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Androgen Receptor mRNA in the Rat Ovary and Uterus

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The distribution of androgen receptor messenger RNA (ARmRNA) in the reproductive tissues of adult rats was examined by Northern blot analysis and *in situ* hybridization using ARcRNA probes corresponding to the androgen binding domain of the receptor. About 10-kilobase rat ARmRNA was observed in all tissues examined in the Northern blot analysis. The amount of ARmRNA in the ovary, uterus and testis was less than that in the prostate. In the *in situ* hybridization study, extensive labeling was observed in the theca cells of the ovary (proestrous) and the endometrium and endometrial glands of the uterus (proestrous). Moderate labeling was observed in the granulosa cells and stromal cells of the ovary and in the myometrium of the uterus. These results were largely in agreement with the distribution of AR previously reported by ligand binding studies. This present *in situ* hybridization study may provide a useful tool for the analysis of the regional regulation of AR synthesis in the rat female reproductive tissues.

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INTRODUCTION

Androgens exert profound influences on sexual behavior and physiological responses. It is well known that androgen actions on the target tissues are mediated by the androgen receptor (AR), which is a ligand-activated regulator of androgen-responsive genes. AR has been demonstrated in the reproductive organs [1], brain [2], and other organs [3] by biochemical assay.

Histological studies were necessary to determine the cellular concentration of AR. Autoradiographic methods and immunohistochemistry have been used to localize AR [4, 5]. These methods are useful in detecting AR at a cellular level. In fact, a previous report demonstrated AR in the rat ovary and uterus using immunohistochemistry [4].

Recently, a complementary DNA (cDNA) clone encoding the rat AR has been isolated [6, 6a] which has made it possible to detect AR messenger RNA (ARmRNA). The tissue distribution of ARmRNA has been demonstrated in both male reproductive and non-reproductive tissues, such as the prostate, testis, epididymis, brain and kidney by Northern blot analysis [7–9]. The tissue concentrations of ARmRNA appear to match the amounts of AR in organs, such as the rat testis [10–14], prostate [15–17], brain [18] and human genital skin fibroblast [19]. Although AR is detectable by the ligand-binding assay and immunohistochemistry in the rat ovary and uterus [4, 20–22], little information is available about ARmRNA in the female reproductive tissues. Androgens may play an essential role in female reproductive functions because AR exists in the ovary and uterus. In the present study, we have thus attempted to detect and quantify the transcript levels of ARmRNA in the rat female reproductive tissues by Northern blot analysis and *in situ* hybridization.

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EXPERIMENTAL

Animals

Adult Wistar strain rats (body wt 200–300 g) were purchased from Japan SLC Ltd. (Shizuoka, Japan). They were kept under a regular day and night cycle (12 h light, 12 h dark) and were given food pellets and water *ad libitum*. In the *in situ* hybridization study, proestrous female rats were used. The stages of the estrous cycle were determined by vaginal smears.

RNA extraction and Northern blot analysis

The ovary, uterus, testis and ventral prostate were removed from rats immediately after they had been killed by decapitation. These tissues were dissected and then frozen in liquid nitrogen and stored at −80°C until RNA extraction. Total RNA was extracted from each tissue by the guanidium–cesium chloride ultracentrifugation method [23, 24]. Poly(A)$^+$ RNA from the testis was isolated by oligo(dT) cellulose chromatography [25]. The RNA concentration was determined by UV absorption. The RNA was denatured in formaldehyde at 60°C for 15 min and size-fractionated by electrophoresis in 1.0% agarose gel containing 6% formaldehyde. After the electrophoresis, the RNA was transferred to a nylon membrane (Hybond-N+, Amersham, Bucks., England) using 0.05 N NaOH as a transfer solution. The membrane was incubated in the prehybridization buffer containing 50% formamide, 5 × SSC, 50 mM sodium phosphate (pH 6.8), 1% sodium dodecylsulfate (SDS), 150 μg/ml salmon testis DNA and 150 μg/ml yeast transfer RNA [26] at 42°C for 3 h. The membrane was then hybridized with a ^{32}P-labeled rat ARcRNA probe in the same buffer at 65°C for 12 h. After the hybridization, the membrane was washed at 65°C using 0.1 × SSC, 0.1% SDS solution, followed by RNase digestion (10 μg/ml) for 3 h. The membrane was exposed to X-ray film (Fuji X-ray film RXO-H) at −80°C using intensifying screen.

In situ hybridization

The hybridization protocol was modified from the method of Swanson and co-workers [27]. Briefly, anesthetized animals were perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M acetate buffer at pH 6.5, followed by 4% paraformaldehyde in 0.1 M borate buffer at pH 9.5. The fixed tissues were removed and postfixed overnight in the second fixative at 4 C. Cryostat sections (10 μm) were mounted onto gelatin-coated, poly-L-lysine-coated slides. After proteinase K digestion (5 μg/ml), the sections were dehydrated in ascending alcohols and dried under vacuum. The ^{35}S-labeled ARcRNA probe was heated at 65 C for 5 min with 500 μg/ml yeast tRNA and 50 μM dithiothreitol (DTT) in water before being diluted to an activity of 1.5×10^7 dpm/ml with a hybridization buffer containing 50% formamide, 0.25 M sodium chloride, 1 × Denhardt's solution, and 10% dextran sulfate. The 75-μl hybridization buffer was applied to each slide, covered with a glass coverslip, and then sealed with DPX (Aldrich) and incubated for 18–20 h at 56°C. The sections were then washed by rinsing four times in 4 × SSC prior to RNase digestion (20 μg/ml), followed by washing in decreasing concentrations of SSC containing 1 mM DTT (2 ×, 1 ×, 0.5 ×; 10 min each) at room temperature to a final stringency of 0.1 × SSC at 75°C for 30 min, and were then dehydrated with ethanol. The slides were coated with Kodak NTB-2 emulsion (diluted 1:1 in distilled water), dried, and exposed at 4°C for 14 days. Autoradiograms were developed with Kodak D-19 developer and fixed. The slides were counterstained with hematoxylin-eosin and mounted with Entellan (Merck). The sections were examined under light-field and dark-field illumination.

In situ hybridization specificity controls

Specificity of hybridization was determined by the following three control experiments in the rat adrenal gland: (1) sections were incubated with hybridization solution containing a sense strand probe; (2) sections were pretreated with RNase (20 μg/ml) prior to

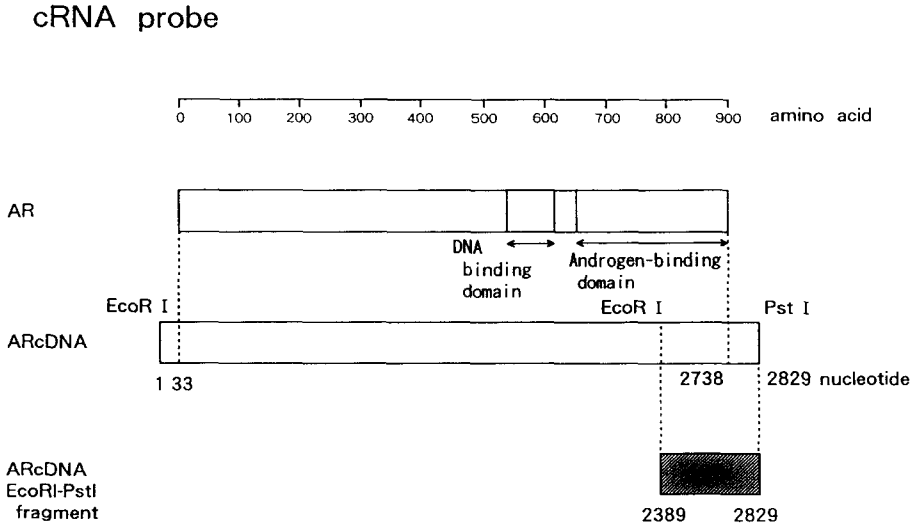

Fig. 1. The structure of the rat ARcDNA. The positions of the DNA- and androgen-binding domains are shown. The ARcRNA probe used for hybridization was synthesized from the EcoRI–PstI fragment (nucleotides 2389–2829, as numbered by Chang *et al.*) of the ARcDNA [6a].

hybridization; (3) sections were incubated with a mixture of unlabeled AR probe and labeled probe. No specific hybridization signals were observed in experiment (1), and the specific signals were reduced in experiments (2) and (3) (data not shown).

RNA probes

Probes were synthesized and labeled with [^{32}P]CTP or [^{35}S]CTP by T3 or T7 RNA polymerase transcription using the cDNA inserts in pBSM13+ vector (pBSrAREF+) as a template [28]. To obtain the pBSrAREF+, we subcloned androgen binding domain-corresponding fragments of the rat ARcDNA into pBSM13+ (see Fig. 1).

RESULTS

Northern blot analysis

The tissue distribution of ARmRNA was examined in the adult female and male rats by using Northern blot analysis. In order to validate identification of ARmRNA by the RNA probes, we screened the total RNA of the rat ventral prostate using ARcRNA antisense strand and sense strand probes. As shown in Fig. 2, about 10-kilobase (kb) mRNA was detected clearly as a single band by an antisense strand probe, but not by a sense strand probe. These results, which were validated by the antisense strand probe, indicate that a band of about 10-kb is specific for ARmRNA.

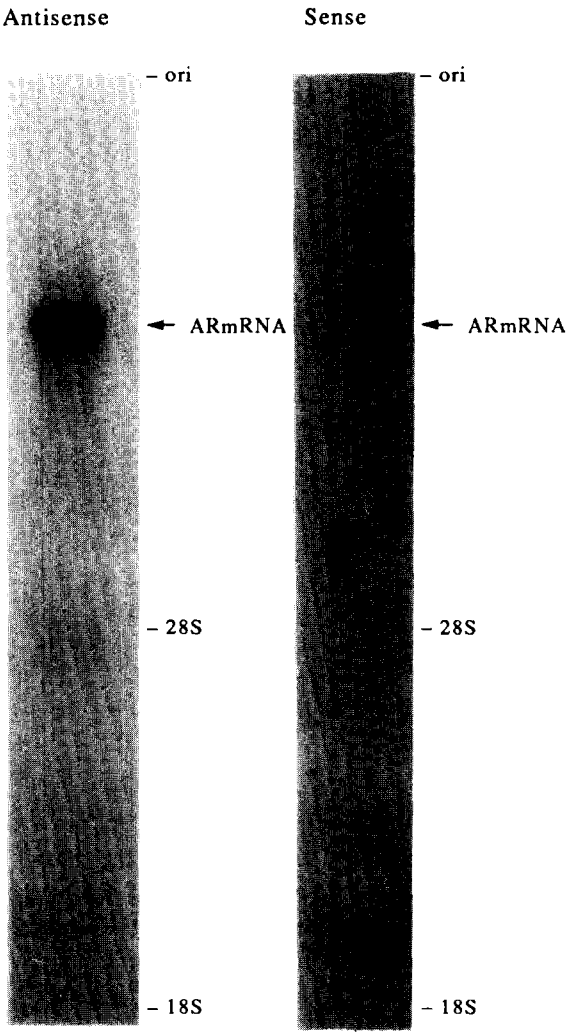

Fig. 2. Northern blot analysis of total RNA (30 μg/track) from the rat ventral prostate. About 10-kb mRNA was observed by an antisense probe (left panel) but not by a sense probe (right panel). The origin, ARmRNA, 28 and 18S ribosomal RNA are shown on the right-hand side.

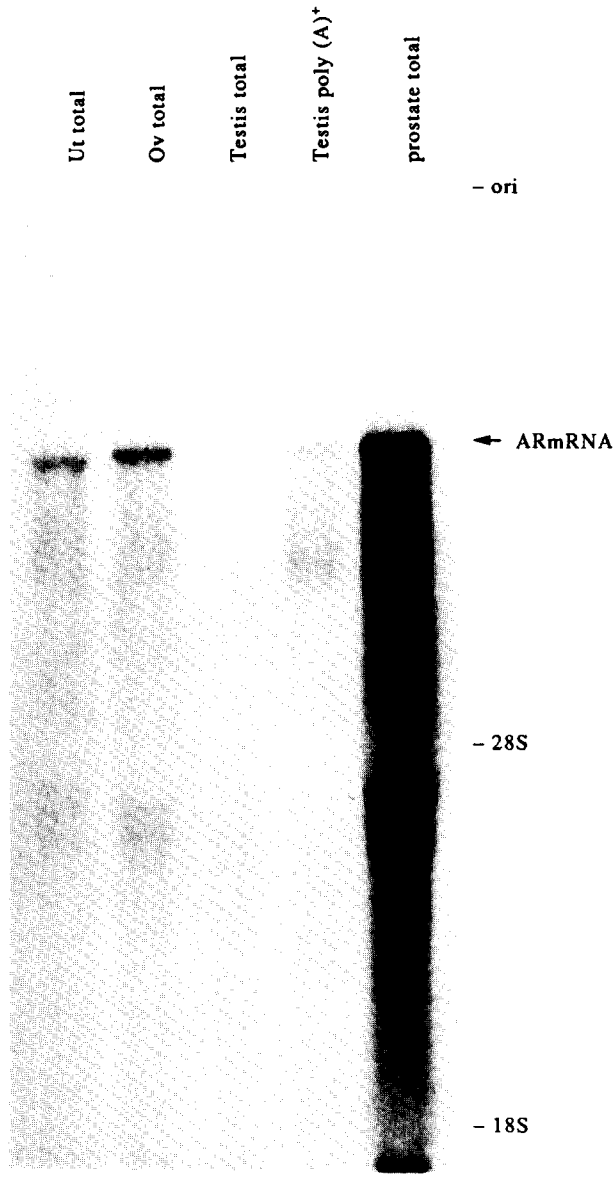

Fig. 3. Northern blot analysis of the rat uterus, ovary, testis and ventral prostate. Total RNA (30 μg/track) from each tissue and poly(A)$^+$ RNA (10 μg/track) from the testis were analyzed by Northern blot with an ARcRNA antisense probe.

Figure 3 shows the result of Northern blot analysis of ARmRNA in the rat ovary, uterus, testis and ventral prostate. Total RNA from the ventral prostate was used as a positive control for ARmRNA. The same amount of total RNA from each tissue was applied for

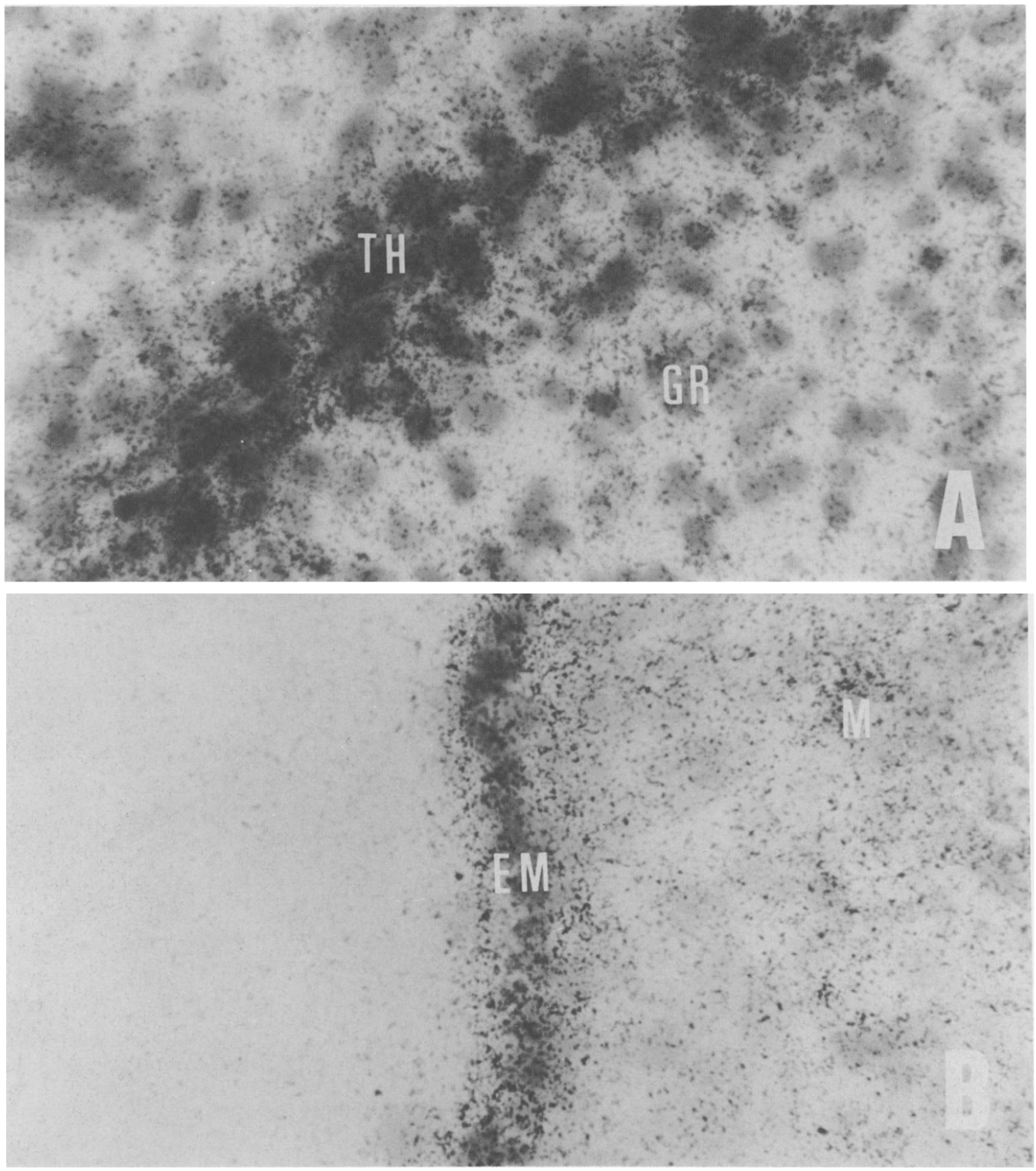

Fig. 4. Light-field micrographs showing ARmRNA in the ovary (A) and uterus (B). GR, glanulosa cells; TH, theca cells; EM, endometrium; M, myometrium. ×100.

hybridization. A single mRNA band of about 10-kb was detected in each of the tissues examined except for the testis. Although the ARmRNA band was not detected when the total testis RNA was used, a 10-kb band was observed with poly(A)$^+$ RNA from the testis. The levels of ARmRNA in the rat ovary and uterus were lower than in the ventral prostate, and considerably lower in the testis.

In situ hybridization

The distribution of ARmRNA in the rat ovary and uterus was investigated by *in situ* hybridization. ARmRNA-containing cells were found to be widely distributed in the ovary and uterus. Cells containing ARmRNA were identified by clusters of silver grains over single cells such as the theca, and granulosa cells of the ovary, and endometrial cells of the uterus (Fig. 4).

Ovary. Strong labeling was observed in the theca cells. Moderate labeling was present in the granulosa cells and the stromal cells [Figs 4(A) and 5].

Uterus. Strong labeling was found in the endometrium and the endometrial glands. Moderate

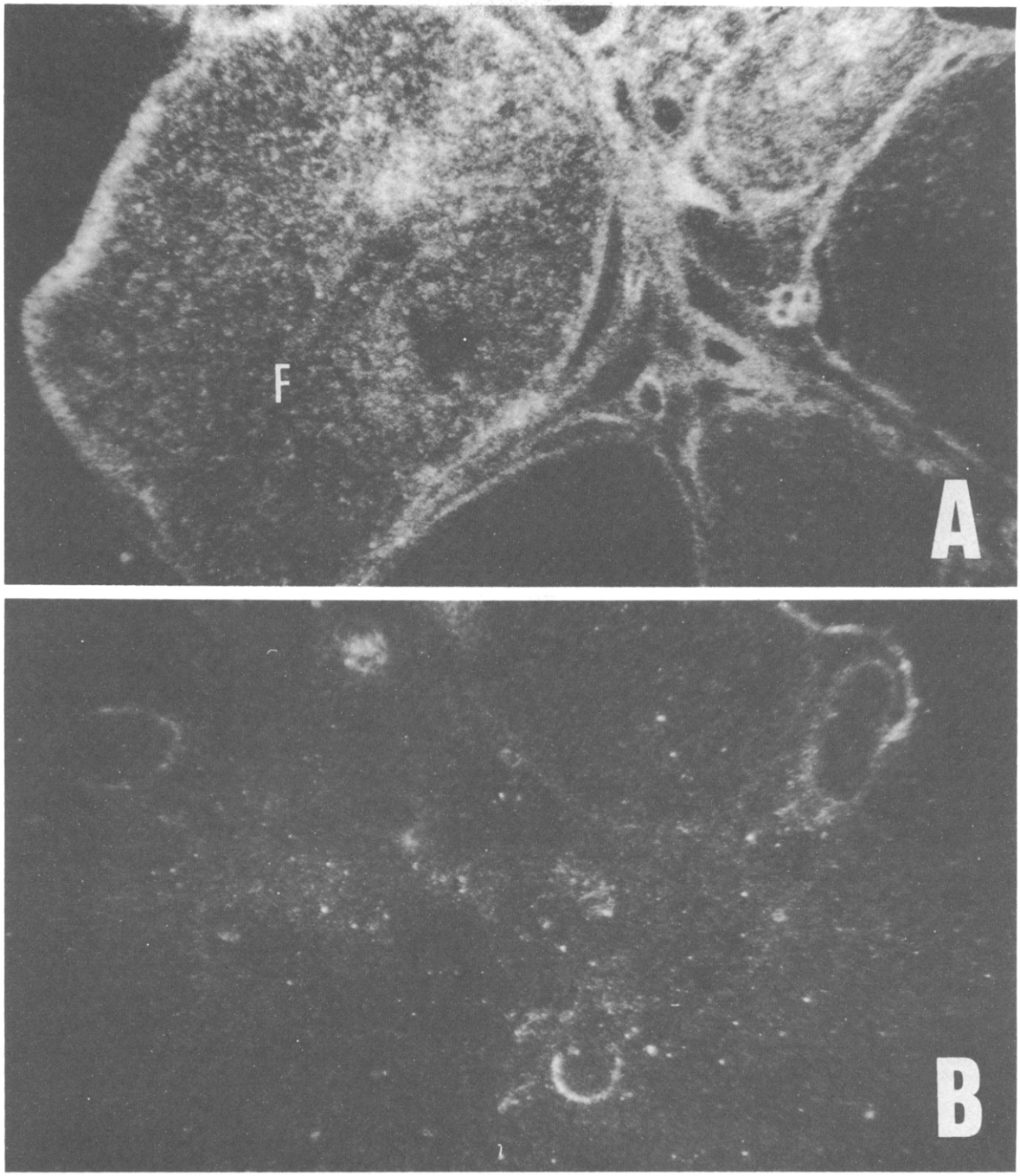

Fig. 5. Dark-field microscopy of the ovary hybridized with an AR anti-sense probe (A) and a sense probe (B). Hybridization signals are seen in the theca, granulosa and stromal cells. No specific signals are observed by the sense probe. F, follicle. ×10.

labeling was present in the myometrium [Figs 4(B) and 6].

DISCUSSION

We have detected a single RNA band of about 10-kb in the rat ovary, uterus, testis, and ventral prostate by Northern blot analysis using a ^{32}P-labeled cRNA anti-sense strand probe. Since the molecular size of the ARmRNA that we detected in the rat reproductive tissues corresponded to that in previous reports [7–9] on the ARmRNA in male tissues, the 10-kb RNA band is considered to be specific for ARmRNA.

The present report is the first demonstration of ARmRNA in the ovary and uterus. The amount of ARmRNA in the ovary and uterus was much less than that in the prostate, though it was much more than that in the testis. These results were largely in agreement with the results of the previous biochemical studies on AR [15–17, 20–22, 29]. The AR protein level may be mainly regulated by the transcription or stability of the ARmRNA. Moreover, these results indicate that

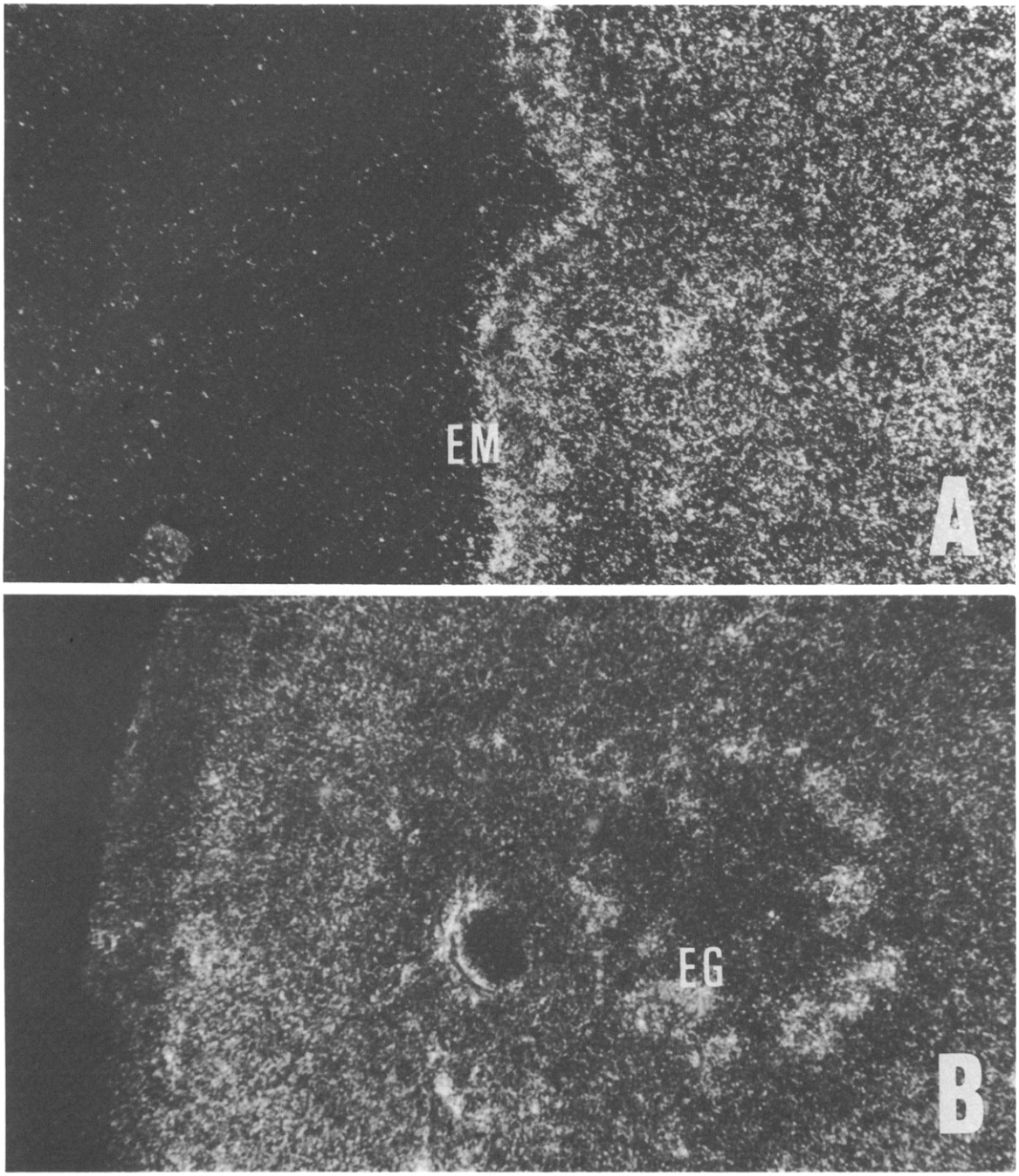

Fig. 6. Dark-field micrographs showing ARmRNA in the uterus. Strong hybridization signals are observed in the endometrium (A) and endometrial glands (B). EM, endometrium; EG, endometrial gland. A; ×50, B; ×20.

androgens appear to play an essential role in mediating female reproductive functions.

In contrast to the male reproductive tissues [10–17], there are fewer reports of androgen actions in female reproductive tissues [20–22, 28–31]. Androgens inhibit granulosa cell proliferation, and increase follicular atresia [31]. Armstrong *et al.* [30] reported that the non-aromatizable androgen dihydrotestosterone (DHT) inhibited the FSH-stimulated aromatase activity in the hypophysectomized immature rat ovary. In addition, androgens stimulate the synthesis of progesterone in cultured granulosa cells [28]. These previous data suggest that (1) androgens may be involved in the follicular development and atresia in cooperation with estrogen and progesterone and that (2) the granulosa cell is considered to be the target cell of androgens. Since AR exists in the ovary and isolated granulosa cells [21, 22], androgen action on the granulosa cells may be mediated directly by the hormone.

AR has been detected in the human endometrium and the rat uterus by biochemical assay [20, 29]. Although androgens significantly increase uterine weight [30], little is known about the mechanism of the action of androgens on the uterus.

Recently, immunohistochemistry has been used to localize AR [4]. AR-positive cells were demonstrated in the corpus luteum of the ovary and in the myometrium and stromal cells of the uterus, but the AR was negative in the granulosa, theca and stromal cells of the ovary, and in the endometrium and endometrial glands of the uterus. In the present study, however, ARmRNA could be detected in the theca, granulosa and stromal cells of the ovary, and in the endometrium, endometrial glands and myometrium of the uterus. Such discrepancies might be explained by two factors. First, we used intact "proestrous" rats in the present study, while intact cycling female rats were used in the immunohistochemical study. Second, due to sensitivity, AR could not be detected in the ARmRNA-detectable regions. The *in situ* hybridization results described here are in good agreement with the previous biochemical reports [10–17, 20–22, 29]. It is interesting that there is ARmRNA in the granulosa cells and androgens stimulate the synthesis of progesterone in cultured granulosa cells [28]. These data suggest that androgens might induce an enzyme which synthesizes progesterone in the granulosa cells.

The extent and significance of androgen action in the female reproductive tissues is unclear. However, further research on the expression and distribution of ARmRNA using *in situ* hybridization should provide a useful tool for more detailed evaluation of the role of androgens in these tissues.

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