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Cellular Localization of Estrogen Receptor Beta Messenger Ribonucleic Acid in Cynomolgus Monkey Reproductive Organs

G. Pelletier,[1] V. Luu-The, A. Charbonneau, and F. Labrie

Laboratory of Molecular Endocrinology, Laval University Medical Research Center and Laval University, Québec, Canada G1V 4G2

ABSTRACT

There is now evidence that the recently identified estrogen receptor (ER) β is more widely distributed in the body than is ER-α. In order to gain more information about the role of ER-β in reproduction, we have investigated by in situ hybridization the localization of mRNA expression of this ER subtype in adult monkey reproductive organs. In the pituitary gland of animals of both sexes, in both the anterior and intermediate lobes, a large number of cells were positive. No specific signal was observed in the posterior lobe. In the ovary, granulosa cells in primary and growing follicles highly expressed ER-β mRNA. The theca interna cells were also strongly labeled. In some corpora lutea, the luteal cells were strongly labeled, while in other ones, the signal was weak. A hybridization signal was also detected in the ovarian surface epithelium. In the uterus, ER-β mRNA was found in high concentration in glandular epithelial cells and stromal cells of the endometrium, while weaker labeling was consistently observed in smooth muscle cells. In the mammary gland, labeling was detected in the epithelial cells of acini and interlobular ducts as well as stromal cells. In the testis, specific labeling was detected in the seminiferous epithelium whereas the interstitial Leydig cells were unlabeled. Although it was not possible to clearly identify all the positive cell types, it appears that Sertoli cells as well as the vast majority of germinal cells express ER-β mRNA. In the prostate, the secretory epithelial cells exhibited a specific autoradiographic reaction while the stromal cells did not show mRNA expression. The epithelial cells of the prostatic urethra showed a strong labeling. No hybridization signal was detected in the seminal vesicles. It then appears quite clear that ER-β is expressed in a cell-specific manner in all the monkey reproductive organs studied. In the female, the wide distribution of these receptors in the ovary and uterus suggests that ER-β may play an important role in the mediation of the known effects of estrogen in reproduction functions. In the male testis and prostate, ER-β has been found in cells that contain very little or no ER-α. The role of circulating or locally produced estrogens in the male reproductive system remains to be clarified.

INTRODUCTION

It is well documented that estrogens play an important role in the growth, differentiation, and function of peripheral reproductive tissues, including the ovary, mammary gland, uterus, vagina, testis, and prostate (for review see [1]). Estrogens exert several other actions, especially at the level of the brain, where they modulate physiological parameters regulating reproduction function. Estrogens also play a role in bone maintenance and in the cardiovascular system, in which they have certain cardio-protective effects [2, 3]. The effects of estrogens are mediated through an intracellular estrogen receptor (ER) that has been well characterized during the last three decades [1]. Recently, a second ER called ER-β has been cloned from a rat prostate library entry, and the original is now designated ER-α [4].

The expression of ER-β appears to be specific for a wide range of tissues in several species, including the human. ER-β has been found in organs that contain little or no ER-α and that were not considered as targets for estrogens. This is the case, for example, for bladder, kidney, intestine, and lung [2, 3]. In the rat ovary and prostate, which contain both ER-α and ER-β, a cell-specific localization has been reported for each type of ER [5, 6]. Recently the cloning of human ER-β [7] has been reported, thus providing further evidence that ER-β shows many of the functional characteristics of ER-α.

The distribution as well as the regulation of the ER-α gene has been extensively studied in several mammalian species [1]. However, very little is known about the cellular localization of ER-β in primates. Thus, in order to identify cells that produce ER-β in primate reproductive tissues and therefore could be considered as targets for circulating and/or locally produced estrogens, we have investigated the histological distribution of ER-β mRNA by in situ hybridization in the Cynomolgus monkey reproductive organs.

MATERIALS AND METHODS

Animals

The two 21-yr-old male and two 20-yr-old female Cynomolgus monkeys (*Macaca fascicularis*) used were proven breeders; they had been maintained for 12 years at the Animal Resources Division, Sir Frederick Banting Research Center of the Health Protection Branch of the Government of Canada, Ottawa, under the supervision of Dr. J. Fournier, in a facility accredited by the Canadian Council on Animal Care. Animals were exsanguinated under isoflurane anesthesia. Since the average life span of these animals kept in captivity is 30 years, they could be considered as moderately aged. The phase of the menstrual cycle of the two females was not determined at the time they were killed, but the presence of growing follicles and corpora lutea in the ovaries of both monkeys (see *Results*) indicated that they were cycling animals.

Histological Techniques

Tissues, namely pituitary, testis, prostate, seminal vesicles, ovary, uterus, and breast, were rapidly excised without any fat and frozen on dry ice in support medium (OCT, Bayer Corp., Elkhart, IN) and then kept at −80°C. Ten-micrometer serial sections were then cut at −20°C and mounted on Superfrost/Plus Microscope slides (Fisher Scientific, Montréal, PQ, Canada). The sections were thereafter fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at 20°C.

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[1]Correspondence: Georges Pelletier, Laboratory of Molecular Endocrinology, CHUL Research Center, 2705, Laurier Boulevard, T3–67, PQ, Canada G1V 4G2. FAX: 418 654 2761; e-mail: lrem@crchul.ulaval.ca

Human ER-β Probe Preparation

Sense and antisense riboprobes were generated by in vitro transcription from the p-Bluescript KSII$^+$ phagemid (Stratagene, La Jolla, CA) containing a DNA insert of 30 nucleotides of ER-β using [^{35}S]UTP (NEN Life Science Products, Boston, MA) as the radioactive source. These 30 nucleotides correspond to a nucleotide sequence spanning positions 1473 to 1502 of the human ER-β (hER-β) cDNA sequence [7]. This sequence was carefully chosen to avoid cross-hybridization with hER-α. We have shown previously that cRNA probes of about 35 nucleotides could be successfully used to perform in situ hybridization [8]. This approach using a short probe is very convenient since it allows selection of a nonhomologous sequence, thus preventing cross-hybridization with other mRNA species that share high homology. To construct the plasmid described above, we synthesized two complementary oligonucleotides (5′-AAT-TCC-TGG-CCC-ACA-GAG-GTC-ACA-AGC-TGA-AGC-GTG-GG-3′ and 5′-GAT-CCC-CAC-GCT-TCA-GCT-TGT-GAC-CTC-TGT-GGG-CCA-GG-3′) containing sequences corresponding to the sequence of hER-β described above, and cohesive overhanging end sequences of the restriction site *Eco*RI and *Bam*HI (underlined). These two synthetic oligonucleotides were mixed together to form a double-stranded DNA fragment containing 30 nucleotides of hER-β. Upon phosphorylation of the 5′-end nucleotide using T4 polynucleotide kinase, the DNA fragment was ligated into the p-Bluescript KSII vector, previously prepared by digestion with *Eco*RI and *Bam*HI restriction enzymes. The oligonucleotides were synthesized using a DNA synthesizer ABI-394 (Perkin-Elmer-Cetus, Emeryville, CA).

In Situ Hybridization

In situ hybridization of frozen tissue sections with the hER-β cRNA probe was adapted from previously described methods [9]. 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-strength SSPE (single-strength SSPE is 0.1 M NaCl, 10 mM NaH_2PO_4 pH 7.4, 1 mM EDTA), 5-strength Denhardt's buffer, 200 mg/ml denatured salmon testis DNA (Sigma, St. Louis, MO), 200 μg/ml yeast-tRNA, 2 μg/ml poly A (Boehringer-Manheim, Montréal, PQ, Canada), and 4% dextran sulfate. After prehybridization treatment, 100 μl of hybridization mixture (prehybridization buffer containing in addition 1 M dithiothreitol and ^{35}S-labeled cRNA probe at a concentration of 20×10^6 cpm/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 double-strength SSC (single-strength SSC is 0.15 M sodium chloride, 0.015 M sodium citrate) at room temperature for 30 min. Sections were digested by ribonuclease A (20 μg/ml in double-strength SSC) at 37°C for 30 min; rinsed in decreasing concentrations of SSC (double-strength SSC and single-strength SSC) for 30 min at room temperature; and washed in 0.5-strength SSC for 30 min at 37°C and for 90 min at room temperature, and in 0.1-strength SSC for 90 min at 60°C and for 30 min at room temperature.

After the posthybridization treatments, the sections were dehydrated and exposed onto Kodak X-Omat films (Eastman Kodak, Rochester, NY) for 4–5 days before being coated with liquid photographic emulsion (Kodak-NTB2; diluted 1:1 with water). Slides were exposed for 14–18 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 eosin (H&E), and rapidly dehydrated through graded concentrations of ethanol, cleared in toluene, and coverslipped with Permount (Fisher Scientific, Montréal, PQ, Canada).

RESULTS

In the pituitary gland, specific labeling, as observed at the light microscopic level, was detected over a large number of the anterior pituitary cells (Fig. 1). Because of the rather diffuse distribution of silver grains, it was not possible to determine the percentage of labeled cells. Most of the cells of the pars intermedia, which is very well developed in the monkey, were also labeled, while the posterior pituitary was devoid of any specific reaction. No sex difference could be recorded.

In the testis, the hybridization signal could be observed in the tubular epithelium (Fig. 2). Because of a lack of high resolution of grains, it was not possible to clearly identify all the labeled cellular elements. Silver grains probably are associated with round spermatogonia and developing spermatids, as well as Sertoli cells. The interstitial Leydig cells did not exhibit any positive signal.

As shown by x-ray autoradiograph, the prostatic lobes were uniformly labeled (Fig. 3), while hybridization of the labeled sense probe did not produce any labeling. At the light microscopic level, the secretory epithelial cells of tubulo-alveoli exhibited a specific autoradiographic reaction, whereas no labeling could be observed in the stroma surrounding the alveoli (Fig. 4). Since, in the monkey prostate, there is no basal cell in the alveoli (unpublished observation), it can be concluded that ER-β mRNA is expressed in luminal cells. In the prostatic urethra, a strong reaction was detected in the pseudostratified epithelium, while the adjacent stroma was weakly labeled or appeared unlabeled (data not shown). The seminal vesicles did not show any specific labeling.

In the ovary, ER-β mRNA was found in the granulosa cells of follicles at different stages of development, including small growing and secondary (antral) follicles (Fig. 5). The theca interna cells were also strongly labeled. The primordial follicles did not show any labeling in the four ovaries from two monkeys that we examined. It was not possible to identify any preovulatory follicle. The corpora lutea appeared either strongly labeled or weakly labeled (Fig. 6). The interstitial cells were consistently unlabeled. A strong autoradiographic reaction was also detected in the ovarian capsule, particularly in the surface epithelium and the stromal cells (Fig. 7).

In the uterus, as illustrated by x-ray autoradiographs,

FIG. 1. **A**) Section through the anterior (AL) and intermediate (IL) pituitary lobes of a female monkey. In the anterior and intermediate lobes, most of the cells appear to be labeled. **B**) Section consecutive to that shown in Figure 2A, which has been hybridized with the labeled (arrow) sense probe. Only a few dispersed silver grains can be observed. Bars = 20 μm.

FIG. 2. **A**) Section through a testis. All the cellular elements at the periphery and center of the tubule (T) appear to be labeled (arrow). Almost no silver grains can be seen over the interstitial (I) cellular elements. **B**) Control section hybridized with the labeled sense probe. Very few silver grains are present over the different structures. Bars = 20 μm.

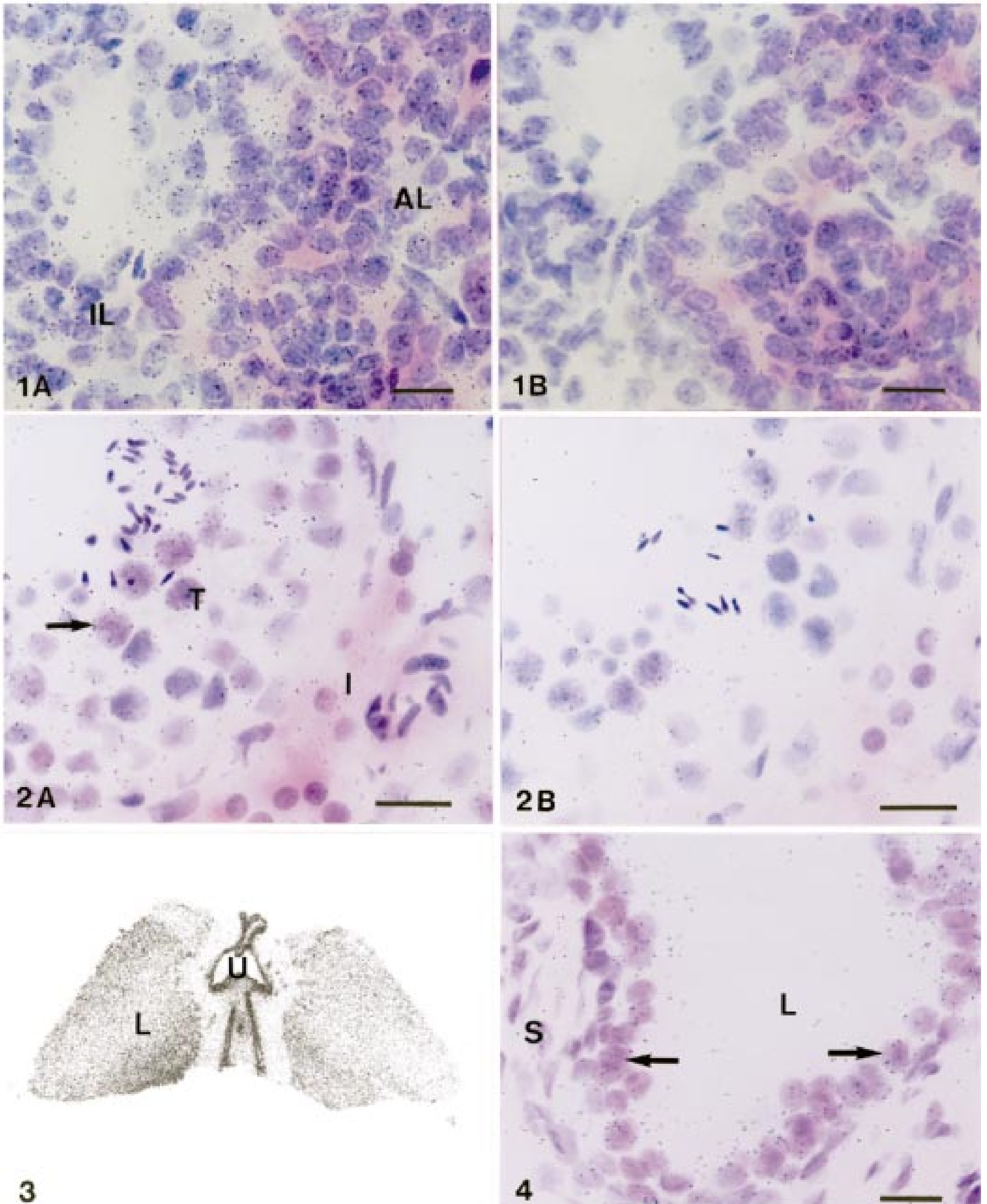

FIG. 3. X-Ray autoradiograph of a section through the prostate. The lobes (L) are relatively uniformly labeled. Note the strong labeling of the urethra (U).

FIG. 4. Section through the prostate. Silver grains are exclusively located over the secretory epithelial cells (arrows) of a tubulo-alveolus. No significant labeling can be observed in the stroma (S). L, Lumen. Bar = 20 μm.

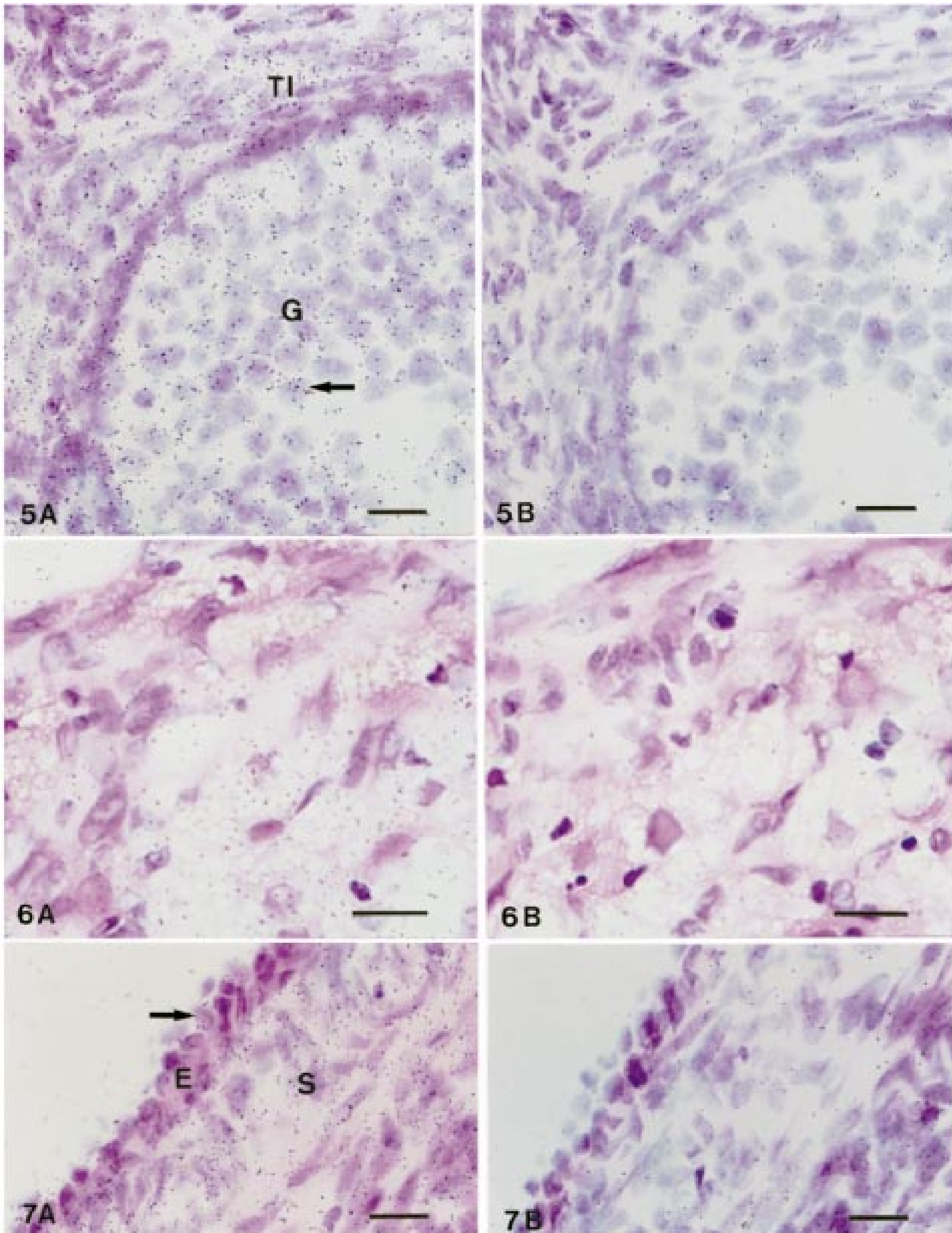

FIG. 5. **A**) Section through an antral ovarian follicle. Silver grains (arrow) are present over granulosa (G) and theca interna (TI) cells. **B**) Control section hybridized with the sense probe. Silver grains are diffusively located. Bars = 20 μm.

FIG. 6. **A**) Section through a corpus luteum. Luteal cells are labeled. **B**) Adjacent control section hybridized with the labeled sense probe. Only a few dispersed grains are present. Bars = 20 μm.

FIG. 7. **A**) Section through the ovarian capsule. The surface epithelium (E) cells (arrow) as well as stromal cells (S) are labeled. **B**) Control section consecutive to that shown in **A** hybridized with the sense probe. Few dispersed grains can be detected. Bars = 20 μm.

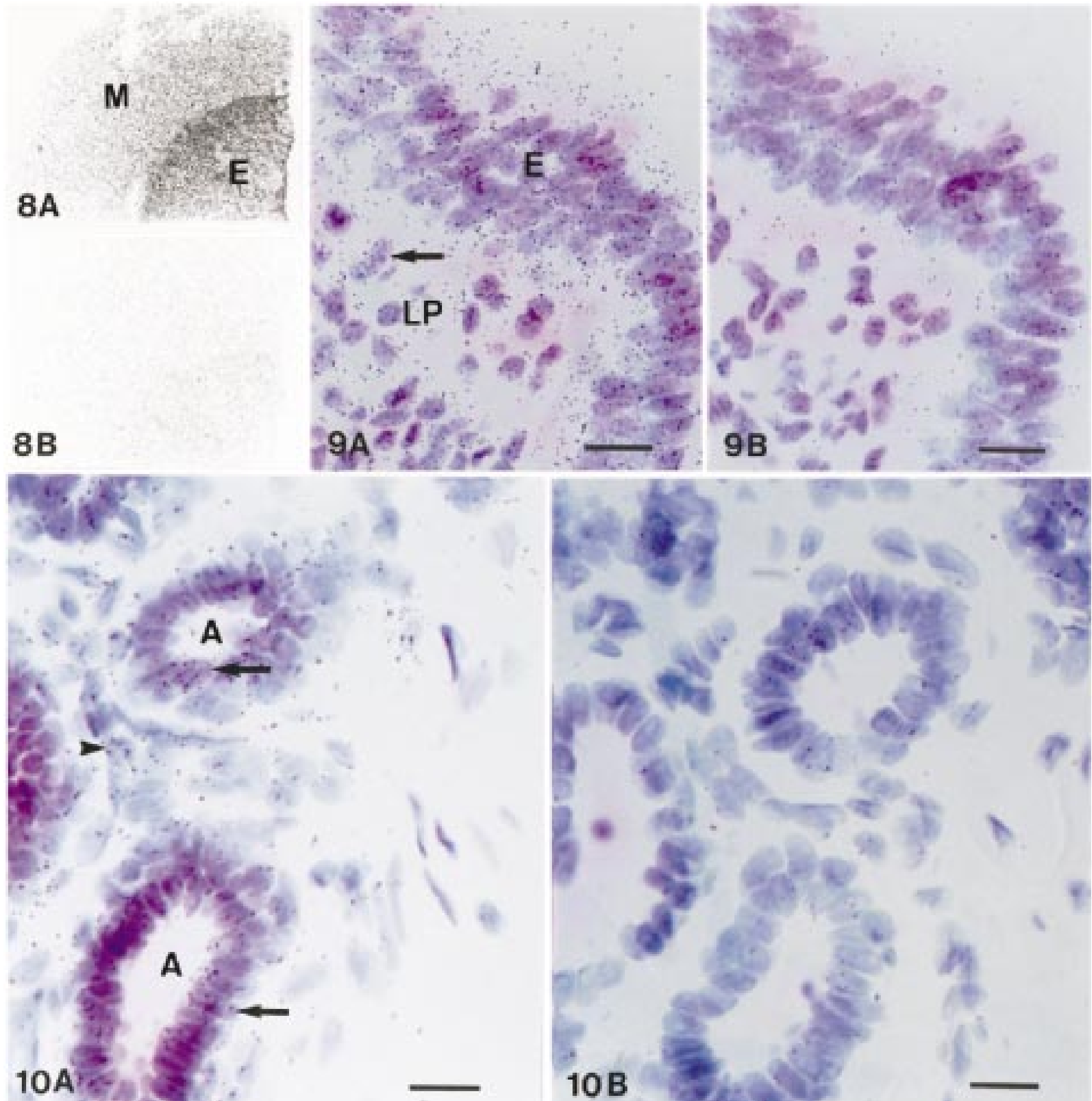

FIG. 8. **A**) X-ray autoradiograph of a fragment of the uterus. A strong reaction was observed in the endometrium (E), while in the myometrium (M) the labeling was weaker. **B**) Control section hybridized with the labeled sense probe. A very weak and uniform reaction can be seen.

FIG. 9. **A**) Section through the endometrium. Silver grains are overlying the glandular epithelium (E). Stromal cells (arrow) in the lamina propria (LP) are also labeled although to a lesser degree. Magnification ×600. **B**) Control section. A few silver grains corresponding to background can be detected. Bars = 20 μm.

FIG. 10. **A**) Section through a mammary gland. Silver grains are overlying the cuboidal epithelial cells (arrows) bordering acini (A). Stromal cells (arrowhead) are also labeled. **B**) Semi-adjacent control section hybridized with the sense probe. Very few silver grains can be observed. Bars = 20 μm.

ER-β mRNA was found to be expressed in both myometrium and endometrium (Fig. 8). In the endometrium, both epithelial cells lining the glands and those covering the surface were labeled (Fig. 9). In the stroma, in which glands are embedded, labeled cells were consistently observed. In the mammary gland, specific labeling was observed in the epithelial cells in intralobular acini and terminal ducts as well in the stromal cells (Fig. 10). ER-β mRNA was also found in the epithelial cells of interlobular ducts.

DISCUSSION

In order to precisely determine the structures and cells producing ER-β, we used in situ hybridization to localize ER-β mRNA expression. Since the ER-β mRNA sequence is not known in the monkey, we used a probe from human ER-β. The specificity of the probe is supported by the high levels of ER-β mRNA expressed in organs such as the ovary, prostate, and urethra, which have been shown to express

high amounts of ER-β and no or very low levels of ER-α [2–4]. Moreover, hybridization with labeled sense probe produced weak and diffuse labeling, without any localization to specific structures or cells (see, for example, Figs. 2B, 3B, 4B, 5B, and 6B).

The detection of ER-β mRNA in a large number of anterior pituitary cells is in agreement with a recent report from Mitchner et al. [10], who showed that both ER-α and ER-β mRNAs were expressed in gonadotrophs, lactotrophs, corticotrophs, and folliculo-stellate cells. Apparently the authors did not study the localization of ER mRNAs in somatotrophs and thyrotrophs. They also reported that 23% of melanotrophs in the intermediate lobe expressed ER-β mRNA. Using an oligonucleotide probe to detect ER-α mRNA in the rat pituitary, we previously demonstrated that ERs were highly expressed in most of the anterior pituitary cells and in all the cells of the pars intermedia [11]. The finding that a large number of cells in the anterior lobe contain ERs agrees well with physiological studies indicating that all the pituitary hormones can be directly modulated by estradiol [12–14]. The present data suggest that this effect of estradiol may be mediated not only by ER-α but also by ER-β. The role of ERs in the intermediate lobe remains to be clarified.

In the testis, the hybridization signal was observed within seminiferous tubules, with no labeling of Leydig cells. The resolution was not high enough to clearly identify all the cell types containing ER-β mRNA, although it was clear that grains were overlying Sertoli cells as well as spermatogonia and spermatids. Enmark et al. [15], using in situ hybridization, have recently reported that ER-β mRNA was found in developing spermatids in human testis, particularly in primary spermatocytes and early round spermatogonia, and could not be detected in Leydig cells. In the rat testis, immunocytochemical studies have demonstrated immunoreactive ER-β in several cell types including Leydig cells, Sertoli cells, and spermatocytes at all stages of spermatogenesis [16]. This discrepancy between the results from primate and rat testis might be explained by a species difference or by the different techniques used. The presence of ER-β in Sertoli and germ cells strongly suggests that estrogens might have a direct influence on germ cell function and maturation.

In the prostate, ER-β mRNA was found only in the epithelial cells of tubulo-alveoli, the stromal cells being negative. These results are in good agreement with the recent data showing that in rat prostate, ER-β is localized in epithelial cells, which contrasts with the stromal (smooth muscle cells) localization of ER-α [5, 6]. Localization of ER-β mRNA in the prostatic epithelial compartment as opposed to the stromal localization of ER-α suggests that this receptor may activate genes that are different from those regulated by ER-α in response to estrogenic stimulation. In the prostatic urethra, a high hybridization signal was mostly detected in the epithelial cells, with a low labeling in the adjacent stroma. This preferential localization of ER-β mRNA agrees with recent data indicating that, in rodent bladder and urethra, ER-β is present in the epithelium while ER-α is only weakly expressed in the underlying connective tissue [4, 5].

In the ovary of adult monkeys, strong labeling was observed in all the follicles with the exception of primordial follicles. This finding is in agreement with previous reports indicating high levels of ER-β mRNA in granulosa cells of rat follicles [17] and cultured human granulosa cells from patients undergoing in vitro fertilization [18]. These ERs are probably involved in the effects of estrogens, which have been shown to stimulate the proliferation of granulosa cells from small follicles, increase granulosa cell gonadotropin receptor levels, and modulate progesterone production by the granulosa cells [19, 20]. Disruption of the ER-α gene in vivo could not eliminate the ability of small follicles to grow, as evidenced by the presence of secondary and antral follicles in ER-α-knockout mice [21]. Such a finding strongly supports the hypothesis that ER-β is involved in the trophic influence of estrogens on follicular development. In female mice lacking ER-β, it has been shown that this ER is essential for normal ovulation efficiency but is not essential for fertility [22]. ER-β mRNA was also detected in corpora lutea, with variations in the intensity of the labeling. The presence of low amounts of ER-β mRNA in a subset of corpora lutea that express LH receptor mRNA has been reported [17]. In the pregnant rat, both ER-α and ER-β mRNAs were observed in corpora lutea by in situ hybridization [23]. No changes in ER-β mRNA were observed throughout pregnancy, while ER-α mRNA was expressed at low levels early in pregnancy and increased thereafter [23]. The exact function of ER in the regulation of luteal cell function remains to be elucidated. An interesting finding is the presence of ER-β mRNA in the ovarian surface epithelium, from which most of the human ovarian cancers originate [24]. Very recently, Brandenberger et al. [25] reported a decrease in ER-β mRNA in carcinomas of the human ovary. The role of ER receptors in the development of ovarian cancer remains to be investigated.

In the uterus, a strong labeling was observed in the epithelial cells bordering the uterine lumen and the glandular luminal cells as well as in stromal cells of the endometrium. In smooth muscle cells, a weaker but very significant hybridization signal was observed. Although both ER subtypes of mRNA have been found to be expressed in rat uterus [5], this is the first report on the histological localization of ER-β mRNA in primate uterus. The present data agree well with recent results obtained by immunocytochemistry indicating that in the rat uterus, both ER-α and ER-β were present in luminal and glandular epithelial as well as stromal cells [26]. Moreover, in ER-α knock-out mice, estradiol binding could be observed in the uterus, probably because of the presence of ER-β in this tissue [21]. The relative physiological role of each ER subtype in the uterus remains to be evaluated.

In the mammary gland, ER-β was detected in the epithelial cells of both alveoli and ductules as well as in stromal cells of large ducts. This observation is in agreement with previous results showing the presence of the classical ER (probably ER-α) in both epithelial and stromal cells [1]. The present results also agree well with recent findings indicating that ER-β is expressed in normal human breast tissue as well as in breast tumors [15]. Moreover, ER-β mRNA has been found in several human breast epithelial cell lines, which were both ER-α-positive and ER-β-positive [27]. The respective functions of the two receptor subtypes in normal breast physiology and in the development of breast cancer are still unknown.

In summary, the present findings have clearly established a cell-specific localization of ER-β mRNA in several reproductive tissues in the monkey of both sexes. Most of the data are in complete agreement with those available on the distribution of this receptor in rodents and humans. They also agree well with recent results from Pau et al. [28], who used reverse transcription-polymerase chain re-

action to demonstrate the presence of ER-β mRNA in male and female monkey reproductive organs. In the female monkey, the wide distribution of ER-β in the ovary and uterus suggests that this receptor may play a major role in the effects of estrogens in reproduction function. In the male reproductive organs, ER-β appears to be highly expressed in cell types that contain very little or no ER-α. The role of estrogens in the male reproductive system is still largely unknown. However, the presence of aromatase, which converts testosterone to estradiol in the male genital tract [2], suggests that locally produced estrogens may play a role more important than previously thought. The different mechanisms involved in the control of ER-α and ER-β activation of transcription [29, 30] and the tissue-specific expression of these two ERs provide means for fine control of estrogen-sensitive gene expression.

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