

# Localization and estrogenic regulation of androgen receptor mRNA expression in the mouse uterus and vagina

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## Abstract

Androgen receptors (AR) are highly expressed in female reproductive organs. In order to define the possible involvement of estrogens in the regulation of AR expression in the uterus and vagina, we have studied the effect of short-term administration of 17 $\beta$ -estradiol (E<sub>2</sub>) to ovariectomized adult mice on AR mRNA levels. Seven days after ovariectomy, the mice received a single injection of E<sub>2</sub> (0.05  $\mu$ g/mouse) 3, 12 or 24 h before they were killed. The levels of AR mRNA were measured in the different uterine and vaginal compartments using quantitative *in situ* hybridization. In the uterus, AR mRNA was expressed in the luminal and glandular epithelial cells, stromal cells and smooth muscle cells. In the vagina, AR mRNA was localized in both epithelial and stromal cells. In the uterus after ovariectomy, AR mRNA levels were decreased by 18% in the epithelial cells, 23% in the stromal cells and 50% in the myometrial cells. AR mRNA levels were completely restored as early as 3 h after E<sub>2</sub> administration

in the epithelium and stroma, and at the 12-h time-interval in the myometrium. In the vaginal epithelium, ovariectomy induced a 70% decrease in AR mRNA expression. No effect could be detected 3 h after E<sub>2</sub> administration, while at the longest time-intervals (12 and 24 h) there was an increase in mRNA levels corresponding to 70% of the levels observed in intact animals. In the vaginal stroma, ovariectomy was responsible for a 55% decrease in mRNA levels. While no significant changes were observed at the 3-h time-interval, a complete restoration of AR mRNA levels in stromal cells could be recorded at the longest time-intervals after E<sub>2</sub> administration. The data obtained indicated that, in adult mice, estrogens exert a positive regulation of AR mRNA expression in the different compartments of both the uterus and the vagina.

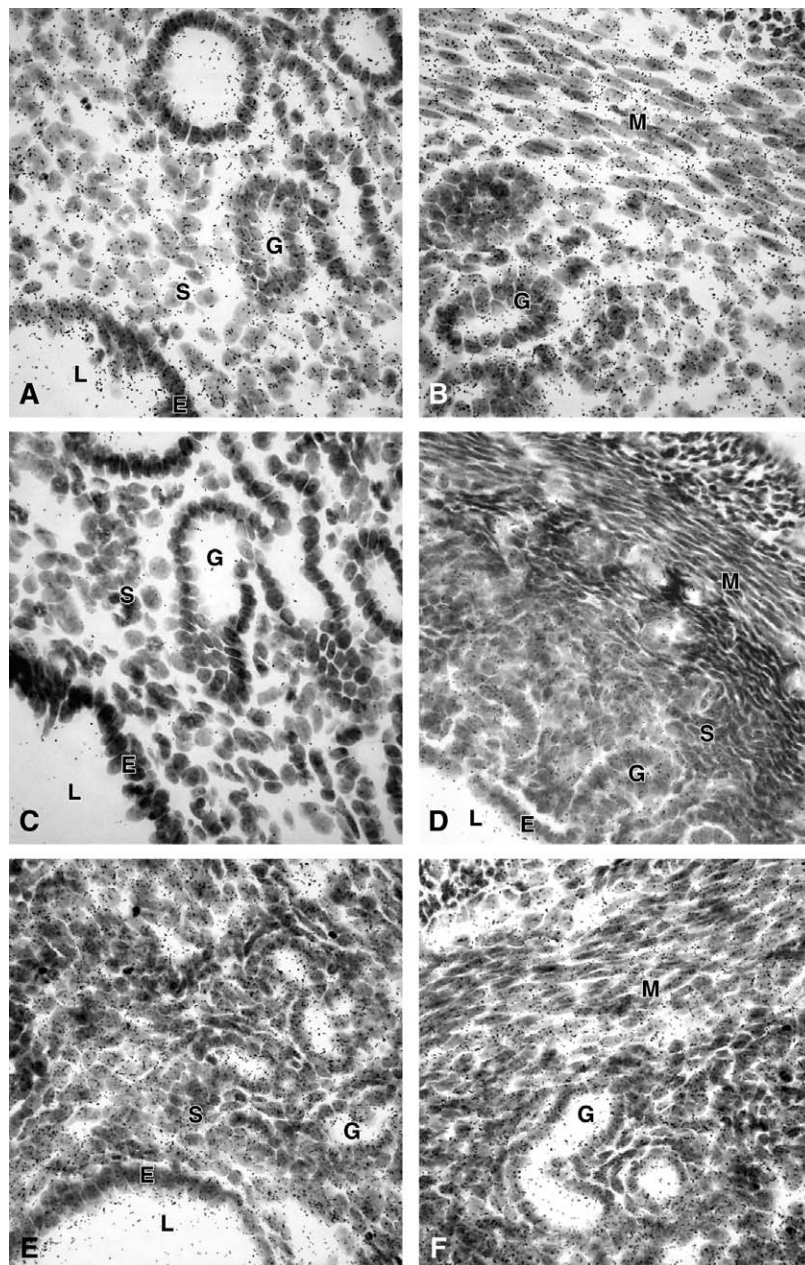
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## Introduction

Androgens play a predominant role in the development and physiological function of male accessory sex organs as well as the functioning of several other organs and tissues in both sexes (Carson-Jurica *et al.* 1990, Clark *et al.* 1992). The action of androgens is mediated by androgen receptors (AR) which belong to the superfamily of ligand-responsive transcription regulators (Evans 1988, Carson-Jurica *et al.* 1990). By immunocytochemistry and *in situ* hybridization, AR has been found to be expressed in a large variety of tissues, including male and female reproductive organs (Ruizeveld de Winter *et al.* 1991, Kimura *et al.* 1993, Tetsuka *et al.* 1995, Adesanya-Famuyiwa *et al.* 1999, Pelletier 2000, Pelletier *et al.* 2000). In the rat uterus, AR has been localized by both immunocytochemistry and *in situ* hybridization in the epithelial, stromal and smooth muscle cells (Hirai *et al.* 1994, Pelletier 2000). Similar results have been reported in the human and monkey uterus (Kimura *et al.* 1993, Adesanya-

Famuyiwa *et al.* 1999, Pelletier 2000). In the human vagina, AR immunoreactivity was found in nuclei of both epithelial and stromal cells (Hodgins *et al.* 1998, Pelletier 2000). In the mouse, the localization of AR in female reproductive organs has not so far been reported.

Very little is known about the physiological role of androgens and the regulation of AR in the uterus and vagina. In immature hypophysectomized rats, Armstrong & Papkoff (1976) have shown that administration of testosterone or dihydrotestosterone induced an increase in uterine weight. In the ovariectomized monkey, a 3-day estradiol (E<sub>2</sub>) treatment was shown to increase AR mRNA levels in uterine stromal cells (Adesanya-Famuyiwa *et al.* 1999). It has also been reported that estrogens can induce AR expression in the chick oviduct magnum mucosa (Tokarz *et al.* 1979). In order to further define the role of estrogens in the regulation of AR expression, we have studied the effects of short-term E<sub>2</sub> administration to ovariectomized adult mice on uterine and vaginal AR mRNA levels using *in situ* hybridization.



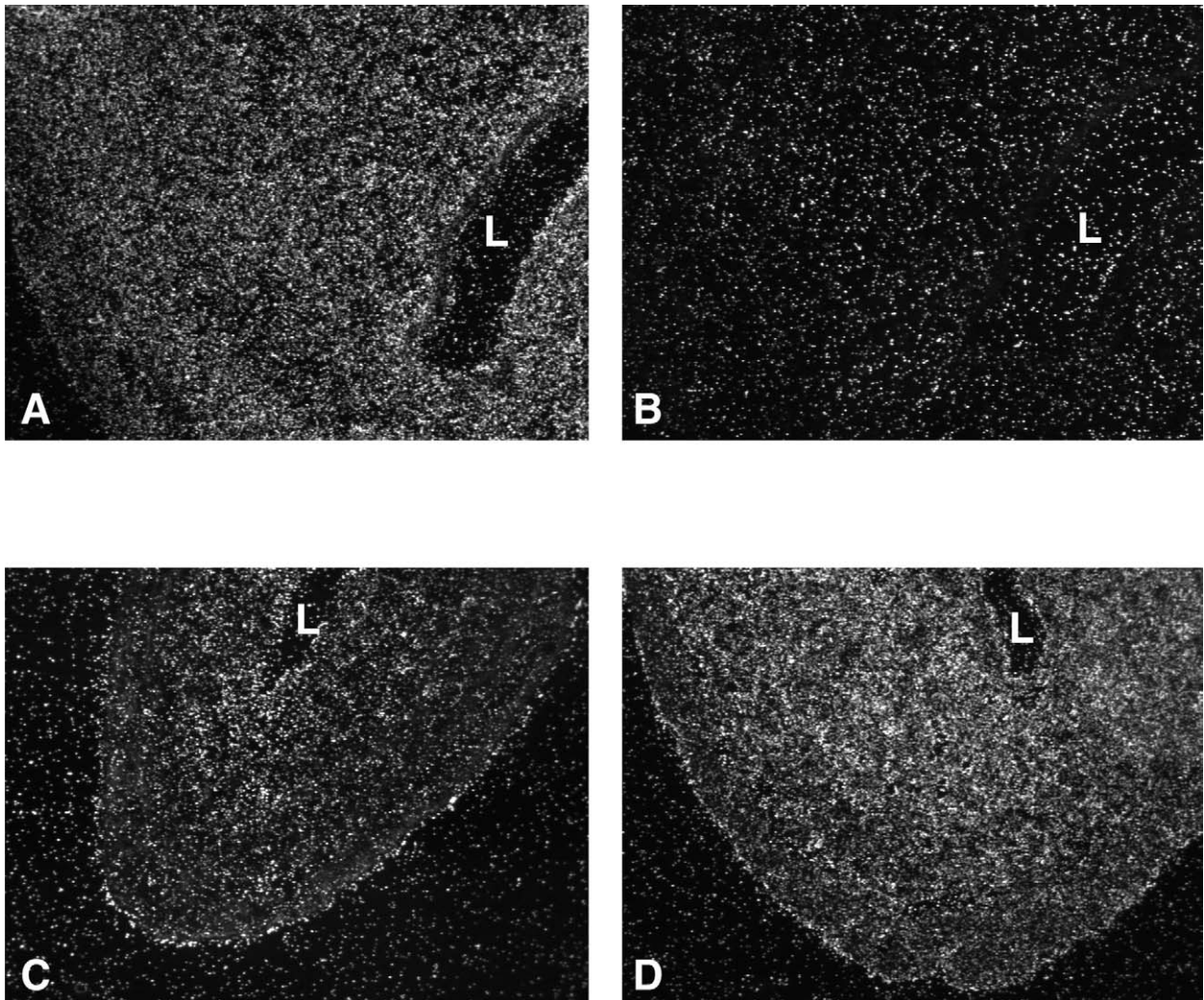
**Figure 1** Representative brightfield micrographs illustrating the localization of AR mRNA in the mouse uterus after hybridization with the antisense riboprobe. (A and B) Intact mouse. (C) Section consecutive to that shown in (A) hybridized with the sense probe (negative control). (D) Ovariectomized mice. (E and F) Ovariectomized mice which received a single injection of  $E_2$  24 h before they were killed. L: lumen; E: epithelial luminal cells; G: glands; S: stroma; M: myometrium ( $\times 450$ ).

## Materials and Methods

### *Animals and treatment*

Eleven- to twelve-week-old female C57BL6 mice were received from Charles River (St Constant, Quebec,

Canada) and were allowed to acclimate for 3 weeks. The animals were housed individually under constant temperature ( $22 \pm 3$  °C) and light (lights on from 0600 to 2000 h) regimens. The mice had free access to tap water and a certified rodent feed (Lab Diet 5002 (pellet);



**Figure 2** Representative darkfield micrographs illustrating AR mRNA hybridization signal in the mouse uterus. (A) Intact mice. (B) Section consecutive to that shown in (A) hybridized with the sense riboprobe (negative control). (C) Ovariectomized mice. (D) Ovariectomized mice which received a single injection of  $E_2$  24 h before they were killed. L: lumen ( $\times 11$ ).

Ralston Purina, St Louis, MO, USA). The experiment was conducted in an animal facility approved by the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care. The study was performed in accordance with the CCAC Guide for Care and Use of Experimental Animals.

Animals, weighing between 23 and 26 g were randomized according to their body weights and were assigned to five groups of five animals each as follows: (1) intact control; (2) ovariectomized control; (3–5) ovariectomized +  $E_2$  (0.05  $\mu\text{g}/\text{mouse}$ ). On day 1 of the study, animals of groups 2 to 5 were bilaterally ovariectomized under isoflurane anesthesia. Mice of group 1 were sham-operated (intact). Before the animals were killed on day 8 of the

study, they received a single subcutaneous injection (0.2 ml/mouse) of the vehicle alone (5% ethanol–0.4% methylcellulose; groups 1 and 2) or  $E_2$  (groups 3–5). The injection was performed 3 h (group 3), 12 h (group 4) or 24 h (groups 1, 2 and 5) before the animals were killed.

#### Tissue collection

On day 8 of the study, mice were anesthetized with ketamine/xylazine and perfused transcardially with 90 ml 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Prior to the perfusion, a vaginal smear was collected from intact female mice of group 1 in order to determine the estrous cycle using Papanicolaou staining. Mice in estrus were selected. The uterus and vagina were

excised and post-fixed in the same fixative for 24 h at 4 °C. The tissues were placed in 15% sucrose in 0.1 M phosphate buffer before being quickly frozen in isopentane chilled in liquid nitrogen.

#### *In situ hybridization*

Frozen sections (10 µm thick) were serially cut at -20 °C and mounted onto gelatin- and poly-L-lysine-coated slides. The vector used for production of the cRNA probe was constructed by insertion into a pCR-Blunt II-TOPO (Invitrogen, Ontario, Canada) of a cDNA fragment of 249 pb mouse AR (Genebank no. NM-013476). The cDNA fragment located at position 26–275 downstream from the ATG start codon was obtained by amplification using polymerase chain reaction. *In situ* hybridization with the antisense and sense <sup>35</sup>S-labeled cRNA probes was performed as previously described (Givalois *et al.* 1997). Briefly, the sections were prehybridized at room temperature in a humid chamber for 2 h in 450 µl/slide of a prehybridization buffer containing 50% formamide, 5 × SSPE (1 × SSPE being 0.1 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4, mM EDTA), 5 × Denhart's buffer, 200 mg/ml denatured salmon testis DNA (Sigma, St Louis, MO, USA), 200 µg/ml yeast tRNA, 2 µg/ml Poly A (Boehringer-Mannheim, Montreal, Canada) and 4% dextran sulphate. After the prehybridization procedure, a 100 µl hybridization mixture (prehybridization buffer containing 10 nM dithiothreitol and the <sup>35</sup>S-labeled cRNA probe at a concentration of 10 × 10<sup>6</sup> c.p.m./ml) was spotted on each slide, sealed under a coverslip and incubated at 37 °C overnight (15–20 h) in a humid chamber.

After hybridization, coverslips were removed and slides were rinsed in 2 × SSC at room temperature for 30 min. Sections were digested by RNase A (20 µg/ml in 2 × SSC) at 37 °C for 30 min at room temperature, washed in 0.5 × SSC for 30 min at 37 °C, followed by 90 min at room temperature in 0.5 × SSC, at 60 °C in 0.1 × SSC and finally for 30 min at room temperature in 0.1 × SSC.

The sections were then dehydrated and coated with liquid photographic emulsion (Kodak-NTB2; diluted 1:1 with water; Kodak, Rochester, NY, USA). Slides were exposed for 21 days, developed in Dektol developer (Kodak) for 2 min and fixed in rapid fixer (Kodak) for 4 min. Thereafter, tissues were rinsed in running water for 30 min, counterstained with hematoxylin and rapidly dehydrated through graded concentrations of ethanol, cleared in toluene and coverslipped with Permount (Fisher Scientific, Montreal, Canada).

Quantitative analysis of hybridization signals was carried out using a Zeiss optical system coupled to a Macintosh computer image software (version 1.6 non FPU, W Rasband, NIH, USA). The optical density (OD) of the signal was measured under darkfield illumination at a

magnification of × 25. Areas covering the different uterine and vaginal compartments were digitized and submitted to densitometric analysis, yielding measurements of integrated OD (area × average OD) as previously described (Givalois *et al.* 1998). The OD of each compartment was then corrected for the background signal which was determined on a consecutive section which had been hybridized with the labeled sense probe.

## Results

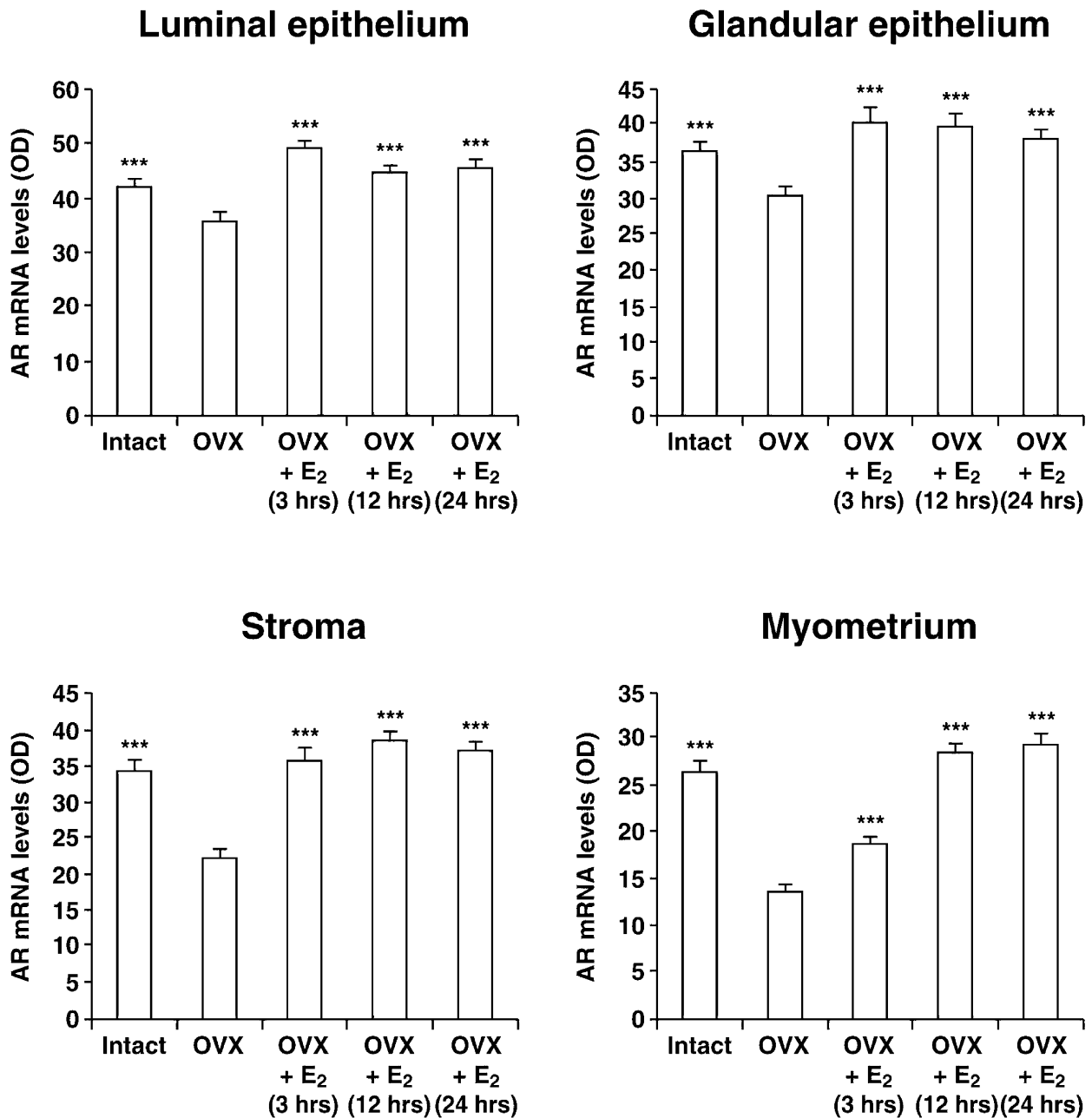
### *Uterus*

In the uterus of sham-ovariectomized (intact) mice, the hybridization signal was detected on epithelial cells (both luminal and glandular), stromal cells as well as myometrial smooth muscle cells (Figs 1A and B and 2A). As observed by brightfield (Fig. 1A and B) and darkfield (Fig. 2A) light microscopy, the labeling was uniformly distributed throughout the different uterine compartments. No significant autoradiographic reaction was observed after hybridization with the labeled sense probe (Figs 1C and 2B). Seven days after ovariectomy, a marked atrophy of epithelial, stromal and smooth muscle cells (Figs 1D and 2C) was noted. At the longest time-interval studied (24 h), the different compartments appeared in the process of reorganization but with cells of smaller size than those observed in intact animals (Fig. 1E and F). Quantitative analysis of AR mRNA levels revealed that ovariectomy induced a decrease of approximately 18% in OD ( $P < 0.01$ ) in both luminal epithelial and glandular epithelial cells (Fig. 3). E<sub>2</sub> administration to 7-day ovariectomized mice induced a significant increase in AR mRNA levels as early as 3 h after injection. Similar AR mRNA levels were measured at the two other time-intervals (12 and 24 h).

In the stroma, a 23% decrease in the hybridization signal was observed 7 days after ovariectomy. AR mRNA levels were restored to the levels observed in intact mice at the earliest time-interval studied (3 h) and remained constant thereafter (Figs 1E, 2D and 3). In the myometrium, after ovariectomy, AR mRNA levels were depressed by 50% (Figs 1D, 2C and 3). Three hours after E<sub>2</sub> administration, there was a 37% increase in AR mRNA levels as compared with the levels observed in ovariectomized mice. Complete restoration of AR mRNA levels in smooth muscle cells occurred at both the 12- and 24-h time-intervals (Figs 1F, 2D and 3).

### *Vagina*

In the vagina of intact mice, the hybridization signal was detected in both epithelial and stromal cells, with a higher density of labeling over the epithelium (Figs 4A and 5A). In vaginal sections hybridized with the sense probe, only a

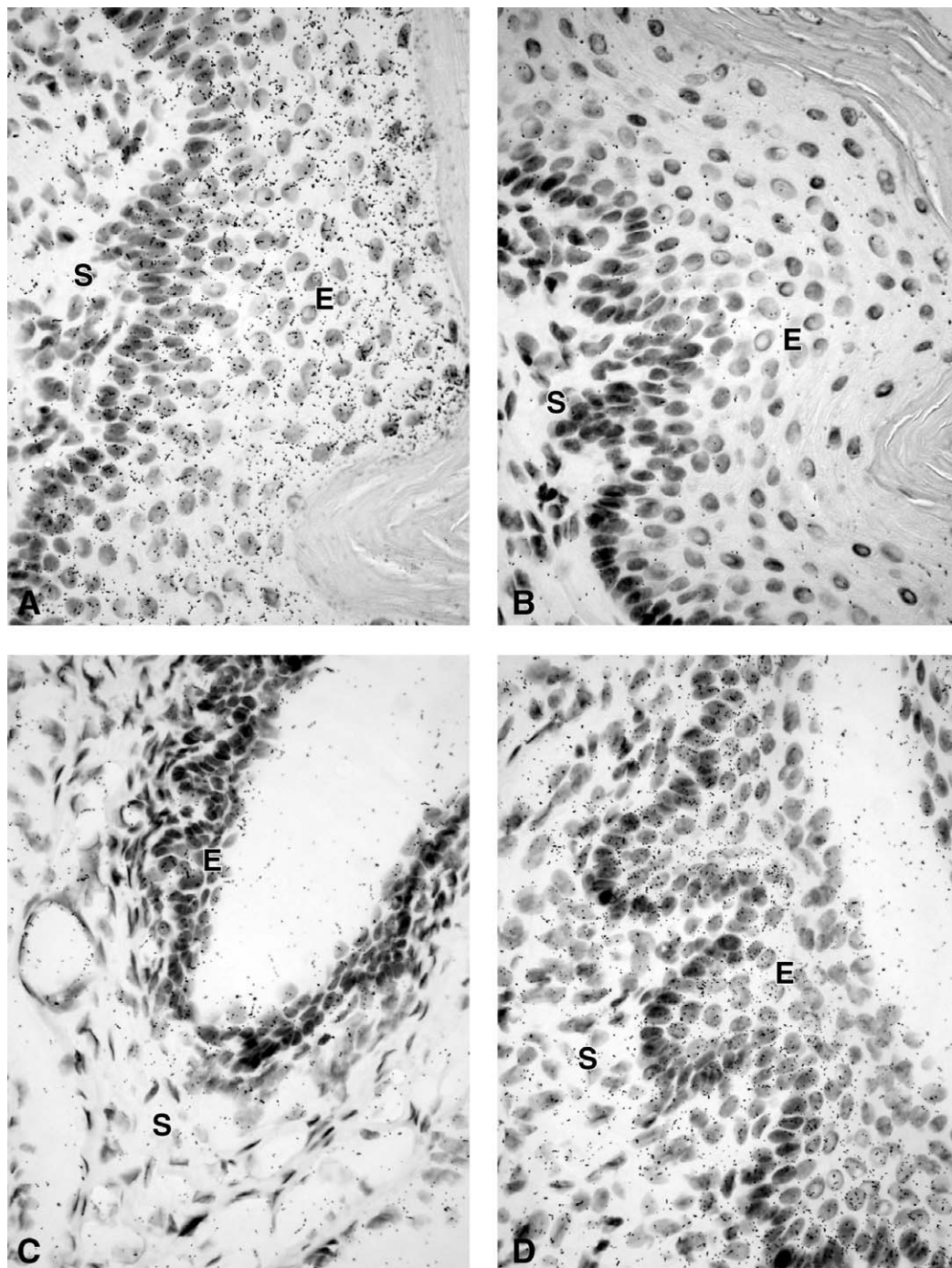


**Figure 3** Mean  $\pm$  S.E.M. effects of ovariectomy and a single injection of E<sub>2</sub> to 7-day ovariectomized mice (OVX) on AR mRNA levels (OD) in the different uterine compartments. \*\*\* $P < 0.001$  vs OVX mice. The group of OVX mice which received E<sub>2</sub> 3 h before they were killed is already significantly different from the group of intact mice ( $P < 0.01$ ).

weak and uniform labeling could be observed (Figs 4B and 5B). Seven days after ovariectomy, both the epithelium and stroma were markedly atrophied (Figs 4C and 5C). In the epithelium only atrophied cells probably belonging to the basal layer could be seen. Twelve and twenty-four hours after E<sub>2</sub> administration, partial restoration of both epithelial and stromal compartments was observed, the

epithelium height at the 24-h time-interval being clearly thicker than observed in ovariectomized animals.

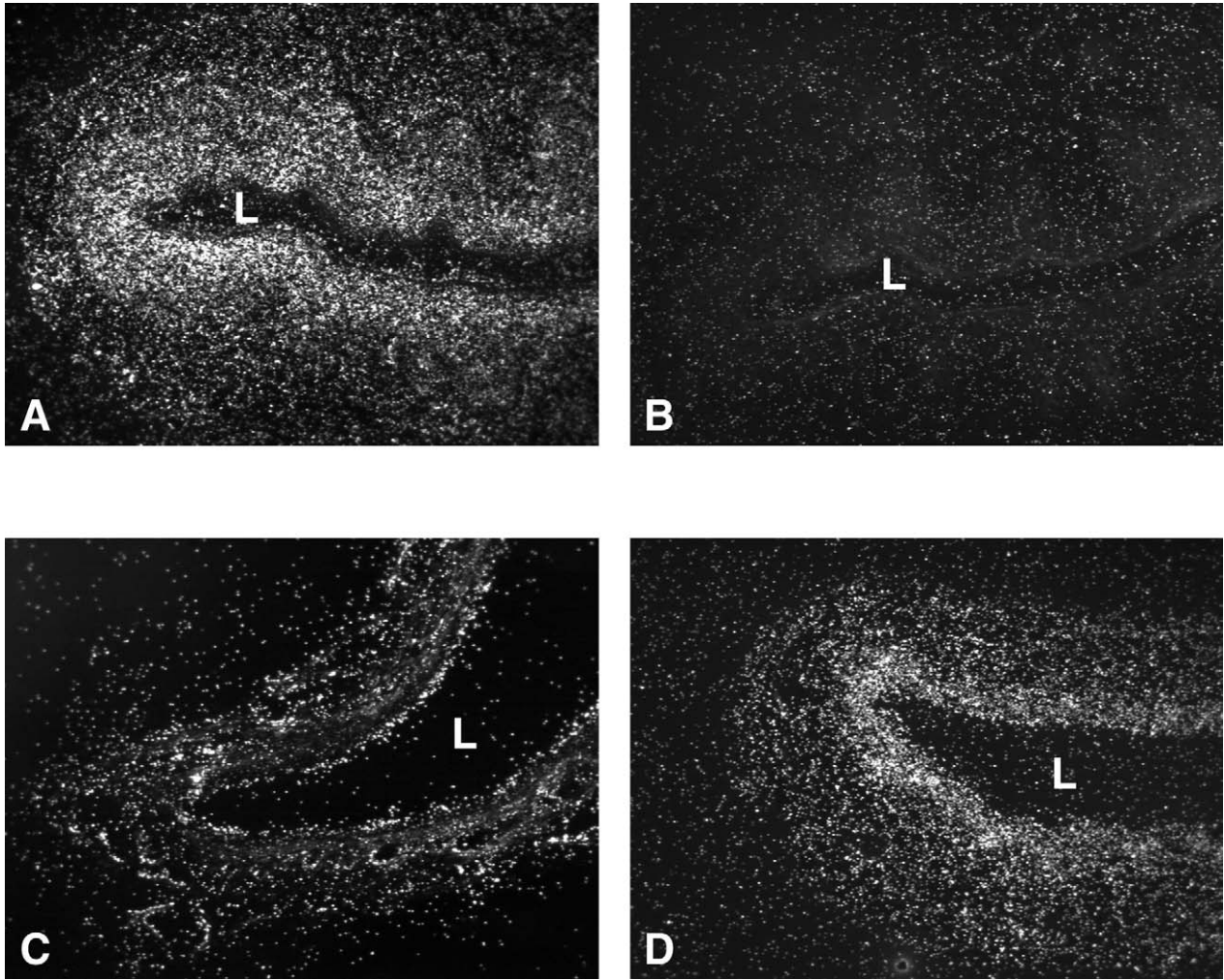
In epithelial cells, ovariectomy induced a 30% decrease in AR mRNA levels compared with levels measured in intact animals (Figs 4C, 5C and 6). No significant changes were observed 3 h after E<sub>2</sub> administration. At longer time-intervals (12 and 24 h), E<sub>2</sub> induced an increase of



**Figure 4** Representative brightfield micrographs illustrating the AR hybridization signal obtained in the mouse vagina. (A) Intact mice. (B) Section consecutive to that shown in (A) hybridized with the sense riboprobe (negative control). (C) Ovariectomized mice. (D) Ovariectomized mice which received a single injection of  $E_2$  24 h before they were killed. E: epithelium; S: stroma ( $\times 730$ ).

approximately 125% in mRNA levels compared with ovariectomized mice (Figs 4D, 5D and 6). This increase corresponded to about 70% of the values observed in intact

animals. In the stroma, ovariectomy induced a 55% decrease in AR mRNA levels (Figs 4C, 5C and 6). While no changes in AR mRNA expression were recorded at



**Figure 5** Representative darkfield micrographs showing AR mRNA expression in the mouse vagina. (A) Intact mice. (B) Section consecutive to that shown in (A) hybridized with the sense riboprobe (negative control). (C) Ovariectomized mice. (D) Ovariectomized mice which received a single injection of  $E_2$  24 h before they were killed. L: lumen ( $\times 13$ ).

the 3-h time-interval, AR mRNA levels were completely restored at the longest time-intervals after  $E_2$  administration (12 and 24 h) (Figs 4D, 5D and 6).

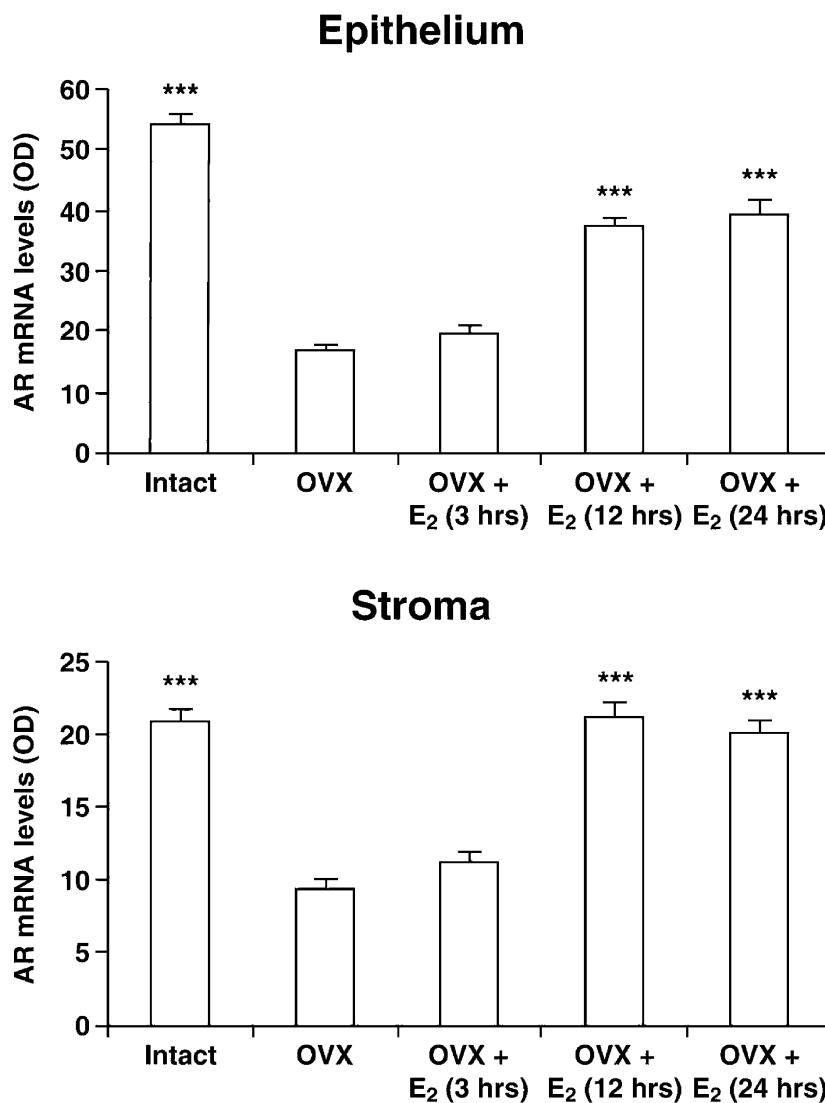
## Discussion

The presence of AR mRNA in epithelial, stromal and smooth muscle cells in the mouse uterus confirms previous results obtained by immunocytochemistry in human, monkey and rat uterus (Kimura *et al.* 1993, Hirai *et al.* 1994, Adesanya-Famuyiwa *et al.* 1999, Pelletier 2000, Pelletier *et al.* 2000). It is noteworthy that, in the mouse uterus, the intensity of AR mRNA labeling is quite uniform throughout the different uterine compartments. In the monkey uterus, the expression of AR mRNA was

lower in epithelial cells than in stroma or muscle cells (Adesanya-Famuyiwa *et al.* 1999).

The present data clearly demonstrated that, in all the compartments, AR mRNA levels were decreased by ovariectomy, the effect being more striking in the stroma and myometrium. AR mRNA levels were completely restored within 3 h in the epithelial and stromal cells and 12 h in the smooth muscle cells. These data indicated that estrogen can positively modulate AR mRNA levels in all the uterine compartments. It has been shown that administration of  $E_2$  to ovariectomized monkeys for 3 days induced an increase in AR mRNA in endometrial stromal cells but not in the other uterine compartments (Adesanya-Famuyiwa *et al.* 1999).

In the uterus, estrogen receptor (ER)- $\alpha$  is the predominant ER (Kuiper *et al.* 1997, Couse *et al.* 2000) and has



**Figure 6** Mean  $\pm$  S.E.M. effects of ovariectomy and a single injection of E<sub>2</sub> to ovariectomized mice (OVX) on AR mRNA levels (OD) in vaginal epithelium and stroma. \*\*\* $p < 0.001$  vs OVX mice.

been localized in the epithelial, stromal and smooth muscle cells (Couse *et al.* 2000, Pelletier 2000, Pelletier *et al.* 2000). In adult ER $\alpha$  knockout (KO) mice, a severe uterine atrophy is observed, while the uteri of ER $\beta$ KO mice have a normal appearance and undergo the cyclic changes associated with ovarian hormones (Couse *et al.* 2000). It is, then, likely that the observed effects of E<sub>2</sub> on AR mRNA are mediated by ER $\alpha$ .

The role of androgens in the regulation of uterine function is still unclear. It has been previously shown that estrogen can induce AR in the chick oviduct magnum mucosa (Tokarz *et al.* 1979). In immature hypophysectomized rats, the administration of testosterone or the

non-aromatizable androgen dihydrotestosterone for 3 days can induce a marked increase in uterine weights (Armstrong & Papkoff 1976). Very recently, Weihua *et al.* (2002) have shown that, in the immature rat uterus, 24 h after administration of E<sub>2</sub>, there was an increase in the levels of AR and proliferation of luminal epithelial cells. Since the E<sub>2</sub>-induced proliferation of epithelial cells in the immature uterus was blocked by the pure antiandrogen flutamide, an involvement of androgens in the effect of estradiol on the uterus has been proposed (Weihua *et al.* 2002).

In the mouse vagina, we have observed that AR mRNA was expressed in both the epithelial and stromal



cells, with a highest level of expression in the epithelial compartment. In the human vagina, AR was localized by immunocytochemistry in both epithelial and stromal cells (Hodgins *et al.* 1998, Pelletier 2000). In both mouse vaginal compartments, ovariectomy induced a marked depression in AR mRNA, while E<sub>2</sub> induced a significant increase in AR mRNA at both 12- and 24-h time-intervals. These results clearly indicate a robust positive influence of circulating estrogens on the expression of AR mRNA. ER $\alpha$  is expressed in both epithelial and stromal cells in the mouse vagina (authors' unpublished observations). Moreover, in ER $\alpha$ KO mice, but not in ER $\beta$ KO mice, there is a marked vaginal hypoplasia and the vaginal tissue is insensitive to estradiol (Couse *et al.* 2000). These observations strongly suggest that, in the vagina, ER $\alpha$  is involved in the stimulatory influence of E<sub>2</sub> on AR mRNA expression. The exact role of androgens in the development and function of the vagina remains to be fully explored.

In summary, the present data clearly showed that estrogens stimulate AR mRNA expression in both the uterus and vagina in adult mice. The effect is rapid, being observed within 3 h for the uterus and 12 h for the vagina after a single injection of E<sub>2</sub> to 7-day ovariectomized mice, and is probably mediated through ER $\alpha$ . In the uterus, AR could well be involved in the action of E<sub>2</sub> in the different uterine cell types. The effect of ER $\alpha$  on the expression of the AR gene might be mediated by the AP-1 site since there are several AP-1 sites in the promoter region of the AR gene (Roy & Chatterjee 1995).

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