situ* hybridization. An increase of AMHRII mRNA expression was found, using RNase protection assay in DES-exposed fetuses at E13, although this increase was less evident, compared with AMH and SF-1 mRNAs expression. At E14 and E15, DES-exposed fetuses showed a higher expression of SF-1, AMH, and AMHRII mRNAs than control fetuses, although less pronounced than at E13. From E15 onwards, changes in mRNA expression of SF-1, AMH, and AMHRII were limited to a slight increase at E19.

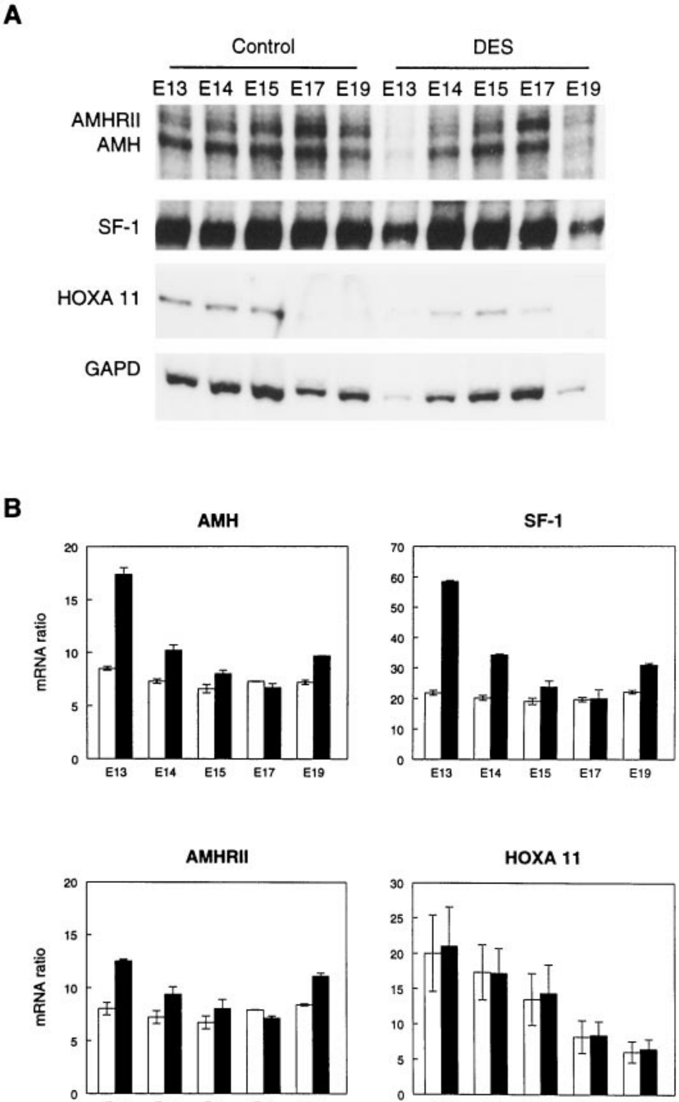

FIG. 4. Quantitative analysis of the expression of AMH, AMHRII, SF-1, and Hoxa 11 mRNAs in control and DES-exposed fetuses, studied by RNase protection assay. Expression in whole fetuses was determined at E13, E14, E15, E17, and E19. A, Results of the RNase protection assay; B, quantitative analysis of the expression levels. The mRNA expression levels are expressed as the ratios: AMH/GAPD, AMHRII/GAPD, SF1/GAPD, and Hoxa 11/GAPD. *Open bars*, Control fetuses; *filled bars*, DES-exposed fetuses. The *bars* and *error bars* represent the mean and SD of two fetuses, isolated from different treatments.

In addition to the expression of SF-1 mRNA, the Hoxa 11 mRNA expression level was determined, as a control for a possible effect of DES treatment on the general rate of fetal development. It was observed that DES-treated fetuses were born 1 day later, compared with control fetuses. This might indicate that the DES-induced delay in Müllerian duct formation would reflect a general delay in fetal development of DES fetuses. However, DES-treated and control fetuses did not show a difference in fetal Hoxa 11 mRNA expression at all time points studied (Fig. 4), indicating similar rates of general fetal development in the two treatment groups. Furthermore, no differences in length, width, or digit differentiation were observed between control and DES-treated fetuses during fetal development (results not shown).

## Discussion

This paper describes the effect of prenatal DES exposure on regression of the Müllerian ducts of fetal male mice. In agreement with previous studies (23, 24), we found incomplete Müllerian duct regression upon DES exposure. In male fetuses from DES-treated mice, regression had initiated in the cranial part of the Müllerian ducts but did not progress completely caudally, leaving remnants of Müllerian ducts at the position of the urogenital sinus. The nonregressed parts of the Müllerian ducts showed female-like differentiation, indicating that the Müllerian duct remnants might be responsive to estrogens. In female mice, prenatal exposure to DES also causes uterine epithelial cell hypertrophy (37). These findings indicate that the Müllerian ducts are a target for DES action in both male and female fetuses.

In addition to the appearance of Müllerian duct remnants, we observed that DES exposure resulted in a delay in Müllerian duct formation of approximately 2 days. In control fetuses, the complete Müllerian ducts were present at E13, whereas in DES-exposed fetuses fully formed Müllerian ducts were not found before E15. In addition, DES-exposed fetuses were born 1 day later, compared with control fetuses. These observations suggest that DES causes a delay in general embryonic development. Also, in rats, exposure to estrogens during pregnancy leads to a prolonged gestation (38), but this is explained by an inhibiting effect of DES on the onset of uterine contraction. Cesarean sections, performed to rescue the litter, revealed no difference in size of fetuses from control and DES-treated mothers (38). Transgenic mice, overexpressing the estrogen receptor $\alpha$ (ER$\alpha$), have similar problems with birth, with gestation lengths prolonged up to 4 days (39). Exposure to DES in neonatal mice results in an increase of ER$\alpha$ mRNA expression in uterine cells (40), suggesting that the longer gestation time in

DES-exposed mice may be a phenocopy of the change in pregnancy in ER$\alpha$ transgenic mice, and this reflects a maternal effect rather than a delay in fetal development. No differences in body size or digit differentiation were observed between control and DES-treated fetuses during fetal development (results not shown). Furthermore, the expression of Hoxa 11 mRNA was studied as a marker for general fetal development (26, 27). Hoxa 11 mRNA is expressed in the limbs, in the kidneys, and in the stromal cells surrounding the Müllerian and Wolffian ducts, and this expression is detected at E10 several days before reproductive tract differentiation (26). In the present study, no difference in Hoxa 11 mRNA expression between control and DES-exposed fetuses was observed, at all embryonic stages studied. This indicates that the rate of general fetal development is not affected but that DES elicits a specific effect on reproductive tract development. The variation in the results with the Hoxa 11 probe is caused by the large differences in specific activity of the probe in different experiments.

The anlagen of the reproductive tract, the Wolffian and Müllerian ducts, are formed separately. The Wolffian duct is formed as an excretory duct of the mesonephros and is recognizable before the gonads are formed. At the time of gonad formation, the Müllerian ducts develop in a cranial-to-caudal direction along the Wolffian ducts, which function as a guiding structure for early growth of the Müllerian ducts (Ref. 41, and references therein). The genes involved in Müllerian duct formation have not been identified yet. It has been suggested that the Wolffian ducts release epithelial cells, which contribute to the developing Müllerian ducts (42). It has also been suggested that the growth of Müllerian ducts is autonomous (43). In our studies, DES treatment affects the formation of the Müllerian ducts rather than formation of the Wolffian ducts, because DES was administered after completion of Wolffian duct formation. However, an effect of estrogens on Wolffian duct formation cannot be ruled out. It has been observed that the Wolffian ducts are affected by exogenous estrogen exposure, resulting in several abnormalities, such as seminal vesicle tumors and prostate inflammation (44). Also in female fetuses, the Wolffian ducts are a target for DES action. Retention of Wolffian ducts, postnatally, was observed in females, both in humans and in mice (45, 46). These effects of DES on Wolffian and Müllerian duct differentiation may point to a common mechanism in the development of these duct systems. Both Wolffian and Müllerian ducts can respond to estrogens, because the ER$\alpha$ is present in both structures during development (47). The identification of a novel estrogen receptor, ER$\beta$ (48), may contribute to our understanding of the mechanism of DES action. Recently, it was reported that ER$\alpha$ and ER$\beta$, when activated by estradiol, signal in opposite ways from an AP1 site (49). DES, therefore, may cause different effects, depending on the tissue studied. ER$\beta$ is highly expressed in prostate and ovary, whereas ER$\alpha$ shows a higher expression in the uterus (50). Studying the effects of prenatal DES exposure in *ER$\alpha$*, *ER$\beta$*, or double-knockout mice, will reveal which ER type is mainly involved in DES action.

In previous studies, it has been proposed that incomplete regression of the Müllerian ducts in fetuses exposed to exogenous estrogens is a result of a change in sensitivity of the ducts to AMH (25). Therefore, we have studied the effect of DES on AMHRII mRNA expression. The expression of AMH and AMHRII mRNAs was studied by *in situ* hybridization, and the expression levels in total fetuses were quantified by RNase protection. With *in situ* hybridization, a strong increase in AMH mRNA expression in the fetal testes of DES-exposed fetuses was evident. Quantification of the expression revealed a 2-fold increase of AMH mRNA expression in DES-exposed fetuses, compared with controls. This increase was most significant at E13. Nevertheless, this higher AMH mRNA expression did not result in complete Müllerian duct regression. This is in agreement with the observations in *in vitro* studies that addition of a relatively high dose of AMH did not result in full regression of Müllerian ducts from DES-exposed fetuses (Newbold *et al.*, personal communication). The DES-induced increase in AMH mRNA expression implies a direct effect of estrogens on the regulation of AMH mRNA expression. Indeed, a 13-bp palindromic sequence, nearly identical to the estrogen response element (ERE), has been identified in the *AMH* gene promoter (51). In footprinting experiments, this site was shown to bind ER$\alpha$. Furthermore, 39 ERE half-sites were identified in the 5′ flanking sequences of the *AMH* gene (52). Clusters of half-sites or degenerate palindromic sites can be effective, as was shown *in vitro,* where several ERE half-sites can act synergistically to control expression of the ovalbumin gene (53). However, the functionality of the ERE half-sites in the *AMH* gene has not been proven. Recent papers have shown that AMH expression is dependent on SF-1 (54, 55). SF-1, an orphan nuclear receptor expressed in adrenals, gonads, and the gonadotrophes of the pituitary gland, was characterized as a transcription factor that regulates several genes, such as genes encoding steroidogenic enzymes (36). *SF-1* knockout mice lack gonads and adrenals, revealing an essential role for SF-1 in sexual differentiation and formation of primary steroidogenic tissues (28). In *in vivo* experiments, it was demonstrated that the proximal *AMH* gene promoter requires an intact binding site for SF-1 for time- and cell-specific expression (55). We observed a strong increase in SF-1 mRNA expression in DES-exposed fetuses, which was most significant at E13 and decreased toward E17. The increased expression of AMH mRNA in DES-exposed mice was found to have a similar temporal pattern as the SF-1 mRNA expression, corresponding with the role of SF-1 in regulation of AMH gene expression. These data suggest that DES has an effect on fetal gonadal gene expression. An effect of prenatal exposure to estrogenic compounds on SF-1 mRNA expression has been reported previously, although the described effect is a down-regulation of SF-1 mRNA expression (56). In that study by Majdic *et al.* (56), DES or the estrogenic compound 4-octylphenol were injected twice during pregnancy (E11 and E15), and expression of SF-1 mRNA was measured in the fetal testis at E17 (56). The disagreement between their and our results may be explained by the animal model, the experimental procedure, and the time points at which expression was determined.

In the present study, expression of AMHRII mRNA was also found to be increased at E13 in DES fetuses, although this increase was less obvious and could not be detected by *in situ* hybridization. In *in vitro* studies, no direct regulation of the

*AMHRII* promoter by estrogens was found (Visser *et al.*, unpublished results). Therefore, it is likely that DES influences AMHRII mRNA expression indirectly. In the DNA sequence of both the human and mouse *AMHRII* gene promoter, a SF-1 response element was identified (Ref. 12, and results not shown). Although regulation of AMHRII mRNA expression by SF-1 has not been reported, the increased AMHRII mRNA expression in DES-exposed fetuses might be a consequence of an increased SF-1 level. In accordance with developmental changes in SF-1 and AMH mRNA expression, the most pronounced increase in AMHRII mRNA expression was found at E13, and this increase becomes less evident in older fetuses.

The increased mRNA expression levels of AMH and AMHRII do not directly explain the decreased sensitivity of the Müllerian ducts to AMH. However, a DES-induced effect on factors downstream of AMHRII, such as a type I receptor or Smad proteins, cannot be excluded. One can hypothesize that a DES-induced inhibition of downstream signaling factors influences a negative feedback loop, resulting in an increased expression of AMH and AMHRII mRNA, although the existence of such a feedback system for AMH has not been reported yet. Furthermore, whether the increase mRNA expression levels result in higher protein levels remains to be studied.

The *in situ* hybridization demonstrated that AMHRII mRNA expression can be detected along the entire axis of the Müllerian ducts in control fetuses at E13, but it decreases in caudal direction toward the urogenital sinus. At E15, expression of AMHRII mRNA could not be detected in the regressed cranial part of the Müllerian ducts. However, also in the caudal part of the Müllerian ducts, AMHRII mRNA expression could hardly be detected. These observations suggest that the onset of the critical period for AMH sensitivity of the Müllerian ducts (E13) is at the time point when Müllerian ducts are completed and express the AMHRII, whereas the end of this critical period (E15) is demarcated by disappearance of the receptor. In DES-exposed fetuses at E13, AMHRII mRNA expression was found in the cranial part of the Müllerian ducts. The caudal parts have not been formed, and expression could not be detected at this site, suggesting that AMHRII mRNA expression is dependent on the presence of a formed Müllerian duct. At E15, a time point at which the Müllerian ducts have completely formed in the DES-exposed fetuses, AMHRII mRNA expression was detectable in the cranial ducts, although expression was much lower, compared with E13. Caudally, expression could hardly be detected, comparable with expression in control E15 fetuses. In DES-exposed mice just before birth (E19), the Müllerian duct remnants had lost expression of AMHRII mRNA and, therefore, are unable to respond to AMH at this late developmental time point. Although the formation of the Müllerian ducts is delayed in DES-treated fetuses, the timing of AMHRII mRNA expression is not delayed. This probably leads to a temporal asynchrony between the presence of the Müllerian ducts and the onset of the critical period of Müllerian duct regression.

The present observation on the DES-induced delay in Müllerian duct formation contributes to our understanding of the diversity of developmental defects in affected DES-sons. In humans, exposure of mothers to DES during early pregnancy results in a 2-fold increase in the prevalence of malformations in their sons (57). The formation of the Müllerian ducts is completed before the 11th week of gestation, and Müllerian duct regression is initiated at the 11th week. Exposure to DES after this period results in less abnormalities, whereas exposure before the 11th week results in a higher incidence of Müllerian duct remnants in the DES-sons (57). This is in concordance with the present observations in mice, and we suggest that, also in humans, administration of DES during early pregnancy causes an asynchrony between Müllerian duct formation and the critical period of Müllerian duct regression.

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