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Estrogen Receptor (ER) and Its Messenger Ribonucleic Acid Expression in the Genital Tract of Female Mice Exposed Neonatally to Tamoxifen and Diethylstilbestrol

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ABSTRACT ***Background:*** **Tamoxifen (Tx) is known as an antiestrogen because of its competitive inhibition of estrogen binding to estrogen receptor (ER), and it is used as an estrogen antagonist in the human breast. However, Tx is known to have estrogen agonist activity in the human fetal reproductive tracts and vaginal epithelium and endometrium of postmenopausal women as has been known in the mouse uterus. Therefore, we examined estrogenic potency of Tx on the uterus and vagina in newborn mice and adult ovariectomized mice.**

Methods: **Using immunohistochemistry and in situ hybridization, we studied changes in expression of ER protein and ER mRNA in the uterus and vagina of C57BL/Tw mice exposed neonatally to 100 μg Tx and 0.03–3 μg diethylstilbestrol (DES), and changes in expression of ER mRNA in the ovariectomized adult mice given injections of 100 μg Tx and 3 μg DES.**

Results: **Nuclei of the epithelial and stromal cells in the vagina and of the stromal cells in the uterus showed strong ER immunostaining on the day of birth (= day 0), whereas nuclei of the epithelial cells in the uterus exhibited the ER immunostaining by day 5. In uterine epithelial cells, however, ER was induced by DES, 17β-estradiol, testosterone or Tx 24 h after a single injection on day 0, but not by the injection of 5α-dihydrotestosterone, progesterone, or epidermal growth factor. ER in uterine epithelial cells was detected even 12 h after a single injection of 3 μg DES on day 0. ER mRNA expression of uterine and vaginal epithelial cells of newborn mice increased 4 h after a single injection of 3 μg DES. ER mRNA expression of uterine and vaginal stromal cells in neonatal mice increased 4 h after a single injection of 100 μg Tx. In uterine epithelial and stromal cells and vaginal epithelial cells of ovariectomized adult mice, ER mRNA expression increased 12 h after a single injection of 3 μg DES and 100 μg Tx.**

Conclusions: **The present study indicates that Tx acts as ER inducer in the uterus and vagina of neonatal and ovariectomized adult mice. However, responsiveness of reproductive tracts to Tx is different between newborn and adult mice.**

Key words: Estrogen receptor, Estrogen receptor mRNA, Uterus, Vagina, Tamoxifen, DES, Mouse

Estrogens stimulate cell proliferation, differentiation, and induction of specific proteins in the uterus, vagina, and other target organs in vivo (Katzenellenbogen and Gorski, 1975; Martin, 1980). The mechanism by which estrogens produce these effects, however, is not yet clearly elucidated. Estrogen action is thought to be mediated by an intracellular estrogen receptor (ER) in the target cells (Gorski and Gannon, 1976). ER is a member of a superfamily of ligand-inducible transcription factors (Barton and Shapiro, 1988). In mice, uterine epithelial cells have no ER at least for 3 postnatal days (Korach et al., 1988; Taguchi

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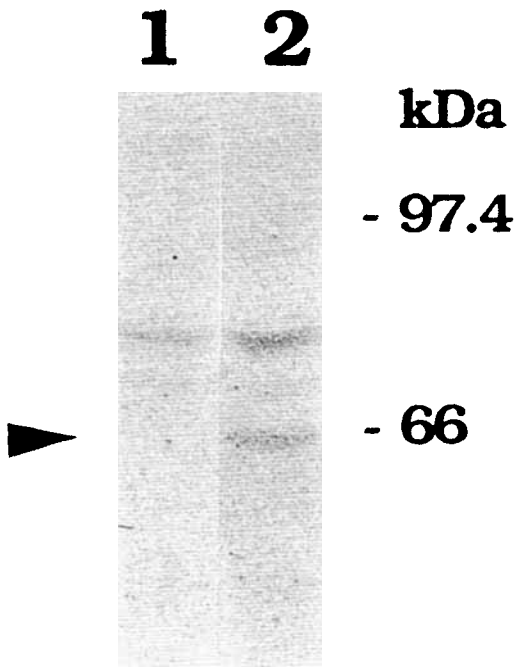

Fig. 1. Immunoblot analysis using anti-ER antibody in adult ovariectomized uterus. Lane 1, uterine proteins stained with non-immune IgG; Lane 2, uterine proteins stained with antibody for ER.

et al., 1988; Yamashita et al., 1989; Bigsby et al., 1990; Greco et al., 1991; Sato et al., 1992), but a single injection of 17β-estradiol (E_2) or diethylstilbestrol (DES) on the day of birth induces ER expression (Taguchi et al., 1988; Sato et al., 1992; Yamashita et al., 1990). However, in the uterus of adult ovariectomized rats, ER mRNA levels were decreased by E_2 (Shupnik et al., 1989; Medlock et al., 1991).

Tamoxifen (Tx) is an antiestrogen because of its competitive inhibition of estrogen binding to ER (Katzenellenbogen et al., 1983; Furr and Jordan, 1984; Jordan, 1984; Jordan and Murphy, 1990) and used as an estrogen antagonist in the human breast (Furr and Jordan, 1984; Baum et al., 1988; Nayfield et al., 1991; Powles, 1992). However, Tx is known to have estrogen agonist activity in human fetal genital tract (Cunha et al., 1987) and in the vaginal epithelium and endometrium of postmenopausal women (Ferrazzi et al., 1977; Boccardo et al., 1981, 1984; Eells et al., 1990; Lahti et al., 1993; Ismail, 1994) as has been known in the mouse uterus (Terenius, 1971; Chou et al., 1992; Iguchi, 1992).

The present study was planned to determine the estrogenic potency in induction of ER of Tx on the uterus and vagina. We compared the changes in expression of ER mRNA and ER protein in the uterus and vagina of mice exposed neonatally to Tx and DES and changes in expression of ER mRNA in the ovariectomized adult mice given Tx and DES injections. We also tested the hypothesis that epidermal growth factor (EGF) acts as a mediator of estrogenic compounds (DiAugustine et al., 1988) by ER induction in newborn mouse uterine epithelium.

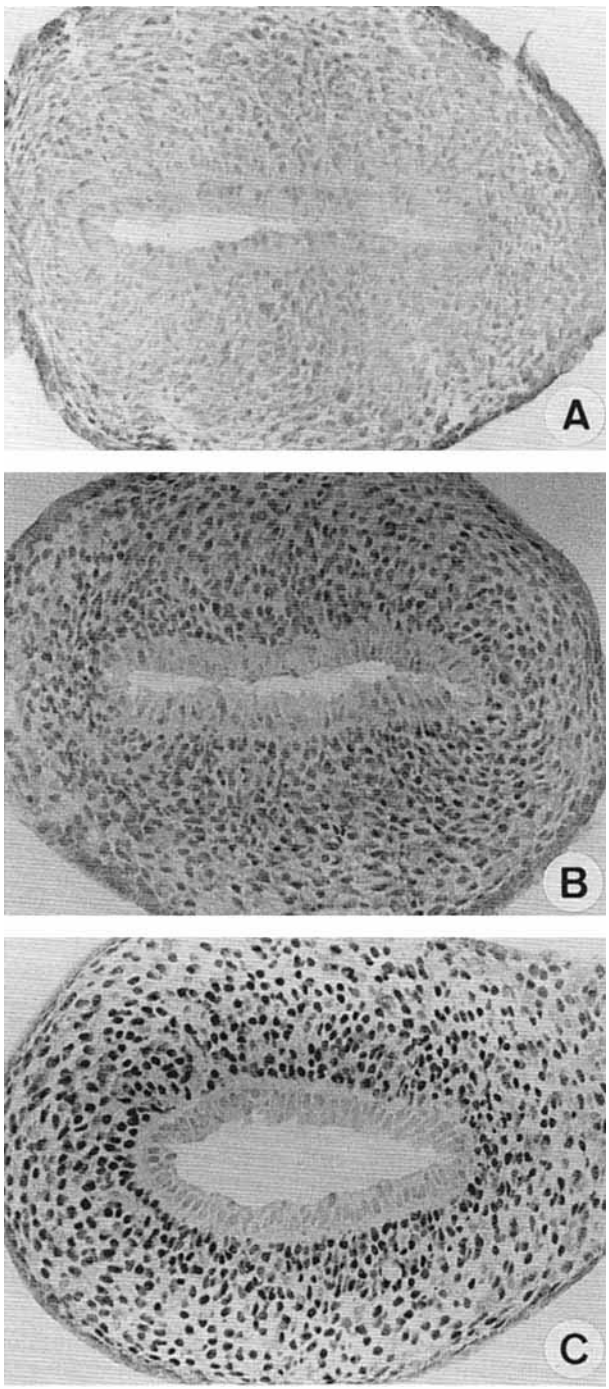

Fig. 2. ER expression in the uterus of newborn mice. Control staining (nonimmune IgG) performed 12 h after a single injection of 3 μg DES (**A**), immunostaining of ER 12 h after a single injection of 3 μg DES (**B**), and 24 h after a single injection of 100 μg Tx (**C**). ×195.

MATERIALS AND METHODS

Animals

Female mice of the C57BL/Tw strain were kept under 12 h light/12 h dark at 23–25°C temperature and fed laboratory chow (CE-2, CLEA, Tokyo, Japan) and

TABLE 1. Induction of estrogen receptor in the uterus and vagina of newborn C57BL female mice by single injection of sex hormone and tamoxifen[1]

Treatment	Hours after injection	Uterus		Vagina	
		epithelium	stroma	epithelium	stroma
Oil	24	−	+ + +	+ + +	+ + +
	48	−	+ + +	+ + +	+ + +
0.03 μg DES	24	−	+ +	+ + +	+ + +
0.3 μg DES	24	+/+ +	+ +/+ + +	+/+ +	+/+ +
3 μg DES	12	−/+	+ +	+/+ +	+/+ +
	24	+ +	+ +	+/+ +	+/+ +
	48	+ +/+ + +	+ + +	+/+ +	+/+ +
1 μg E_2	24	−	+ +	+ +	+ +
	48	−	+/+ +	+ + +	+ + +
10 μg E_2	24	−	+/+ +	+ + +	+ + +
50 μg E_2	24	+/+ +	+ +	+ + +	+ + +
1 μg Tx	24	−	+ + +	+ + +	+ + +
	48	−	+ + +	+ + +	+ + +
10 μg Tx	24	−	+ + +	+ + +	+ + +
	48	−/+	+ + +	+ + +	+ + +
100 μg Tx	24	+	+ + +	+ +	+ + +
	48	+/+ +	+ + +	+/+ +	+ + +
50 μg P	24	−	+ +	+/+ +	+ +
	48	−	+/+ +	+ +/+ + +	+ + +
50 μg T	24	−/+	+ +	+ +	+ +/+ + +
	48	−	+/+ +	+/+ +	+/+ +
50 μg DHT	24	−	+ + +	+ +	+ +/+ + +
	48	−	+ +/+ + +	+ +	+/+ +
5 μg EGF	24	−	+ + +	+ +	+ +

[1]Abbreviations: DES: diethylstilbestrol, E_2: 17β-estradiol, Tx: tamoxifen, P: progesterone, T: testosterone, DHT: 5α-dihydrotestosterone, EGF: epidermal growth factor, −: negative, + to + + +: slight, medium, and strong immunostaining.

tap water ad libitum. All procedures were carried out according to the NIH Guide for the Care and Use of Laboratory Animals. ER induction potency of various compounds was studied in newborn mice. Newborn females were given a single s.c. injection of 0.03, 0.3 or 3 μg DES (Sigma Chemical, St. Louis, MO) dissolved in 0.02 ml sesame oil or of the oil vehicle alone on the day of birth (=day 0). ER expression in the vagina and uterus was examined 24 h and 48 h after the injection. In addition, 0-day-old mice injected with 50 μg 5α-dihydrotestosterone (DHT), 50 μg testosterone (T), 1, 10 or 50 μg 17β-estradiol (E_2), 50 μg progesterone (P), 1, 10, or 100 μg Tx (all from Sigma), 5 μg mouse EGF (Collaborative Research, Bedford, MA) and 5 μg human EGF (Wakunaga, Osaka, Japan) were killed 24 h after the injection.

For the time-course study of ER mRNA expression, newborn mice and 50-day-old ovariectomized mice (10 days after ovariectomy) given a single injection of 3 μg DES or 100 μg Tx dissolved in 0.1 ml sesame oil or suspended in 0.1 ml saline, respectively, were killed 4, 8, 12, or 24 h after the injection.

To determine effects of Tx and DES on mitotic activity in the vagina and uterus, newborn mice and 50-day-old ovariectomized mice given a single injection of 3 μg DES, 100 μg Tx or the vehicle alone were killed 24 h after the injection. A single injection of 1 μg colchicine (Merck, Darmstadt, Germany) dissolved in 0.02 ml saline solution was given 3 h before sacrifice. The genital tract fixed in Bouin's solution was embedded in paraffin and serially sectioned at 8 μm thickness. Sections were stained with hematoxylin and eosin. The number of epithelial and stromal cells at metaphase per 400 cells was counted separately.

Tissues for DNA and protein measurements were frozen and stored at −80°C. DNA and protein contents of uterus and vagina in newborn and 50-day-old ovariectomized adult mice given a single injection of 3 μg

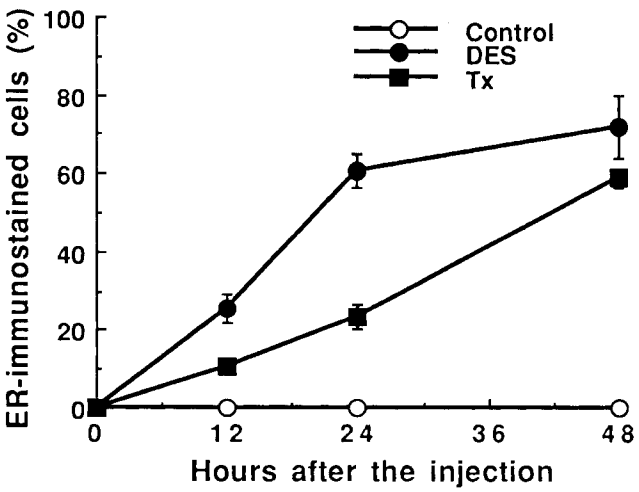

Fig. 3. The incidence of ER immunostained cells in the uterus of newborn mice after a single injection of vehicle only (control), 3 μg DES or 100 μg Tx on day 0.

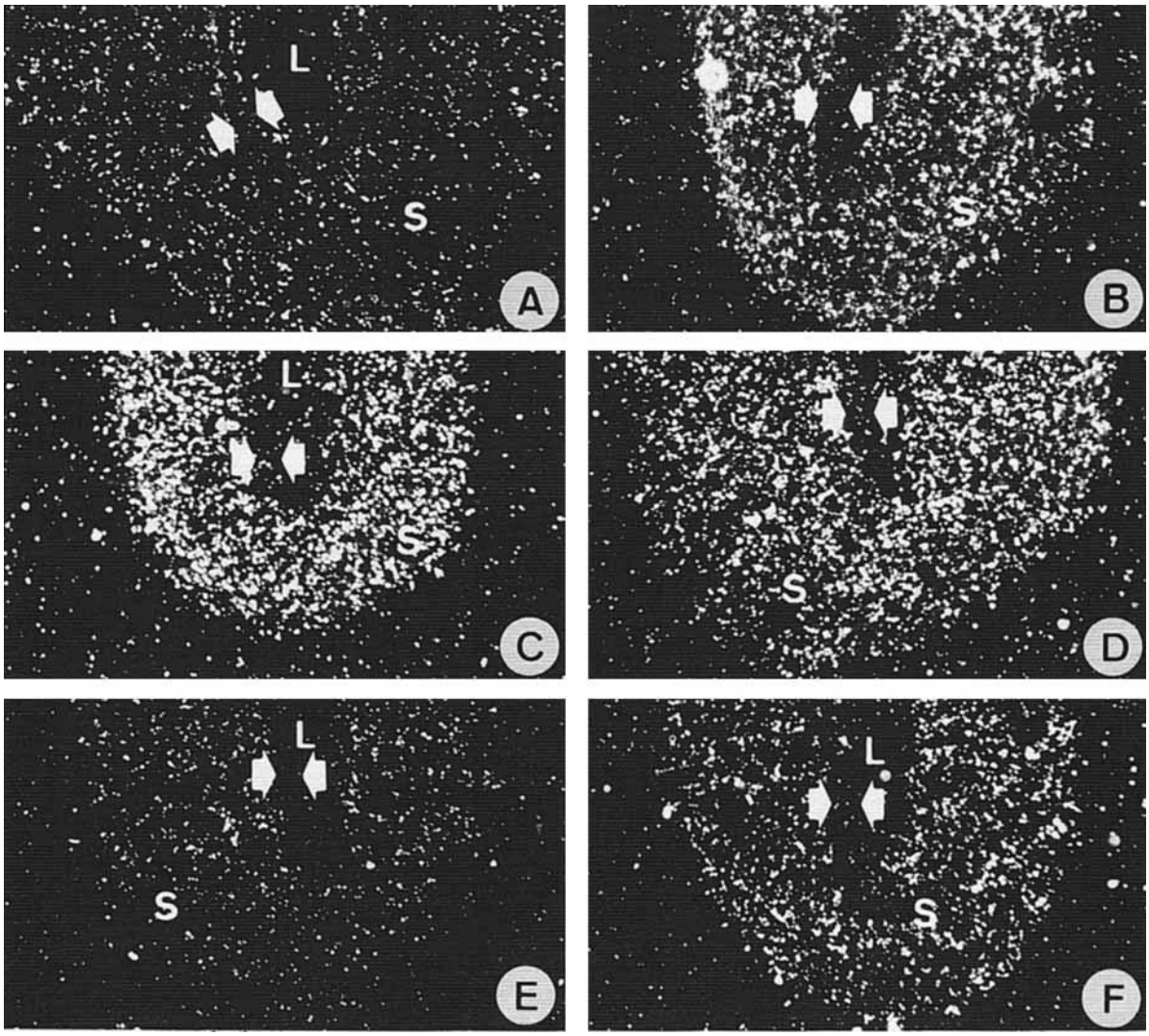

Fig. 4. In situ hybridization of ER mRNA in the uterus of 0-day-old mice performed before and 4 to 24 h after a single injection of 3 μg DES. Uterus hybridized with sense probe (**A**). Uterus hybridized with antisense probe before (**B**), and 4 (**C**), 8 (**D**), 12 (**E**), and 24 (**F**) h after injection. Arrows indicate epithelium; S, stroma; L, lumen. ×195.

DES, 100 μg Tx, or the vehicle alone were determined by the method reported previously (Labarca and Paigen, 1980) using a dye, Hoechst 33258 (Calbiochem, La Jolla, CA), and BCA Protein Assay Reagent (PIERCE, Rockford, IL), respectively.

Seven mice were used for each experimental group. To avoid differences in developmental stages in newborn mice, pups in each experimental group were combined at least from three litters. Data were analyzed by ANOVA.

Immunoblot Analysis and Immunohistochemical Staining of ER

For studies of immunoblots, uterus from adult ovariectomized mice were minced and extracted in 10 mM Tris and 2% sodium dodecyl sulfate (SDS) buffer by sonication and passed through a 25G needle as described previously (Greco et al., 1991). Proteins were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes that were incubated in Tris-buffered saline (TBS) (pH 7.5) containing 5% skim milk (Difco, Detroit, MI) at room temperature for 1 h, then treated with anti-ER monoclonal antibody (Abbott Laboratories, Chicago, IL) or nonimmune IgG (Abbott) at 1:50 dilution overnight. The membrane washed in 0.5% Tween 20-TBS (TTBS) and incubated with biotinylated antirat IgG (1:1000 dilution) (Vector, Burlingame, CA) for 45 min. Then, the membrane was incubated with alkaline phosphatase-conjugated avidin D (1:1000 dilution) (Vector) for 45 min. The alkaline phosphatase-conjugated site was visualized with the phosphatase substrates, nitro blue tetrazolium (Wako Pure Chemicals, Osaka, Japan) and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma).

For studies of immunostaining, anti-ER monoclonal antibody (Abbott) was diluted two times with phos-

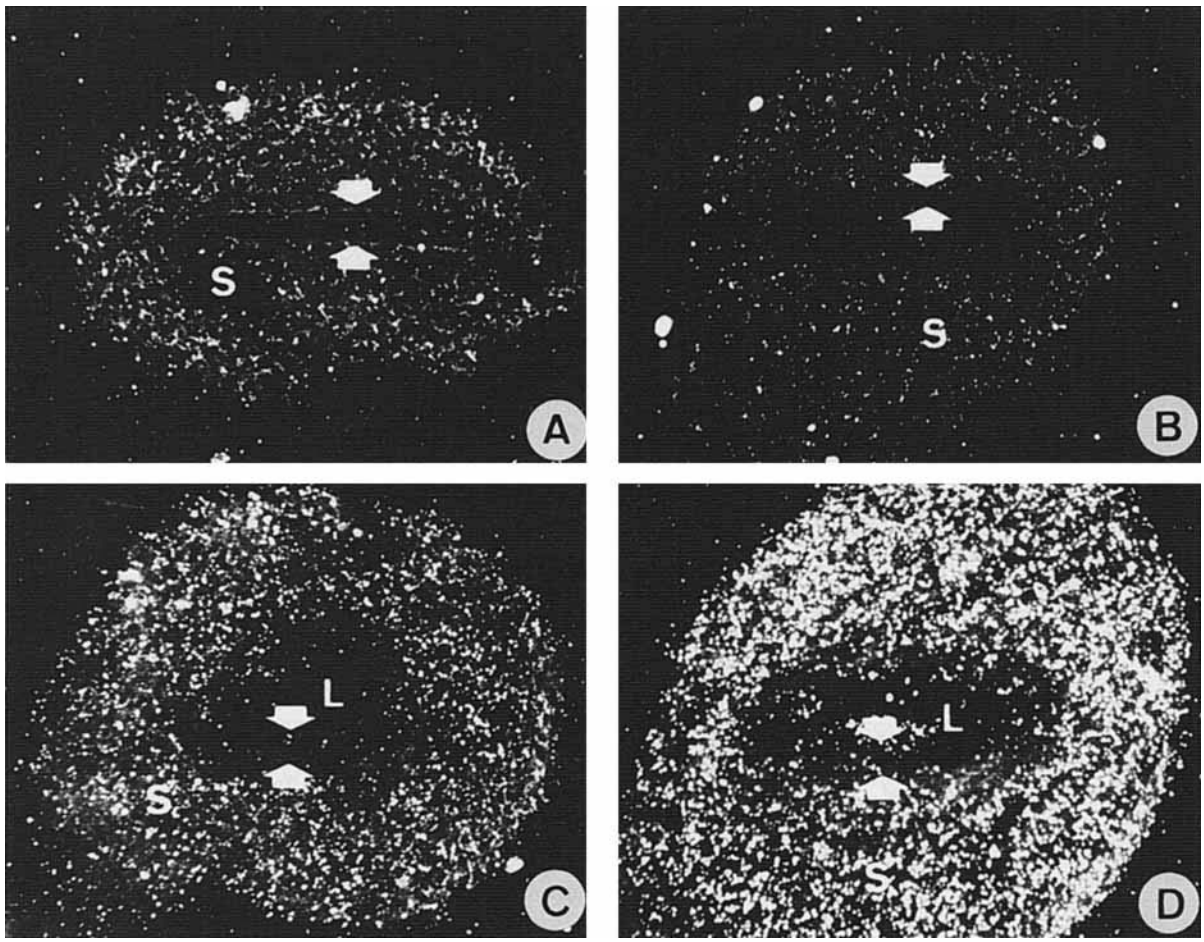

Fig. 5. In situ hybridization of ER mRNA in the uterus of 0-day-old mice performed before and 4 to 24 h after a single injection of 100 μg Tx. Uterus hybridized with antisense probe before (**A**), and 4 (**B**), 8 (**C**), and 24 (**D**) h after the injection. Uterus hybridized with antisense probe as in Figure 4. Arrows indicate epithelium; S, stroma; L, lumen. ×185.

phate-buffered saline (PBS) containing 2% bovine serum albumin fraction V (BSA, Sigma) at pH 7.4. Horseradish peroxidase (HRP)-labeled antirat $F(ab')_2$ fragment (HRP-$F(ab')_2$) was obtained from Amersham (Chicago). HRP-$F(ab')_2$ was diluted 50 times with PBS containing 1% BSA.

Tissues embedded in O.C.T. compound (Miles Laboratories, Elkhardt, IN) were sectioned at 6 μm thickness. The frozen section was mounted on a glass slide and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2 for 15 min at room temperature. The immunohistochemical staining procedure was performed as described previously (Yamashita and Korach, 1989; Iguchi et al., 1991; Sato et al., 1992, 1994). Sections were incubated with 1% BSA in PBS for 30 min and then with anti-ER monoclonal antibody or nonimmune IgG (Abbott) for 2 h at room temperature. After washing with PBS, the sections were incubated with the second antibody HRP-$F(ab')_2$ fragment for 45 min at room temperature. Diaminobenzidine tetrahydrochloride (DAB) reaction with imidazole was carried out for 10 min (Straus, 1980). Intensity of the immunohistochemical reaction was graded from − (negative) to + + + (strongly positive) using a Color Image Analyzer CIA-102 (Olympus, Tokyo) as described previously (Iguchi et al., 1991; Sato et al., 1992, 1994).

In Situ Hybridization

Tissues cut into pieces at the time of sacrifice were fixed at 4°C for 3 h in 4% paraformaldehyde diluted with PBS and then washed with PBS containing 8% sucrose overnight at 4°C and embedded in O.C.T. compound for freezing. The sections cut serially at 6 μm thickness were mounted on a glass slide precoated with 1% BSA and fixed in 25% glutaraldehyde. The cDNA insert encoding rat ER (Koike et al., 1987), which was kindly provided by Professor M. Muramatsu (Department of Biochemistry, Faculty of Medicine, University of Tokyo), was ligated into the *Eco*RI site of plasmid Bluescript (Stratgene, La Jolla, CA). The orientation was confirmed by restriction mapping. After linearization with *Bam*HI, the plasmid was transcribed by T7 RNA polymerase using (α-^{35}S) UTP (Dupont/NEN Research Products, Boston, MA) according to the manufacturer's protocol to produce an antisense RNA probe. For control, a sense RNA probe was transcribed by T3

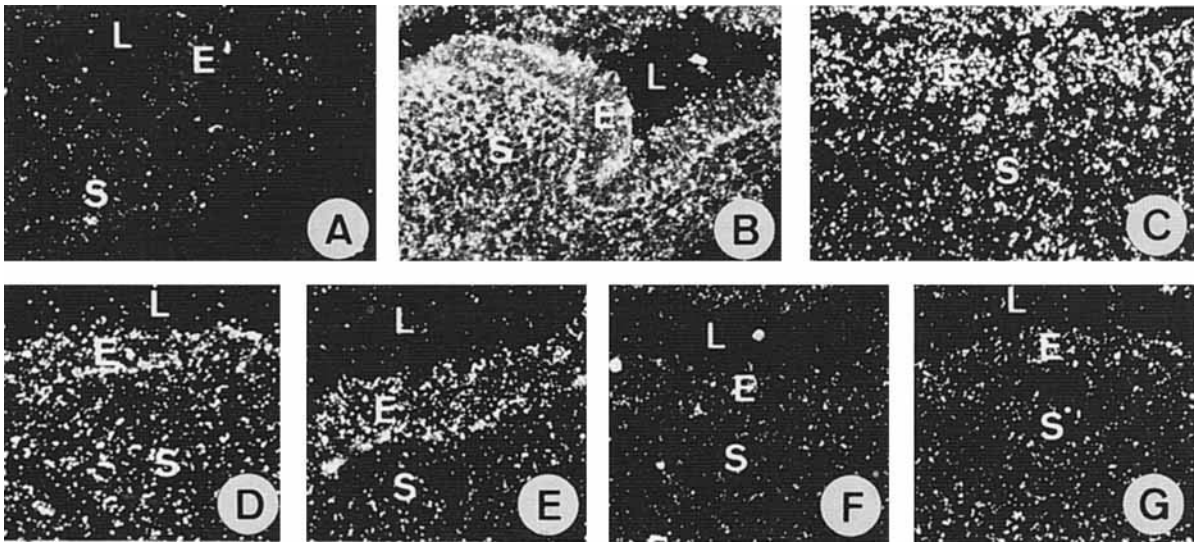

Fig. 6. In situ hybridization of ER mRNA in the vagina of 0-day-old mice performed before (**A**), 8 (**B**), and 24 (**C**) h after a single injection of 100 μg Tx, and 4 (**D**), 8 (**E**), 12 (**F**), and 24 (**G**) h after a single injection of 3 μg DES. Vaginae hybridized with antisense probe as in Figure 4. E, epithelium; S, stroma; L, lumen. ×175.

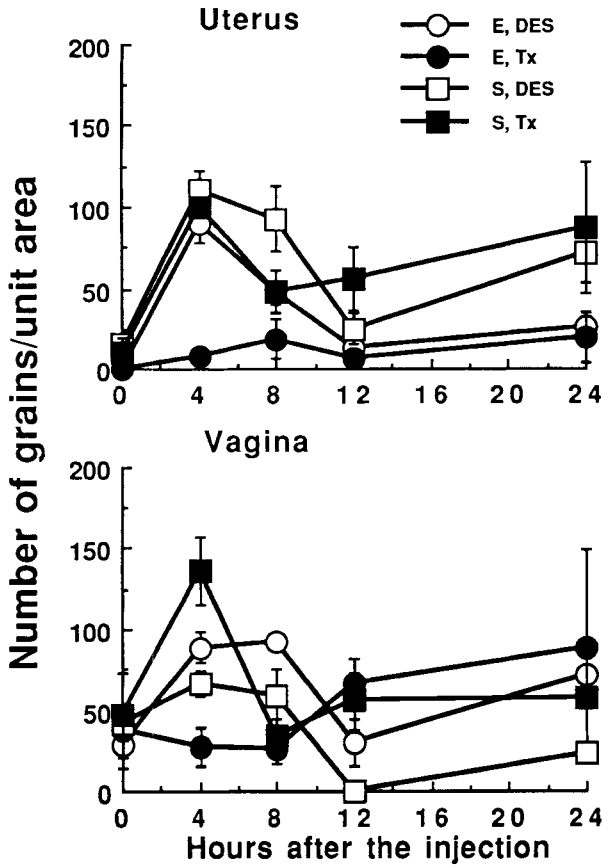

Fig. 7. Number of silver grains per an unit area (5,000 μm^2) of epithelium (E) and stroma (S) in the uterus and vagina of newborn mice given a single injection of 3 μg DES or 100 μg Tx.

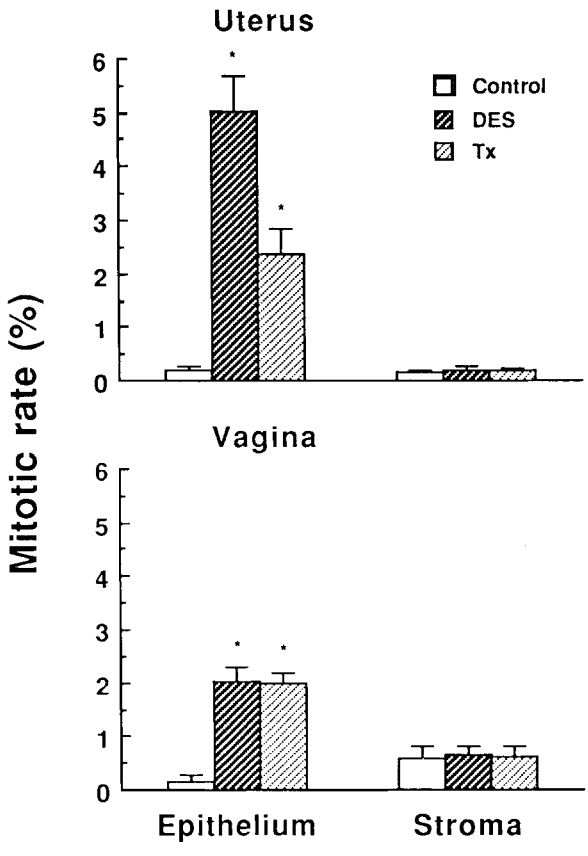

Fig. 8. Mitotic rate in the uterus and vagina of 50-day-old ovariectomized mice 24 h after a single injection of 3 μg DES and 100 μg Tx. *, $P < 0.05$ compared with controls (ANOVA).

RNA polymerase after linearization with *Hind*III. Both probes were truncated to ~75 nucleotides by alkaline treatment. Hybridization was carried out as described previously (Ishii et al., 1990; Sato et al., 1994) with 2 × 10^7 cpm/ml of the ^{35}S-labeled probes at 50°C overnight in a solution containing 50% formamide (Merck), 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 2.5 mM EDTA (Sigma), 10 mM dithiothreitol (Sigma), 0.5 mg/ml tRNA (Boehringer Mannheim, Mannheim, Germany), 1× Denhardt's solution, 10% dextran sulfate, and 0.5 mg/ml sonicated salmon sperm DNA (Sigma). The sections were washed in 2× saline sodium citrate (SSC), 50% formamide including 0.1% mercaptoethanol (Sigma) at 50°C for 1 h, and then treated with heated RNase A (20 μg/ml) (Boehringer Mannheim) in 0.5 M NaCl, 10 mM tris-HCl at pH 8.0 at room temperature for 30 min. Slides were then rinsed in 2× SSC, 50% formamide (Merck) and 0.1% mercaptoethanol at 50°C for 1 h, 1× SSC, 50% formamide and 0.1% mercaptoethanol at 50°C for 40 min, 0.5× SSC, 50% formamide and 0.1% mercaptoethanol at 50°C for 40 min, and finally in 0.1× SSC and 50% formamide at room temperature for 40 min, and dehydrated. The slides were dipped in emulsion (Konica, Tokyo, Japan), air dried, and then exposed at 4°C for 10 days. They were developed in Konicadol X (Konica), counterstained with hematoxylin, and observed by darkfield microscopy. The number of grains per unit area (5,000 μm^2) were estimated in the epithelium and stroma, separately, by a Color Image Analyzer (Olympus) as described previously (Sato et al., 1994).

RESULTS

Effects of Single Injection of Tx or DES on Cell Division, DNA, and Protein Contents of Uterus and Vagina

A single injection of Tx to newborn mice did not stimulate the mitotic rate in uterine (1.4 ± 0.3) and vaginal (2.3 ± 0.2) epithelium compared with that of oil-injected controls (uterine epithelium, 1.5 ± 0.4; vaginal epithelium, 1.9 ± 0.3) 24 h later. The rate was significantly raised only in uterine epithelium (2.8 ± 0.3) but not in vaginal epithelium (2.2 ± 0.3) of newborn mice given injections of 3 μg DES. In the stromal cells of the uterus (vehicle, 0.5 ± 0.1; DES, 0.8 ± 0.1; Tx, 0.9 ± 0.1) and vagina (vehicle, 0.7 ± 0.1; DES, 0.6 ± 0.2; Tx, 0.5 ± 0.1), however, the rate was not altered by neonatal injections of these compounds.

DNA and protein contents of the uterus and vagina in newborn mice were not changed 24 h after a single injection of 100 μg Tx or 3 μg DES.

Effects of Single Injection of Tx, DES, Sex Hormones, or EGF on ER Expression in Newborn Mouse Uterus and Vagina

Immunoblot analysis demonstrated that the antibody recognized a single band of 66 kDa protein extracted from the uterus of ovariectomized adult mice (Fig. 1).

In mice given a single injection of oil vehicle alone, uterine stromal cells showed ER. However, no ER positive cell was found in epithelial cells at least 48 h after the injection (Table 1; see also Fig. 3). A single injection of 100 μg Tx on the day of birth (= day 0) induced ER expression in ~25% of uterine epithelial cells 24 h after the injection (Fig. 2C). Approximately 60% of

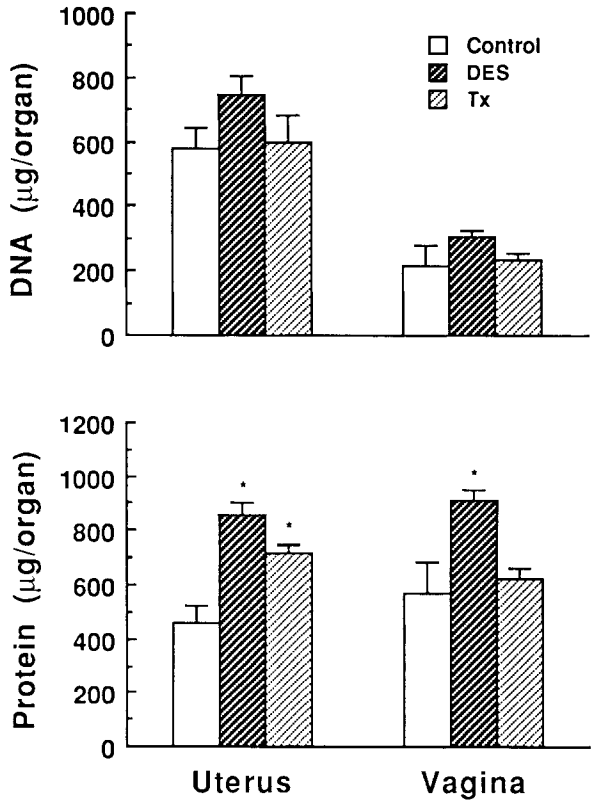

Fig. 9. DNA (A) and protein (B) contents of the uterus and vagina in 50-day-old ovariectomized mice treated with a single injection of 3 μg DES or 100 μg Tx. *, $P < 0.05$ compared with controls (ANOVA).

uterine epithelial cells were stained ER positive 48 h after the 100 μg Tx injection (Fig. 3). A single injection of 3 μg DES on day 0 induced ER in uterine epithelial cells within 12 h after the injection. The incidence of ER-immunostained epithelial cells increased 24 h later (Fig. 3). Staining intensity was increased 12 h after the injection (Fig. 2B). The staining intensity of uterine stromal cells was reduced by the DES injection compared with that in the controls. A single injection of 50 μg E_2, 0.3 μg DES and 50 μg T also induced ER expression in ~85%, 55%, and 50% of uterine epithelial cells 24 h later, respectively. Ten μg Tx induced ER expression in uterine epithelial cells 48 h later. DHT, P, 0.03 μg DES, 1 or 10 μg E_2, 1 μg Tx or 5 μg EGF did not induce ER expression in uterine epithelial cells (Table 1). In the vagina, ER immunostaining was decreased in both epithelial and stromal cells compared to oil-injected controls.

Effect of Tx and DES on ER mRNA Expression of Newborn Mouse Uterus and Vagina

ER mRNA was demonstrated by in situ hybridization in uterine and vaginal stromal cells and in vaginal epithelial cells, but not in uterine epithelial cells on day 0. ER mRNA of uterine and vaginal epithelial cells and of uterine stromal cells significantly increased 4 h, then decreased 12 h, after a single injection of 3 μg DES. A single injection of 100 μg Tx significantly increased ER mRNA expression in uterine and vaginal

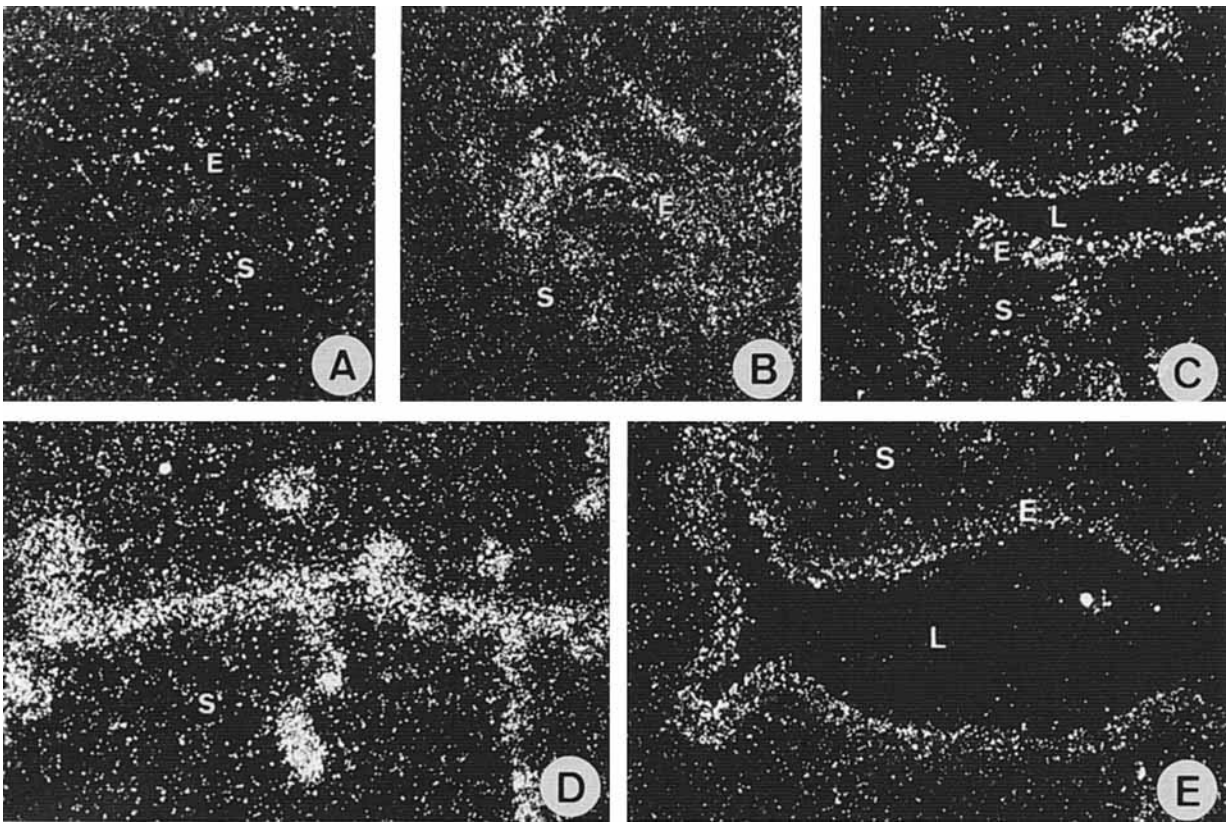

Fig. 10. In situ hybridization of ER mRNA in the uterus of 50-day-old ovariectomized mice performed before and 4 to 24 h after a single injection of 3 μg DES. Uterus hybridized with sense probe (**A**). Uterus hybridized with antisense probe before (**B**), and 4 (**C**), 12 (**D**), and 24 (**E**) h after injection. E, epithelium; S, stroma; L, lumen. ×140.

stromal cells 4 h later (see Fig. 7). However, ER mRNA in uterine and vaginal epithelial cells showed a control level of expression 24 h after Tx injection (Figs. 4–7).

Effects of Single Injection of Tx and DES on Cell Division, DNA, and Protein Contents of Adult Ovariectomized Mouse Uterus and Vagina

A single injection of 3 μg DES and 100 μg Tx to 50-day-old ovariectomized mice significantly stimulated the cell division in uterine and vaginal epithelial cells 24 h later. However, no change was found in the uterine and vaginal stromal cells (Fig. 8).

DNA contents of the uterus and vagina in 50-day-old ovariectomized mice were not changed 24 h after a single injection of 100 μg Tx or 3 μg DES. Protein contents of the uterus in 50-day-old ovariectomized mice treated with 100 μg Tx or 3 μg DES were significantly greater than in controls. Only the injection of 3 μg DES significantly increased protein contents of the vagina (Fig. 9).

Effect of Tx and DES on ER mRNA Expression of Ovariectomized Adult Mouse Uterus and Vagina

In adult ovariectomized mice given a single injection of 3 μg DES, expression of ER mRNA in uterine epithelial and stromal cells and vaginal epithelial cells was significantly augmented 12 h later. In vaginal epithelial cells, ER mRNA expression also significantly increased 12 h after a single injection of 100 μg Tx. ER mRNA expression in vaginal stromal cells was not changed by a single injection of 3 μg DES or 100 μg Tx (Figs. 10–13).

DISCUSSION

Although the uterus of early postnatal mice does not express ER protein in epithelial cells until at least the third postnatal day (Korach et al., 1988; Yamashita et al., 1989; Bigsby et al., 1990; Greco et al., 1991; Sato et al., 1992), a single injection of E_2 or DES on day 0 induces ER protein expression in uterine epithelial cells in mice (Yamashita et al., 1990; Sato et al., 1992). In the present study, ER mRNA and ER protein in uterine epithelial cells were induced 4 h and 12 h later, respectively, by a single injection of DES on day 0. ER protein in uterine epithelial cells was induced by neonatal injection of estradiol or DES in a dose-dependent manner. A single injection of 50 μg E_2 and of 0.3 μg DES similarly induced ER in uterine epithelial cells 24 h after the injection, indicating that the potency of 50 μg E_2 in induction of ER is equivalent to that of 0.3 μg DES. Neonatal injection of 3 μg DES induced ER and also significantly stimulated cell division of the uterine epithelium. Exposure of more than 0.1 μg DES or 10 μg E_2 to newborn mice induced abnormalities in reproductive tracts including uterus in later life (Takasugi,

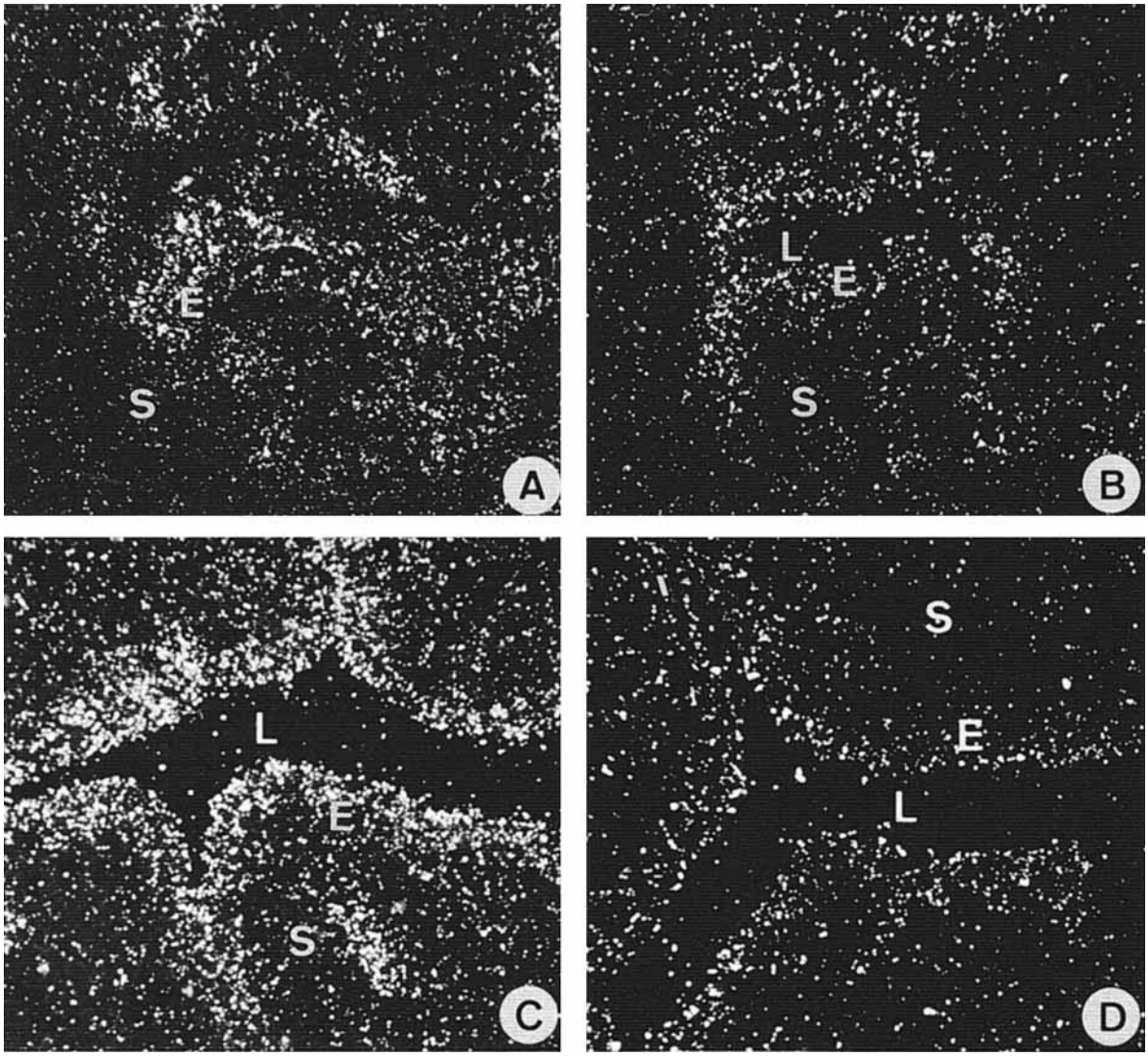

Fig. 11. In situ hybridization of ER mRNA in the uterus of 50-day-old ovariectomized mice performed before (**A**) and 4 (**B**), 12 (**C**), and 24 (**D**) h after a single injection of 100 μg Tx. Uterus hybridized with antisense probe as in Figure 10. E, epithelium; S, stroma; L, lumen. ×220.

1976; Iguchi, 1992). These results suggest that the doses of DES and E_2 that induce ER mRNA and ER protein in neonatal mouse uterine epithelial cells are correlated with those that induce persistent changes in reproductive tracts in mice. ER was induced in uterine epithelial cells by T given on day 0, but not by DHT, suggesting that T acted as ER inducer directly or after conversion into estrogen (Ryan et al., 1972; Iguchi et al., 1988).

Tx is a nonsteroidal triphenylethylene derivative that binds to ER (Katzenellenbogen et al., 1983; Ignar-Trowbridge et al., 1992), acting as an estrogen agonist or antagonist (Gronemeyer et al., 1992). Neonatal 100 μg Tx exposure induced abnormalities in the uterus, ovary, and pelvic bones in mice (Iguchi, 1992). In the present study, a single injection of 100 μg Tx on day 0 induced ER protein expression in uterine epithelial cells 24 h later; the staining intensity increased from 24 h to 48 h after the injection. However, the expression level of ER mRNA in uterine epithelial cells remained low for 24 h after the injection of Tx. ER mRNA expression and the staining intensity of ER induced by DES were greater 4 h and 24 h later, respectively, than were seen after Tx. Thus, Tx also induces ER in uterine epithelial cells, although it is weaker in action than DES. The time course of nuclear binding of E_2 and Tx in rat uterus was different: the peaks were found at 2 h and 8 h, respectively (Ennis and Stumpf, 1988). Binding affinities of Tx to ER and DES to ER are 1/50 and 1/1.25 of that of E_2 (Korach et al., 1978; Miller et al., 1986). The differences in time course of nuclear binding and/or affinity for ER between E_2 and Tx may be as-

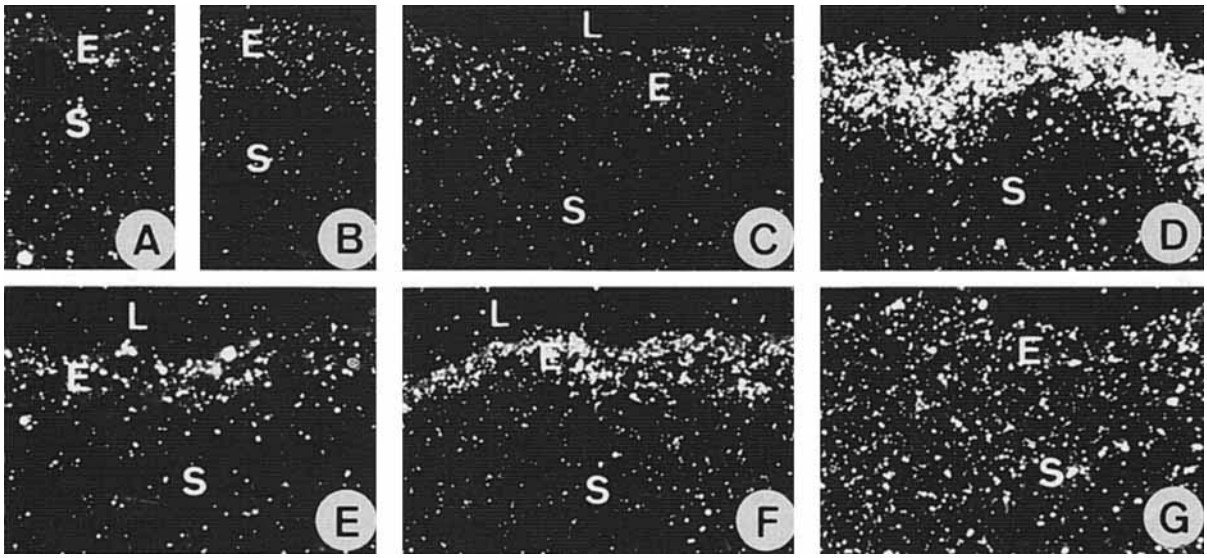

Fig. 12. In situ hybridization of ER mRNA in the vagina of 50-day-old ovariectomized mice performed before (**B**), 4 (**C**), and 12 (**D**) h after a single injection of 100 μg Tx, and 4 (**E**), 12 (**F**), and 24 (**G**) h after a single injection of 3 μg DES. Vagina hybridized with sense probe (**A**). Vaginae hybridized with antisense probe as in Figure 10. E, epithelium; S, stroma; L, lumen. ×180.

cribable to the difference in reaction time and/or amount of ER induced by DES and Tx.

Cunha et al. (1985, 1992) suggested that estrogen-induced female genital tract epithelial cell proliferation is mediated by the stroma. Estrogens cause a rapid increase in EGF of the uterus (DiAugustine et al., 1988), and EGF applied by pellet stimulated proliferation of uterine epithelial cells in vivo (Ignar-Trowbridge et al., 1992) and in vitro (Tomooka et al., 1986; Uchima et al., 1991). Therefore, expression of ER in uterine epithelial cells of neonatal mice may be mediated by EGF induced by estrogen. However, a single injection of 5 μg EGF did not induce ER expression or cell division in the present study, providing no support for the hypothesis that EGF acts as a mediator of estrogen in induction of cell division.

In situ hybridization revealed that both DES and Tx induce ER mRNA in uterine and vaginal epithelial cells 12 h after injection in ovariectomized adult mice. And the injection of DES or Tx to 50-day-old ovariectomized mice significantly stimulated the cell division in uterine and vaginal epithelial cells. Shupnik et al. (1989) and Medlock et al. (1991) showed that ER mRNA level in the uterus of ovariectomized adult rats is reduced by E_2. Our results, however, indicate that both DES and Tx act as ER inducer in the uterus and vagina in both neonatal and ovariectomized adult mice, although ER mRNA induction time of Tx is slower than that of DES in neonatal mice. Estrogens including DES evoke the early response of genes, *jun* and *fos* proto-oncogenes, whose products control the cell cycle (Loose-Mitchell et al., 1988; Weisz and Bresciani, 1988; Kamiya et al., 1995). Tamoxifen also raises the uterine activity levels of these protooncogenes in rats (Kirkland et al., 1993; Nephew et al., 1993) and in mice (Nishimura et al., 1993). In the present study, Tx shows an estrogenic activity in induction of ER and cell

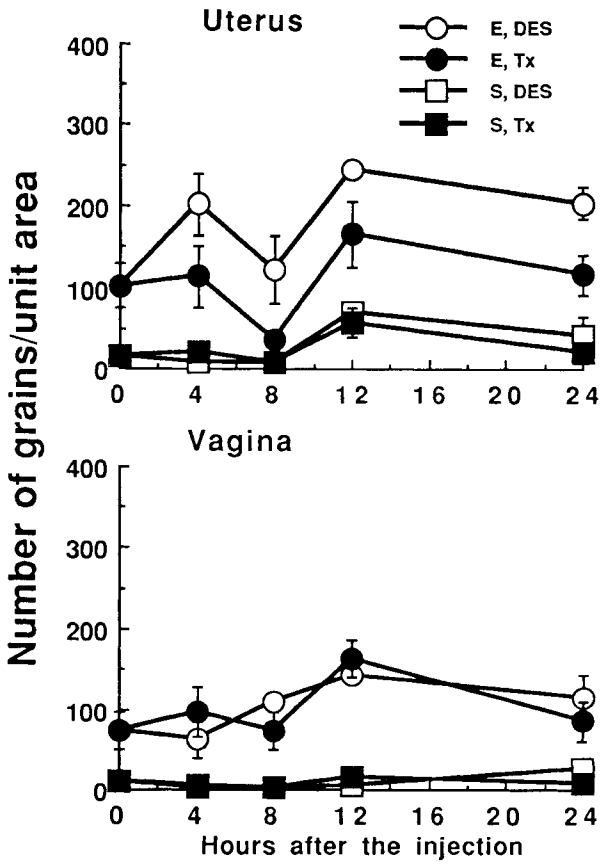

Fig. 13. Number of silver grains per an unit area (5,000 μm^2) of epithelium (E) and stroma (S) in the uterus and vagina of 50-day-old ovariectomized mice given a single injection of 3 μg DES or 100 μg Tx.

division as strong as DES in adult ovariectomized mice. Thus, Tx acts as estrogen agonist in induction of ER and cell division as well as in induction of proto-oncogenes. The responsiveness of reproductive tracts to Tx is different between newborn and adult mice. Further studies are needed to clarify the cause underlying the age differnce in responsiveness to Tx.

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