# Estrogen Receptor mRNA in Uteri of Normal Estrous Cycling and Ovariectomized Rats by *In Situ* Hybridization (44406)

SHIN-ICHI KATSUDA,* MIDORI YOSHIDA,* TAKAO WATANABE,* HIROYUKI KURODA,* JIN ANDO-LU,* MASAKAZU TAKAHASHI,* HIROYUKI HAYASHI, AND AKIHIKO MAEKAWA*,[1]

*Department of Pathology,* Sasaki Institute, Chiyoda-ku, Tokyo, 101–0062; and Department of Histology, Kanagawa Dental College, Yokosuka, Kanagawa, 238–8580, Japan*

*Abstract.* **Biological effects of estrogen are mediated *via* its binding to the estrogen receptor (ER), the contents of its protein and mRNA varying during the estrous cycle. In the present study, the ERα mRNA expression in different cell components of the uterus was investigated in normal estrous cycling rats using nonisotopic *in situ* hybridization. Additionally, ovariectomized (OVX) rats treated with 17β-estradiol (E2: 5 μg/kg, sc injection daily) were also investigated to clarify the effects of exogenous E2. At proestrus and diestrus, and especially the former, the luminal and glandular epithelial (LE and GE) cells were strongly positive, along with stromal cells beneath the luminal epithelium. At estrus, the expression was slightly diminished in LE cells, but almost completely lacking in GE cells. At metestrus, positive signals appeared again in GE cells. In the myometrium, ER mRNA was demonstrated to be constantly positive in all estrous cycle stages. OVX rat uteri underwent marked atrophy, but ER mRNA still remained in all cell types. After 2 consecutive days of E2 treatment, markedly increased intensity was observed, especially in LE and GE cells. The uteri of OVX rats treated with E2 for 14 days, however, showed slightly diminished expression, whereas the serum concentration of E2 was comparable to that in rats after 2 days. These results provide evidence that cell-type specific patterns of ER mRNA expression characterize the uteri of both normal estrous cycling rats and OVX rats after estrogen treatment.** [P.S.E.B.M. 1999, Vol 221]

The rodent uterus undergoes cyclic changes that are dependent on ovarian hormone levels (1). In female adult rats, the average length of an estrous cycle is 4–5 days, with four estrous stages, proestrus, estrus, metestrus and diestrus. The blood level of estrogen peaks at proestrus and is continuously low from estrus to diestrus (1, 2). Definite proestrous uterotrophic effects of estrogen are observed with regard to uterine weight, mitotic activity (3), and luminal epithelium height (4). An inverse correlation between cell death and cell proliferation is also observed at estrus and metestrus (5, 6). It is well known that biological effects of estrogen are mediated *via* its binding to the estrogen receptor (ER) (7, 8), distributed throughout many organs/tissues, including the ovary and reproductive tract, pituitary gland, brain, bone, skin, gut, heart vasculature, and submandibular gland (9–12). The ER protein and mRNA levels in the uterus of normal cycling rodents have been quantitated using whole uterine homogenates by binding assays and Northern blot analysis, respectively (2, 7). Nuclear binding ability of ER was most highly increased at proestrus, when formation of activated estradiol(E2)-ER complexes was highest. The ER mRNA level also tended to show the same pattern. Thus, the plasma E2 level during the estrous cycle is linked with uterine ER protein and ER mRNA expression.

The rat uterus composes endometrial luminal epithelial (LE) cells, endometrial glandular epithelial (GE) cells, stro-

[1] To whom requests for reprints should be addressed at Department of Pathology, Sasaki Institute, 2–2 Kanda-Surugadai, Chiyoda-ku, Tokyo 101–0062, Japan. E-mail: amaekawa@sasaki.or.jp

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mal cells, and myoid cells among others. It is unclear which components are responsible for changes of ER and its mRNA levels in the uterus during the estrous cycle, because whole uterine homogenates were used in the previous assays. However, an ontogeny study of the postnatal rat uterus indicated that there are temporal and cell type–specific patterns of ER mRNA expression (13). Since to our knowledge, there are no reports concerning localization of ER mRNA in the uterus of adult rats with a normal estrous cycle, the present study used *in situ* hybridization (ISH) to investigate ER mRNA. Exogenous estrogen exposure has a paradoxical biological effect on the uterus of rodents. Physiological concentrations increase ER and its mRNA, but supraphysiological levels suppress ER mRNA expression in the uterus of rodents (7). For these reasons, an examination was also conducted to clarify the effects of exogenous E2 on the localization and biosynthesis of ER mRNA in the uteri of ovariectomized (OVX) rats.

Recently, it has been reported that two types of ER, the classic ERα form, and a novel ERβ, exist with differential expression in organs/tissues (14). In the uterus, expression of ERα mRNA is high, whereas mRNA for ERβ is found at relatively low levels as compared to the ovary (15). Actually, E2 treatment did not result in characteristic estrogenic responses in the uteri of ERα knockout (ERαKO) mice generated by gene targeting (8, 16). Thus, in the present study, an oligonucleotide of an antisense sequence for ERα mRNA was employed. In the experiment, we used Donryu strain rats, in which each estrous cycle stage can be identified easily by vaginal smears with a precise 4-day cycle. It is also known that this rat is a high-incidence strain for spontaneous development of endometrial adenocarcinoma (17) and sensitive in terms of endometrial carcinogenesis by hormones/carcinogens (18).

## Materials and Methods

**Experimental Design.** Two experiments were conducted. In the first (Experiment 1), the localization of ER mRNA in the uterus of normal rats during the estrous cycle was determined. The second (Experiment 2) was designed to determine the role of exogenous E2 treatment in the localization and biosynthesis of ER mRNA in the uteri of OVX rats.

**Animals and Tissue Preparation.** Adult virgin female Crj:Donryu rats were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed in plastic cages in an air-conditioned animal room at 24° ± 2°C and 55% ± 10% humidity with a 12-hr light/dark cycle (0800–2000 hr), and maintained on basal diet, CRF-1 (Oriental Yeast Inc., Tokyo) and tap water *ad libitum*. Animal care and use followed the NIH *Guide for the Care and Use of Laboratory Animals*. Vaginal smears were used as an indicator of the stage in the estrous cycle. Five or six animals at each stage, weighing ≈ 250 g, were sacrificed at 1000 hr, and the uteri were removed, weighed, and immersed in 4% paraformaldehyde (PFA; Agar Scientific, Cambridge, UK) in phosphate-buffered saline (PBS) for 24 hr fixation at 4°C (Experiment 1). Blood samples were taken immediately and processed for radioimmunoassays of serum E2 and progesterone (P) (19).

Ovariectomy was performed by the dorsal route under light ether anesthesia. Success of the operation was confirmed by vaginal smears with a diestrus-like appearance for at least 4 days. The rats were then primed with 15 ng E2 (Wako Pure Chemical Ind. Ltd, Osaka, Japan) three times at 13, 14, and 15 days after ovariectomy (20). From 3 weeks after the operation, a dose of 5 μg/kg/day E2 dissolved in dimethylsulfoxide (Wako Pure Chemical) was administered sc daily for 2 or 14 days. Negative controls were treated with vehicle only. Five animals were used in each group. Rats were sacrificed at 24 hr after the last administration of E2, and the uteri were removed, weighed, and fixed in 4% PFA for 24 hr (Experiment 2). Blood samples were also collected.

The uteri fixed in 4% PFA were dehydrated through a graded ethanol series and xylene and embedded in paraffin wax; 3-μm sections were cut and mounted on BIOBOND (British Bio Cell, UK) coated glass slides for ISH and hematoxylin-eosin (HE) staining.

**Oligonucleotides.** The ER antisense oligonucleotide selected was complementary to positions 1405–1435 (estrogen binding domain) of the Wistar rat ER cDNA sequence as follows: 5′-ACA GGA GCT TCC CCG GGT GTT CCA TGG AGC-3′ (21). The ER sense oligo-DNA selected corresponded to the following mRNA sequence: 5′-GCT CCA TGG AAC ACC CGG GGA AGC TCC TGT-3′. This oligo-DNA was completely homologous to the sequence for Sprague-Dawley rats (22). The oligoprobes were synthesized and purified by gel filtration (Greiner Japan, Tokyo). A nonhomologous oligo-DNA sequence selected for control experiments was based on the *lac* Z′ region in pUC and M13 plasmids (Boehringer Mannheim, Germany) as follows: 5′-TTG GGT AAC GCC AGG GTT TTC CCA GTC ACG-3′. The probe sequence was chosen by computer-assisted analysis: the BLASTN sequence alignment program was used to avoid cross-hybridization with any reported sequence of the rat genome, with special attention to those coding for other steroid receptors. The oligonucleotide probes were labeled at their 3′-end with digoxigenin-11-deoxyuridine triphosphate by 3′-terminal deoxynucleotidyl transferase (Boehringer Mannheim).

**Hybridization.** The protocol used for ISH was based on the method described in detail elsewhere (23–27). Briefly, the fixed sections were dewaxed and rehydrated, treated with 0.2 *M* HCl, and digested with 50 μg/ml proteinase K (Boehringer Mannheim) each at 37°C for 30 min. Following post-fixation with 4% PFA in PBS, the sections were acetylated with freshly diluted acetic anhydride. The hybridization was run overnight in a moist chamber at 37°C with a mixture containing 1x Denhardt's solution, 2x standard saline citrate (SSC), and 0.2 mg/ml denatured herring sperm DNA by adding the labeled probe at a final concen-

tration of 1 μg/ml. After washing in graded SSC at 37°C, bound probe was localized with alkaline phosphatase-linked antidigoxigenin antibody (Boehringer Mannheim, 1:500 dilution) for 1 hr at room temperature. The final detection step was carried out overnight using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Sections were then dehydrated and mounted without counterstaining.

**Preliminary Experiment.** In confirmation of the specificity of ER mRNA signals, a preliminary control experiment was performed using serial uterine sections from normal rats in the proestrous stage. First, hybridization without labeled ER antisense probe or with labeled sense probe was used as a negative control. Some sections were also hybridized with ER antisense probe in the presence of an excess amount (10- and 50-fold) of either a homologous unlabeled oligonucleotide or a nonhomologous labeled one to provide definitive evidence of the sequence specificity of the signal. To eliminate possible involvement of proteins and DNA in signal formation, some sections were digested with 100 μg/ml RNase (37°C, 1 hr) prior to the hybridization.

**Statistical Analysis.** Data are expressed as means and standard deviations. Differences between control and treated groups in Experiment II were analyzed by Student's $t$ test with a significant level of $P < 0.05$.

## Results

**Experiment 1.** In the preliminary experiment, strong positive signals for ER mRNA were clearly detected in the perinuclear regions of LE and GE cells in the uterus of normal rats in proestrus, when the uterus was hybridized with ER antisense nucleotide. Stromal cells beneath the luminal epithelium were positive, but negative cells were also scattered in the lamina propria (Fig. 1A). The expression of myometrial muscle cells was relatively weak, and vascular elements, blood cells, and serous membrane were not stained for ER mRNA (Fig. 1B). No signals were evident in sections without labeled ER antisense probe or with labeled sense probe. When serial sections were hybridized with ER antisense nucleotide mixed with an excess of the homologous unlabeled nucleotide, the ER mRNA signals were essentially dose-dependently decreased (Fig. 1C,D), whereas this specific signal was not altered in the presence of nonhomologous labeled oligonucleotide (Fig. 1E). No signals were detected in sections digested with RNase.

Data for uterine weights and serum concentrations of E2 and P in adult female rats at each stage in the estrous cycle are shown in Figures 2 and 3, respectively. The weights were increased with luminal excretion from diestrus to proestrus, showing a peak in proestrus, but decreased in estrus and metestrus. E2 levels were highest in proestrus, corresponding with the increased uterine weights; thereafter, they dropped toward estrus and slightly increased again at diestrus. P-values were slightly increased in metestrus, although they were low from diestrus to estrus.

Histologically, the proestrous LE and GE cells were cuboidal to columnar in shape, and mitotic figures were relatively common in the luminal epithelium (Fig. 4A). In this stage, the ER mRNA signals were strongly expressed in the LE and GE cells, predominately located in the supranuclear cytoplasm. Stromal cells beneath the luminal epithelium were moderately positive (Fig. 5A). In estrus, the uterine lumen was lined with columnar epithelial cells, and many of the LE and GE cells demonstrated vacuolated degeneration (Fig. 4B,C). The glandular epithelium lacked signals although the luminal epithelium was still moderately positive. ER mRNA negative GE cells tended to have vacuolated cytoplasm and apoptotic bodies with nuclear and cytoplasmic fragmentation (Fig. 5B). At metestrus, vacuolated apoptotic cells in both luminal and glandular epithelia were decreased, and mitotic figures were frequently seen (Fig. 4D). Areas of luminal epithelium demonstrated decreased hybridization signals, but some glands again showed positive signals in the supra- or infranuclear region of cytoplasm (Fig. 5C). At diestrus, epithelial cells were cuboidal, and

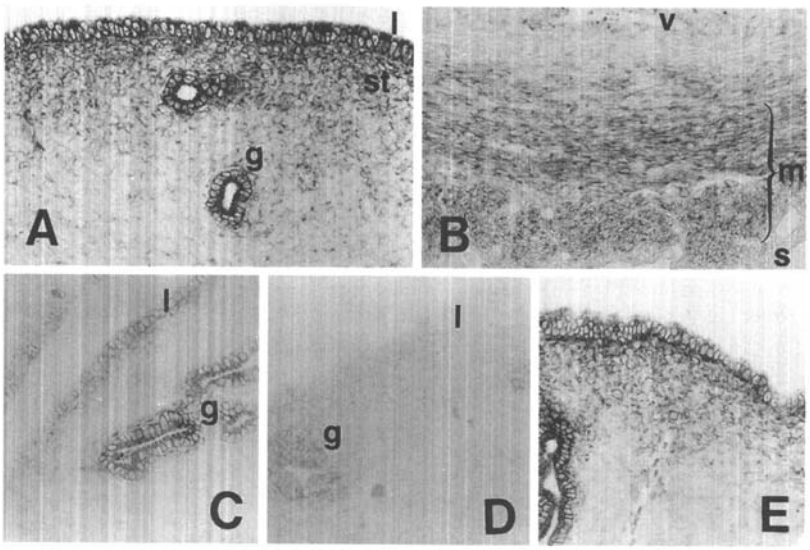


**Figure 1.** *In situ* hybridization signals in various controls. (A) A section of proestrous endometrium hybridized with ER antisense probe. Positive signals are distributed in both luminal and glandular epithelia, as well as in stromal cells beneath the luminal epithelium. (B) Outer portion of the uterus as the same section as Figure 1A: expression of myometrial muscle cells is relatively weak, and vascular element, blood cells, and serous membrane are negative. (C) A semiserial section hybridized with ER antisense probe in the presence of a 10-fold amount of homologous unlabeled oligonucleotide antisense nucleotide; positive signals are diminished. (D) A semiserial section hybridized with ER antisense probe in the presence of a 50-fold amount of unlabeled ER antisense nucleotide; positive signals are much diminished as compared to Figure 1C. (E) A semiserial section hybridized with ER antisense probe in the presence of nonhomologous-labeled oligonucleotide; positive signals are not affected. l, LE cells; g, GE cells; st, stromal cells; v, vascular element; m, myometrial layer; s, serosa. Magnification: 180×.

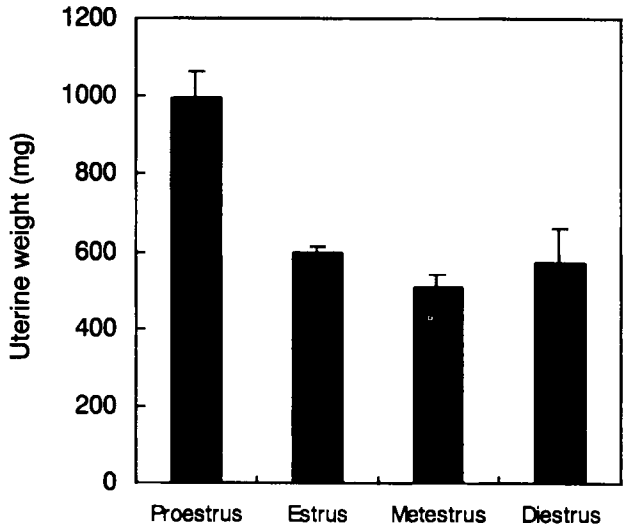


**Figure 2.** Rat uterine weights during the estrous cycle. Values are expressed as means, and standard deviations are shown.

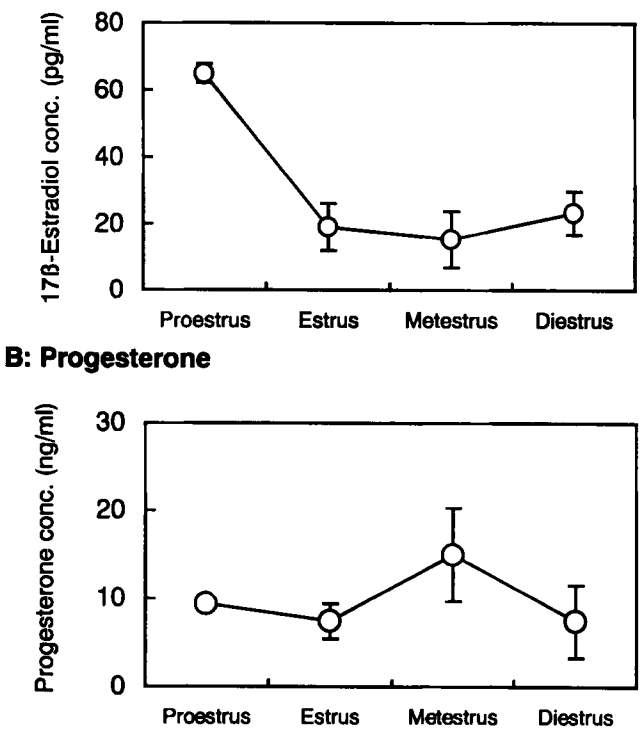


**Figure 3.** Serum concentrations of E2 and P during the estrous cycle. Values are expressed as means, and standard deviations are shown.

stromal cells beneath the luminal epithelium were round or oval in shape (Fig. 4E). These cells demonstrated increased expressions of ER mRNA in their cytoplasm (Fig. 5D).

During the estrous cycle, spindle-shaped stromal cells around the glands demonstrated consistently weak expression of ER mRNA. Hybridization signals in smooth muscle cells of the myometrium were also weak and constant during the cycle. Most other cells, including vascular endothelial cells and infiltrating polymorphonuclear leukocytes, were negative for ER mRNA at all stages of the estrous cycle.

**Experiment 2.** Marked atrophy of the uteri was observed in OVX rats injected with vehicle alone during the experimental period. Two-consecutive-day injections of E2 induced luminal dilation of the uteri filled with liquid, resulting in 4-fold increases in their weights (E2-treated, 618 ± 81 mg; control, 143 ± 18 mg), and serum E2 levels were also elevated to 4-fold (E2-treated, 24.4 ± 8.8 pg/ml; control, 5.9 ± 1.7 pg/ml), although P levels were not different from the OVX control values (E2-treated, 6.9 ± 2.0 ng/ml; control, 9.0 ± 3.6 ng/ml). The E2 level in rats given the hormone for 14 days was comparable to that in animals given for 2 days, but the uterine weight was lower (422 ± 16 mg). There was neither marked luminal dilation nor liquid storage, but the uterine wall had become thick with polymorphonuclear leukocytes infiltrating into the stroma.

Control uteri (uteri of OVX rats with vehicle treatment) showed positive signals for ER mRNA in both LE and GE cells, stromal cells, and muscle cells, as in normal cycling uteri, but their intensities were weak. The signals were located in perinuclear regions of LE cells and basal regions of GE cells. Stromal cells beneath the luminal epithelium, which were small and had chromatin-dense flat nuclei, showed definite but weak ER mRNA signals (Fig. 6A,B). The intensity in the myometrium was also weak.

In the uteri of OVX rats given two-consecutive-day injections of E2, ER mRNA expression was extremely increased in both LE and GE cells, and located in their cytoplasms. Histologically, both LE and GE cells were cuboidal to tall columnar in shape, and mitotic figures were frequent. Stromal cells beneath the luminal epithelium were round or oval in shape, and they demonstrated a moderate level of signals (Fig. 6C). The signals in the myometrium were slightly increased in intensity. In the uteri of rats after 14 consecutive injections of E2, the signal intensity of the luminal epithelium was comparable to that in the 2-day injection case. Histologically, luminal cell height was also comparable, but mitoses were much reduced. In the glandular epithelium, the intensity of the signal was heterogeneously diminished and varied from negative to very strong. Stromal cells were small and had slightly lower intensity than those in rats given E2 for 2 days (Fig. 6D).

## Discussion

Nonisotopic ISH using formalin-fixed and paraffin-embedded specimens is a useful tool for the rapid detection of mRNA without the need for any radioactive handling facilities (24, 27). Although autoradiography may be preferred in a situation where quantitation is required, the nonradioactive ISH procedure is useful for identification of distribution of nucleic acid sequences with a good sensitivity, compared to a radioactive ISH (28, 29). In a preliminary experiment, the ER mRNA signal specificity of the digoxi-

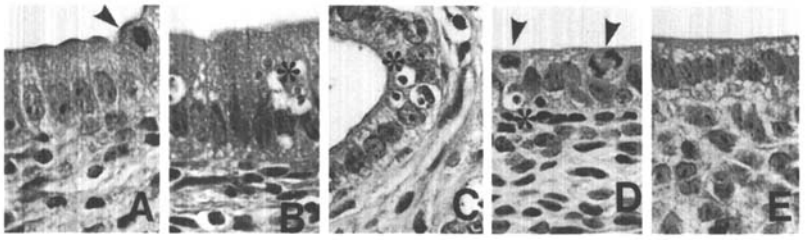


**Figure 4.** Uterine epithelium during estrous cycle stages (HE staining). (A) Columnar LE cells in proestrus. (B) Columnar and/or degenerated LE cells in estrus. (C) Degenerated GE cells in estrus. (D) LE cells in metestrus. (E) Cuboidal LE cells in diestrus. Arrowheads indicate mitotic figures, and asterisks show vacuolated cytoplasm and apoptotic bodies with nuclear and/or cytoplasmic fragmentation. Magnification: 650×.

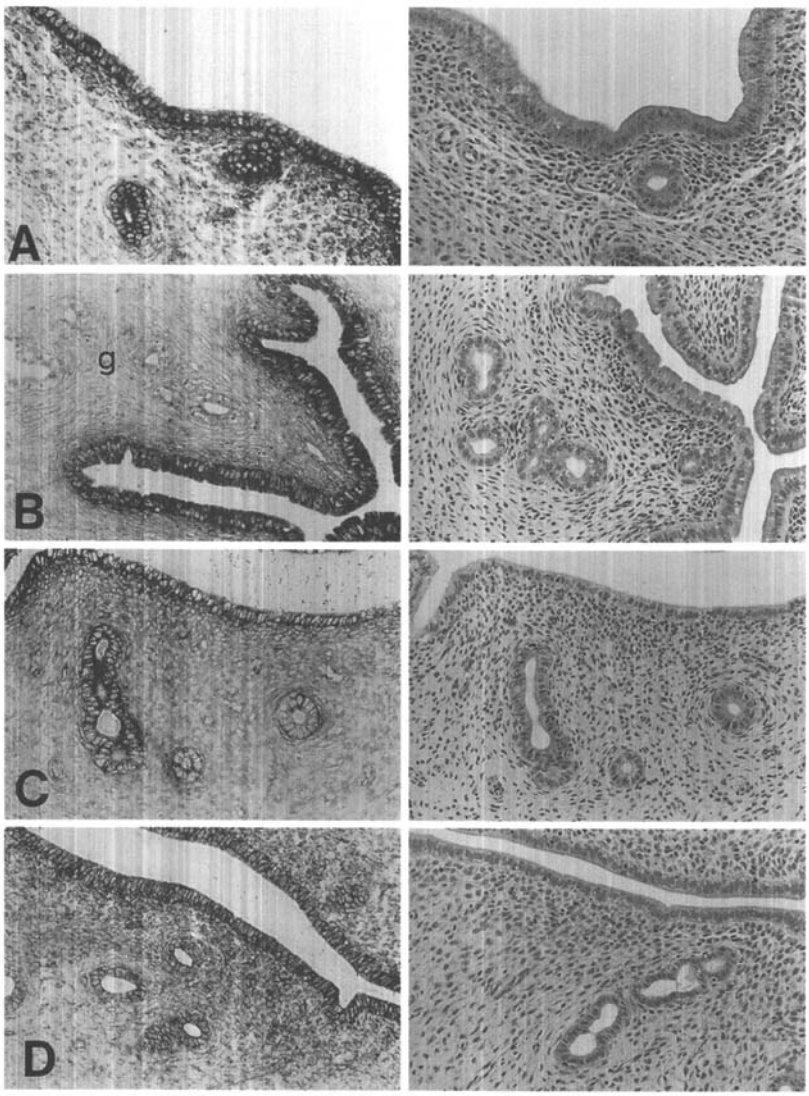


**Figure 5.** Uterine *in situ* hybridization signals (left side) and HE staining (right side) at each stage of the estrous cycle. (A) Proestrous stage. (B) Estrous stage. (C) Metestrous stage. (D) Diestrous stage. g, GE cells. Magnification: 150×.

genin-labeled oligonucleotide probe was confirmed for the uterus of normal cycling rats. The cell type–specific localization and signal intensity of uterine ER mRNA demonstrated in adult rats coincided with those in adult rhesus monkeys (23).

In adult rats, a wide spectrum of cellular changes of the uterus during the estrous cycle are controlled by sex steroid hormones, mainly E2 and P (1, 4). Serum E2 reaches a peak at proestrus and thereafter drops down, but P levels are slightly increased at metestrus and diestrus in Sprague-Dawley (2) and Füllinsdorf albino rats (1). Our results for Donryu rats demonstrated a similar profile of the E2 and P during the estrous cycle as for other strains. As the responsiveness of estrogen-dependent cells to E2 is considered to be controlled by the ER content, the kinetics of receptor expression plays an important role in cyclic changes in the uterus. Examination of whole uterine homogenates has demonstrated ER mRNA to be highest at proestrus and reduced during other estrous stages (2). In the present ISH study, intense expression of ER mRNA was seen in certain cell types of the uterus, such as epithelial and stromal cells, from diestrus to proestrus, especially the latter, correlated with an increase in serum E2. These cells showed cyclic changes in ER mRNA signals in contrast to smooth muscle

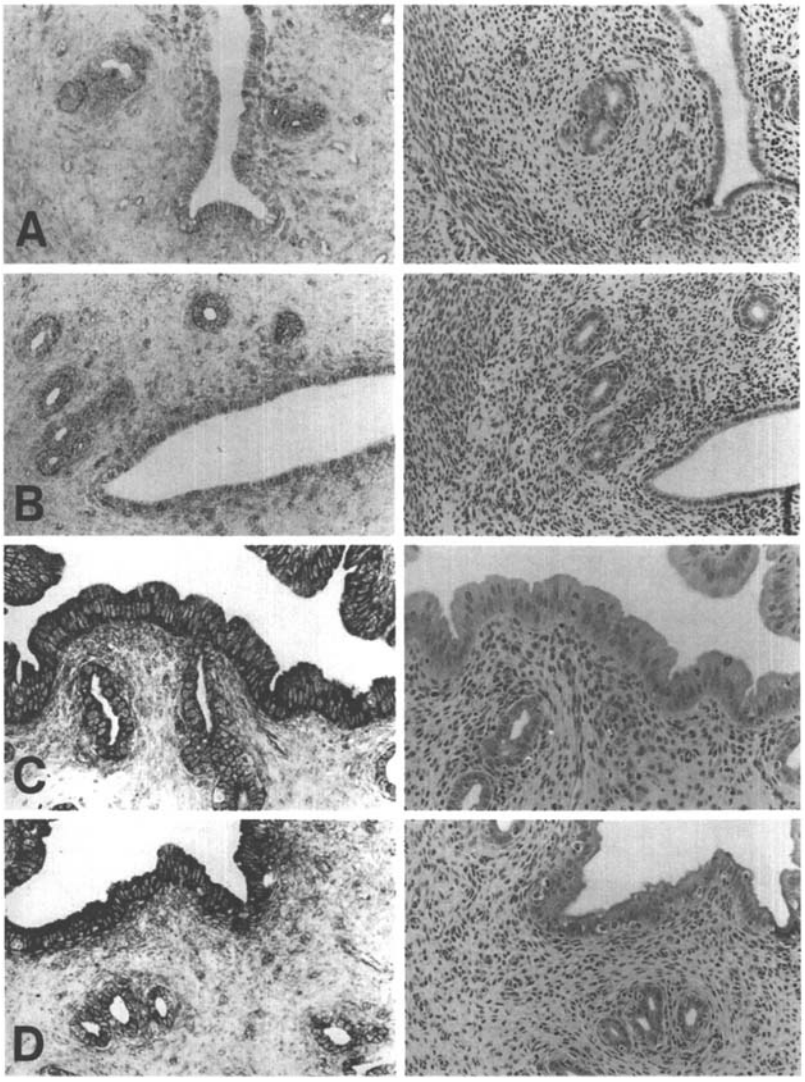


**Figure 6.** Uterine *in situ* hybridization signals (left side) and HE staining (right side) for tissue from OVX rats with or without E2 treatment. (A) 2-day control. (B) 14-day control. (C) 2-day E2 treatment. (D) 14-day E2 treatment. Magnification: 180×.

cells of the myometrium. The results indicated that E2 has upregulatory effects on expression of ER mRNA in certain cell components of the rat uterus during the normal estrous cycle, and that the cell type–specific expression pattern of ER mRNA is associated with cyclic uterine changes.

In the present study, the serum E2 level decreased at estrus, and uterine weight was also reduced. ER mRNA disappeared from the glandular epithelium, although the luminal epithelium continued to show moderate to intense expression. Apoptosis is most frequently seen in the glandular epithelium at estrus (5), and this might partly explain the relative lack of ER mRNA. Apoptotic cells characterized by DNA fragmentation and apoptotic bodies would not be expected to produce functional mRNA. Reduction of serum E2 at estrus may also contribute to the signal reduction. The question, however, still remains as to why surviving GE but not LE cells lack positive signals. It has been well known that P bears an important role in the regulation of ER during the estrous cycle and reduces the level of ER (30, 31). The preovulatory P surge in the day of proestrus has been proven to reduce ER levels (31). We did not confirm the preovulatory P surge since we collected serum samples once in the morning at each cycle stage. A previous ISH study indicated that the ER mRNA expression of the GE cells was greatly reduced in the functionalis but not in the basalis of the OVX rhesus monkey uterus after sequential E2 plus P treatment (23). At metestrus, expression of ER mRNA began to appear again in the glands although the serum P level was highest at metestrus in this study. Thus, further study is needed to investigate the role of P in regulation of ER mRNA in GE cells of rodents. On the other hand, we reported that a significant increase of bromodeoxyuridine-labeled cells occurred in the gland at this stage (6). Expression of ER mRNA may have a relationship with cell mitosis.

In the luminal epithelium, while signal expression was not lost during the estrous cycle, a relative increase was evident from diestrus to proestrus. In addition, the stromal cells beneath the luminal epithelium, which were histologically round or oval in shape, demonstrated a strong expres-

sion in these stages. Tissue recombination of uterine epithelium of ERαKO mice lacking ER and receptor positive stroma of wild type BALB/c mice confirmed that luminal epithelial mitogenesis is a stroma-mediated event (16). Ontogeny studies of rodents have revealed positive signals not in the luminal epithelium but rather limited to stromal cells distributed in the lamina propria of the uterus at birth (13, 32, 33). Our results for adult rat uterus also indicated the possibility that ER mRNA positive stromal cells may mediate E2 signals to the overlying luminal epithelium. On the other hand, spindle-shaped stromal cells around the glands had consistently low expression of ER mRNA during the estrous cycle. Thus, the epithelial-stromal relationship to the glandular epithelium may be different from that of the luminal epithelium, and the reason for this discrepancy remains unclear.

The present results for OVX rats indicated that expression of ER mRNA is fundamentally related to the E2 level. It is well known that endogenous estrogens exert a uterotropic agent during the estrous cycle, but exogenous exposure to estrogen has complex biological effects on the uterus depending on the dose and the duration of treatment (7, 34). Single dosing with low concentrations of exogenous E2, similar to physiological levels, increases ER mRNA (7), whereas continuous treatment with low-dose E2 may exert an overall downregulatory effect on ER mRNA (34). Furthermore, no increases in ER mRNA expression have been found after exposure to high concentrations of E2 (7, 34). In the present study, the rises of serum E2 in OVX rats given the hormone for 2 or 14 days were in the physiological range. The fact that signals were still pronounced in the luminal epithelium and some GE cells after the 14-consecutive-day treatment of E2 demonstrates that continuous exposure does not always have a downregulatory effect on all cell components of the uterus.

Estrogens are considered to be of essential importance for uterine carcinogenesis in humans and experimental animals. In Donryu rats, an age-related rise of the E2/P ratio and a constant high level of proliferative activity of epithelial cells appeared to underlie the spontaneous development of endometrial adenocarcinomas (6, 17, 19). The present data indicated that profiles of ER mRNA expression differ between the luminal and glandular epithelia and their associated stroma. Endometrial adenocarcinomas are primarily derived from the glandular epithelium in Donryu rats (17). Since it is well known that alteration of the epithelial–stromal interaction has a relationship to epithelial carcinogenesis (35–37), further studies of the different cell populations in the uterus are needed. The ISH technique for detecting ER mRNA should contribute to clarification of the histo- and pathogenesis of uterine carcinogenesis.

In conclusion, the present study confirmed the utility of ISH using a nonisotopic oligonucleotide probe for detection of ER mRNA at the cellular level in the uterus of rats. Cell-specific expression of ER mRNA was thereby demonstrated to be fundamentally related to E2 levels.

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