Localization of oestrogen receptor α, oestrogen receptor β and androgen receptors in the rat reproductive organs

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Abstract

There is now evidence that oestrogens and androgens can influence male and female reproductive systems. In order to accurately identify the sites of action of oestrogens and androgens, we have proceeded to the histological localization of the two oestrogen receptor (ER) subtypes, ERα and ERβ, and the androgen receptor (AR) in the reproductive tissues of adult rats of both sexes. AR was detected by immunocytochemistry, while ERα and ERβ were localized by both immunocytochemistry and *in situ* hybridization. In the pituitary gland of animals of both sexes, ERα was found in the majority of nuclei of secretory cells in the anterior pituitary. The intermediate and posterior lobes did not show any staining. ERβ was not found to be expressed in any of the pituitary lobes. Using AR antibodies, nuclear staining was detected in about 50% of secretory cells of the anterior lobe, the intermediate and posterior lobes being completely unstained. In the testis, ERα was localized in nuclei of Leydig cells as well as in round spermatocytes and spermatids, while ERβ could only be detected in Sertoli cell nuclei. AR immunoreactivity was found in nuclei of Sertoli, peritubular myoid and Leydig cells. In the prostate, ERβ was observed in epithelial cells of tubulo-alveoli, while the stroma was unlabelled. ERα was not found to be expressed in any prostate cells. In the prostate, AR was detected in nuclei of epithelial, stromal and endothelial cells. In seminal vesicles, staining of ERα was found in nuclei of epithelial and stromal cells. Similar findings were observed using AR antibodies. While ERβ mRNA could not be detected by *in situ* hybridization, weak staining for ERβ was localized in epithelial cells of seminal vesicles. In the ovary, both ERα and ERβ were found to be expressed. ERβ mRNA was found in granulosa cells of growing follicles, while ERα was present in theca cells, interstitial gland cells and germinal epithelium. AR immunoreactivity was detected in granulosa cell nuclei in growing follicles and also in scattered interstitial cells. In the oviduct and uterus, ERα was observed in nuclei of epithelial cells as well as of stromal and muscle cells. Similarly, AR immunoreactivity was present in nuclei of epithelial cells, stromal and muscle cells in both the oviduct and uterus. ERβ was not detected in the oviduct and uterus. The present findings indicate a cell-specific localization of ERα, ERβ and AR in reproductive tissues in rats of both sexes. By establishing the precise sites of action of oestrogens and androgens they contribute to a better understanding of the respective role of these steroids in reproduction function.

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Introduction

Oestrogens play an important role in the growth, differentiation and function of female and male reproductive tissues (for review see Clark *et al*. 1992, Sharpe 1998). The effects of oestrogens are mediated through an intracellular oestrogen receptor (ER), a member of the steroid/thyroid hormone receptor superfamily which regulates gene transcription via the oestrogen-responsive element (Mangelsdorf *et al*. 1995). Recently, a second ER called ERβ has been cloned from a rat prostate library (Kuiper *et al*. 1996) and the original one is now designated as ERα. RT-PCR analysis and *in situ* hybridization have established that the highest levels of ERβ mRNA are in the rat ovary and prostate (Kuiper *et al*. 1996, 1997). So far, there have been very few reports on the cellular localization of ERβ in the female and male rat reproductive organs (Kuiper *et al*. 1996, 1997, Byers *et al*. 1997, Saunders *et al*. 1998, Sar & Welsch 1999).

Androgens are involved in the development and physiological function of male accessory sex organs as well as in the functioning of several other organs and tissues (Carson-Jurica *et al*. 1990). The androgen action is mediated by the androgen receptor (AR) which also belongs to the superfamily of ligand-responsive transcription regulators (Evans 1988, Carson-Jurica *et al*. 1990). By immunocytochemistry, AR have been localized in a variety of human tissues, including reproductive tissues in both sexes (Ruizeveld de Winter *et al*. 1991, Kimura *et al*. 1993). Similarly, in the rat, AR have also been localized not only in male but also in female reproductive tissues (Sar *et al*. 1990, Hirai *et al*. 1994, Tetsuka *et al*. 1995).

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For a better understanding of the role of androgens and oestrogens in the different reproductive tissues it appears important to define the exact site(s) of action of these sex steroids. Then, in order to accurately determine cells expressing AR, ERα and ERβ in rat reproductive tissues, we have proceeded to the simultaneous localization of these receptors using immunocytochemistry as well as *in situ* hybridization.

Materials and Methods

Animals

Six adult male (225–250 g) and female (175–200 g) Sprague–Dawley rats were housed under constant temperature (21 ± 1 °C) and light (lights on from 0600 to 2000 h) regimens. They received Purina chow (Ralston-Purina, St Louis, MO, USA) and tap water *ad libitum*. They were all perfused between 0900 and 1000 h for histological procedures as described below. The females were on diestrous day 1.

Histological procedures

All the animals were perfused transcardially with 200 ml 4% (w/v) paraformaldehyde in 0·1 M phosphate buffer (pH 7·4). The different tissues, namely pituitary, testis, prostate, seminal vesicle, ovary and uterus were excised and post-fixed in the same fixative for 48 h at 4 °C. For immunocytochemistry, the tissues were embedded in paraffin while, for *in situ* hybridization, the tissues were placed in 15% sucrose in 0·1 M phosphate buffer before being quickly frozen in isopentane chilled in liquid nitrogen.

Immunocytochemistry

Paraffin sections (5 μm) were deparaffinized, hydrated and then treated with 3% H_2O_2 in phosphate-buffered saline (pH 7·6) for 30 min. These steps were followed by heating the sections in a microwave oven for antigen retrieval using citrate buffer (pH 5·5) as previously described (Tacha & Chen 1994). The sections were then incubated overnight at 4 °C with AR antibody (see below for details) at a concentration of 1 μg/ml or ERα antibody (2 μg/ml) (see below for details). Control sections were incubated with preadsorbed antibodies as described below. Sections were then washed in Tris–saline (pH 7·6) and incubated at room temperature for 4 h with peroxidase-labelled goat anti-rabbit γ-globulin (Hyclone, Logan, UT, USA) diluted at 1:500 as previously described (Pelletier *et al.* 1992).

AR An affinity-purified rabbit polyclonal antibody (N-20) against a peptide corresponding to amino acids 299–315 of the AR of human origin (identical to the corresponding mouse sequence) was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). It reacts with the AR of mouse, rat and human origin as tested by Western blotting and immunohistochemistry. Preadsorbed antibody was prepared by incubating 1 μg/ml AR antibody with 20 μg synthetic peptide for 2 h at room temperature.

ERα To localize ERα, we used an affinity-purified rabbit polyclonal antibody (MC-20; Santa Cruz Biotechnology, Inc.) raised against a synthetic peptide corresponding to amino acids 580–599 mapping at the carboxyl-terminus of the ERα of mouse origin. This antibody was used at a concentration of 2 μg/ml. For control experiments, the antibody (2 μg/ml) was adsorbed by preincubation with 20 μg peptide for 2 h at room temperature.

ERβ For ERβ localization, we used a rabbit polyclonal antibody (06–629: Upstate Biotechnology, Lake Placid, NY, USA) directed against a synthetic peptide corresponding to amino acids 54–71 of the rat ERβ. The antibody was used at a concentration of 10 μg/ml. For specific control, the antibody was adsorbed by preincubation with 20 μg synthetic peptide for 2 h at room temperature

In the anterior pituitary gland, the percentage of ERα- and AR-expressing cells was obtained by counting a total number of 1000 cells for each sex. The cells exhibiting clear nuclear staining were considered as positive.

In situ *hybridization*

Frozen sections (10 μm thick) were serially cut at − 20 °C and mounted onto gelatin- and poly-L-lysine-coated slides. *In situ* hybridization with 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_2PO_4 pH 7·4, 1 mM EDTA), 5 × Denhart's buffer, 200 mg/ml denatured salmon testis DNA (Sigma), 200 μg/ml yeast tRNA, 2 μg/ml Poly A (Boehringer-Mannheim, Montreal, Canada) and 4% dextran sulphate. After prehybridization treatment, 100 μl hybridization mixture (prehybridization buffer containing, in addition, 10 mM dithiothreitol and ^{35}S-labelled cRNA probe at a concentration of 20×10^6 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 mg/ml in 2 × SSC) at 37 °C for 30 min, rinsed in decreasing

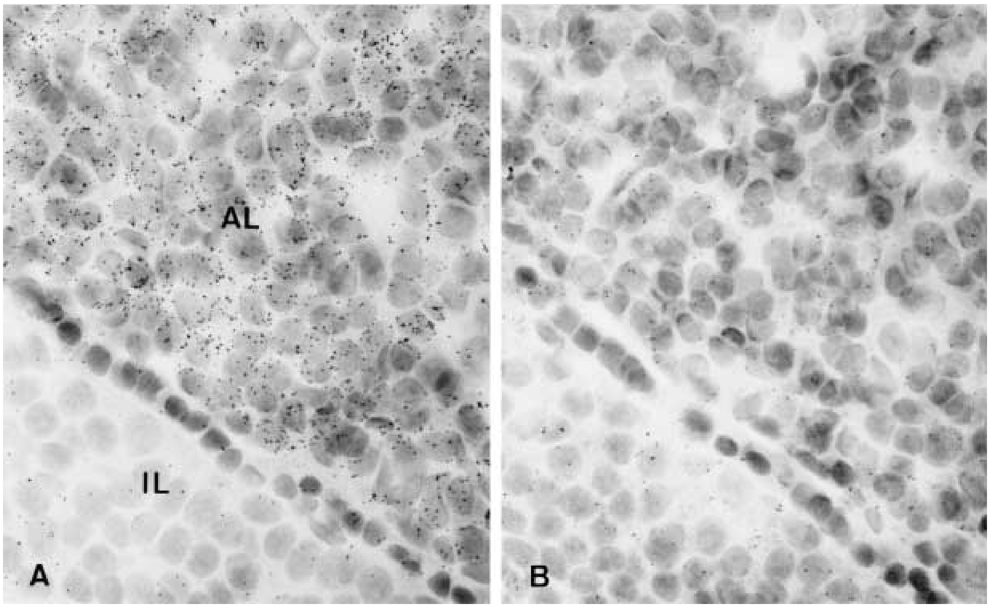

Figure 1 Section through the anterior (AL) and intermediate (IL) pituitary lobe of a male rat. (A) Hybridization with a ^{35}S-labelled ERα antisense probe. In the AL, most of the cells appear to be labelled, while IL cells are unlabelled. (B) Hybridization with a labelled ERβ antisense probe. Only a few dispersed silver grains can be observed. × 620.

concentrations of SSC (2 × SSC and 1 × SSC) 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 exposed onto Kodak X-Omat films 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 haematoxylin and rapidly dehydrated through graded concentrations of ethanol, cleared in toluene and cover-slipped with Permount (Fisher Scientific, Montreal, Canada).

cRNA probe preparation

Specific ERα and ERβ cRNA probes were prepared as previously described (Laflamme *et al.* 1998). Briefly, these probes were generated from their respective linearized rat ER cDNA subcloned into a pBluescript II KS(+) plasmid vector. ERα cDNA templates were linearized with BamHI and HindIII for antisense and sense, whereas XbaI and XhoI were used to linearize antisense and sense ERβ cDNAs respectively. Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 mM $MgCl_2$, 40 mM Tris (pH 7·9), 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0·2 mM ATP/GTP/CTP, 200 μCi [α-^{35}S]UTP (Dupont NEN, Boston, MA, USA), 40 U RNAsin (Promega, Madison, WI, USA) and 20 U of either T3 (antisense probes) or T7 (sense probes) RNA polymerase for 60 min at 37 °C.

Results

In the pituitary gland of both sexes, hybridization signal could be detected only with the ERα cRNA probe (Fig. 1). The autoradiographic reaction was present over the anterior pituitary, the intermediate and posterior lobes being unlabelled. Although a high degree of resolution could not be achieved with the ^{35}S-labelled probe, it could be considered that the majority of the cells in the anterior pituitary were specifically labelled. Hybridization with the labelled ERα sense probe did not generate specific labelling, silver grains being randomly distributed throughout the three lobes (not shown). No sex difference could be observed. Immunostaining performed with an antibody to ERα revealed a nuclear staining in a large number of cells (approximately 90%) in the anterior pituitary (Fig. 2). Weak cytoplasmic staining could be consistently visualized in a few cells. The intermediate and posterior lobes showed no staining. Immunoabsorption of the antibody with the antigen completely abolished the nuclear and cytoplasmic staining (not shown). No immnostaining could be obtained with the antibody to ERβ.

In the anterior lobe, immunolocalization with anti-AR produced nuclear labelling in about 50% of cells (Fig. 3).

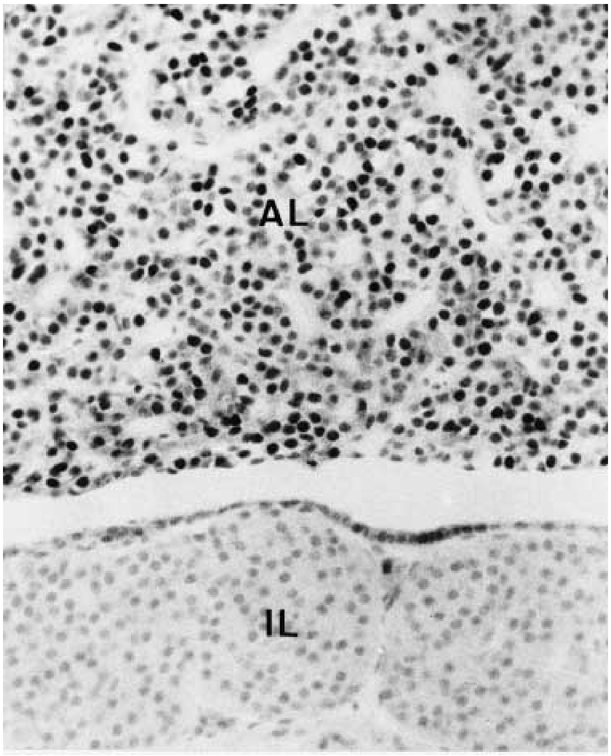

Figure 2 Immunolocalization of ERα in a male rat pituitary. In the anterior lobe (AL), most of the nuclei are stained, while no labelling can be detected in the intermediate lobe (IL). × 300.

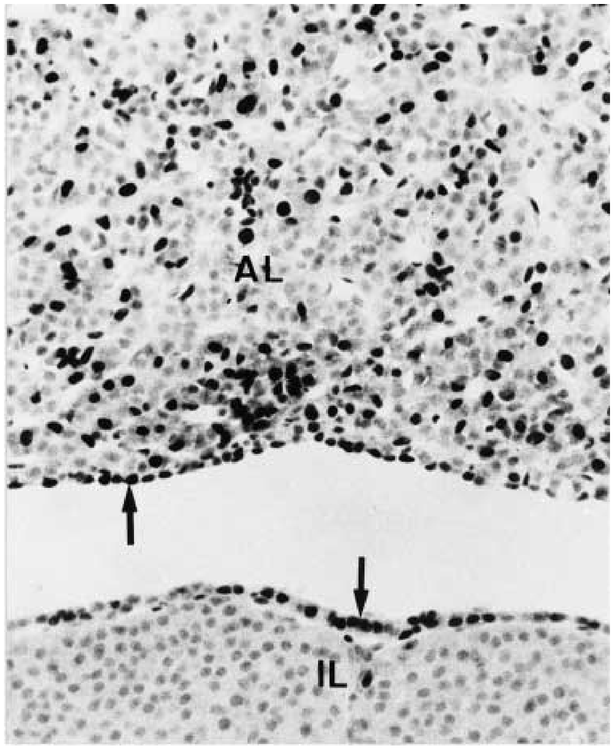

Figure 3 Immunolocalization of AR in a male rat pituitary. In the anterior lobe (AL), several nuclei are immunostained. The nuclei of the epithelial cells lining the pituitary cleft are also labelled (arrows). No labelling can be found in the intermediate lobe (IL). × 300.

Light cytoplasmic staining was also consistently observed in some cells. The nuclei of the epithelial cells lining the pituitary cleft were also stained. No staining was detected in the intermediate and posterior lobes. No difference in staining could observed between male and female pituitaries. Preadsorbed AR antibody did not induce any nuclear or cytoplasmic staining.

Male reproductive organs

In the testis, *in situ* hybridization demonstrated, on X-ray films, a strong signal with the ERα probe (Fig. 4A), and a weak labelling with the ERβ probe (Fig. 4B). At the light microscopic level, the silver grains demonstrating ERα mRNA were associated with the seminiferous epithelium and interstitial cells. In the seminiferous tubules, the grains could be seen over developing spermatids whereas Sertoli cells and germ cells at early stages of differentiation were negative (not shown). Similar results were obtained by immunocytochemistry. ERα immunoreactivity was found in nuclei of Leydig cells as well as in round spermatids and spermatocytes (Fig. 5A). In these germinal cells, the staining was mostly detected in the cytoplasm. Immunostaining for ERβ was detected in nuclei of cells located at the periphery of the tubules, which are likely Sertoli cells (Fig. 6). Similarly, AR immunoreactivity was localized in nuclei of cells, which are presumably Sertoli cells (Fig. 7). Nuclear staining was also present in nuclei of peritubular myoid cells as well as of Leydig cells.

In the prostate, following *in situ* hybridization for ERβ mRNA detection, hybridization signal was associated with the epithelial cells of tubulo-alveoli while the stroma cells appeared to be unlabelled (Fig. 8A). Identical results were obtained with immunocytochemistry. It was not possible to detect ERα expression either by *in situ* hybridization (Fig. 8B) or immunocytochemistry (not shown). ARs were detected in the nuclei of almost all the secretory cells in tubulo-alveoli (Fig. 9). The nuclei of several stromal cells were immunopositive. Endothelial cells in capillaries and larger blood vessels also exhibited nuclear staining.

In seminal vesicles, ERα was detected in the epithelial cells by both *in situ* hybridization and immunocytochemistry (data not shown). Immunostaining was observed in the nucleus and also to a lesser degree in the cytoplasm of epithelial cells. A few stromal cells exhibiting a light nuclear and cytoplasmic staining were consistently observed. No ERβ could be detected by *in situ* hybridization while, by immunocytochemistry, weak nuclear

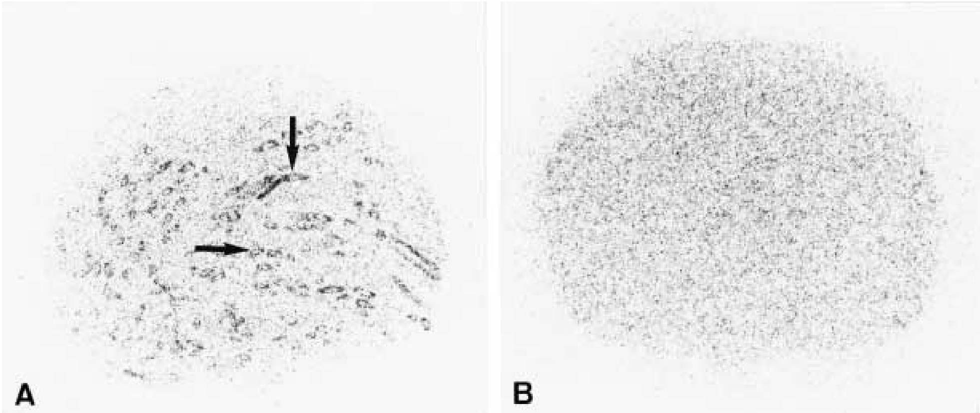

Figure 4 X-ray autoradiographs of a rat testis. (A) Hybridization with a labelled ERα antisense probe. Tubular labelling (arrows) can be observed. (B) Hybridization with a labelled ERβ antisense probe. No specific labelling can be detected.

labelling was detected in nuclei of epithelial cells. Immunostaining for AR revealed nuclear staining in the epithelial and stromal cells.

Female reproductive tract

In the ovary, immunocytochemical studies conducted with the antibodies to ERα revealed that nuclear staining occurred in thecal cells, interstitial gland cells and germinal epithelium (Fig. 10). Granulosa cells in primary, secondary and mature follicles did not exhibit any nuclear staining. Similarly, corpora lutea cells also remained unlabelled. Identical localization of ERα mRNA was obtained by *in situ* hybridization. ERβ mRNA expression was evaluated by *in situ* hybridization. Specific labelling was found in the granulosa cells of growing follicles at all stages from primary to mature follicles, including preovulatory follicles (Fig. 11). Corpora lutea, thecal and interstitial gland cells

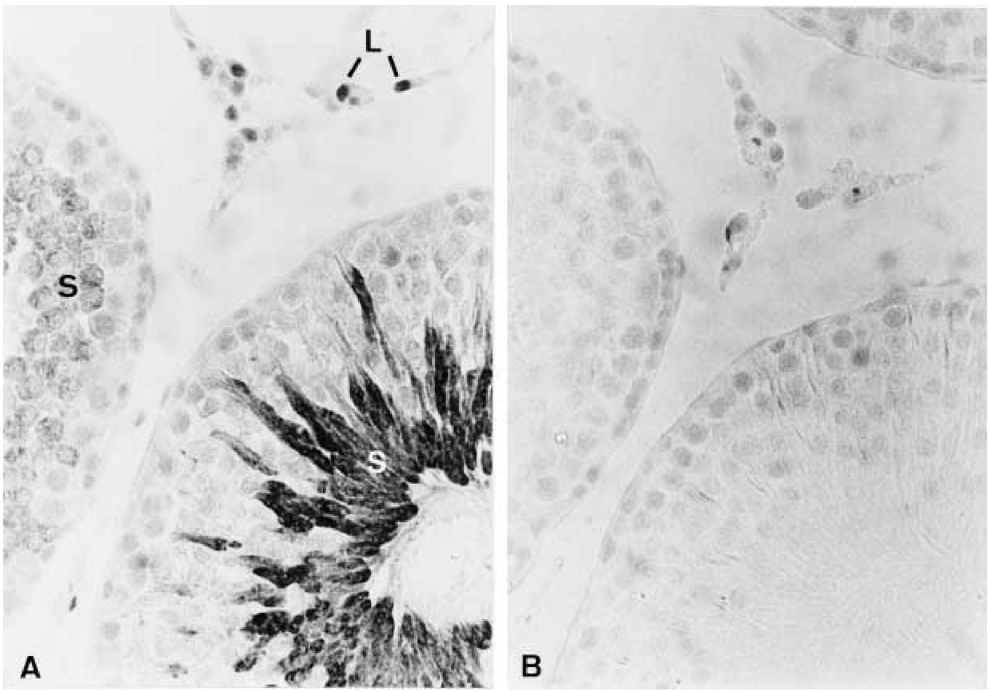

Figure 5 Rat testis. (A) Immunolocalization of ERα. In the tubules, diffuse labelling of spermatocytes (S) can be observed while Sertoli cells are unstained. Nuclear staining can also be detected in Leydig cells (L). (B) Immunoabsorption control. No staining can be detected. × 590.

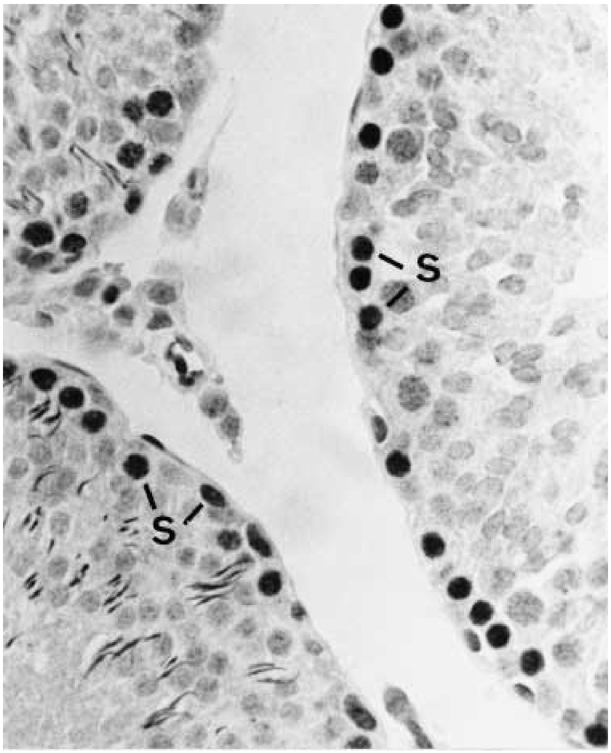

Figure 6 Rat testis. Immunolocalization of ERβ. Nuclear staining is observed in Sertoli cells (S). × 590.

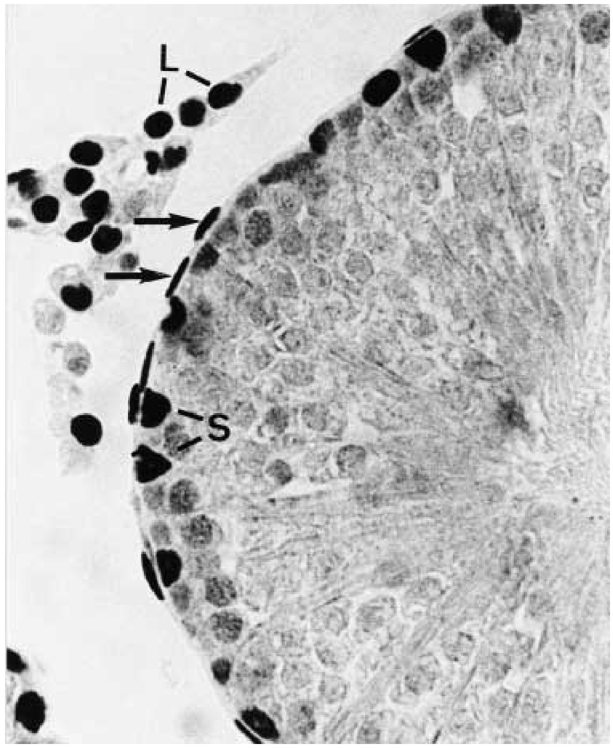

Figure 7 Localization of AR in a section of rat testis. Nuclear staining is present in Sertoli cells (S), peritubular myoid cells (arrows) as well as Leydig cells (L). × 590.

did not exhibit any hybridization signal. The primordial follicles and germinal epithelium also did not appear to express ERβ mRNA. Immunocytochemical studies generated the same results (not shown). AR immunoreactivity was detected in granulosa cell nuclei in primary, secondary and mature follicles (Fig. 12). The primordial follicles and corpora lutea were unstained, but scattered interstitial cells were immunopositive. No staining could be detected in the germinal epithelium.

In the oviduct, the immunostaining for ERα produced a nuclear labelling in the vast majority of the epithelial cells. Staining was also observed in nuclei of muscle cells. In the uterus, nuclear staining was found in both glandular and luminal epithelial cells (Fig. 13). A large number of labelled cells was also observed in the stroma in which glands are embedded. Nuclear staining was consistently detected in muscle cells. By *in situ* hybridization, the same cell types were seen to contain ERα mRNA. No ERβ expression could be detected by either *in situ* hybridization or immunostaining. In the oviduct, immunostaining for AR was observed in the nuclei of epithelial, stromal and muscle cells. In the uterus, AR immunoreactivity was detected in nuclei of both epithelial cells lining the glands and those covering the surface (Fig. 14). A large number of stroma cells in the endometrium and muscle cells also exhibited nuclear staining.

In all the reproductive organs studied, including the pituitary, immunolabelling was completely abolished by immunoabsorption of the antibody with the corresponding antigen (Figs 5B and 12B). Also, *in situ* hybridization with labelled ERα or ERβ sense probe produced weak and diffuse labelling, without any localization to specific structures or cells (Fig. 11B).

Discussion

In the present study, the cellular localization of ERα, ERβ and AR expression in the reproductive tissues of adult male and female rats have been compared. The specificity of the immunostaining was ascertained by preabsorption of the antiserum with the corresponding antigen, and that of the *in situ* hybridization by the use of labelled sense probes as negative controls.

Pituitary gland

In the anterior pituitary gland of rats of both sexes, as evidenced by immunocytochemistry and *in situ* hybridization, only ERα was found to be expressed. There was no evidence for the presence of ERs in the intermediate and posterior lobes. These results are in agreement with a

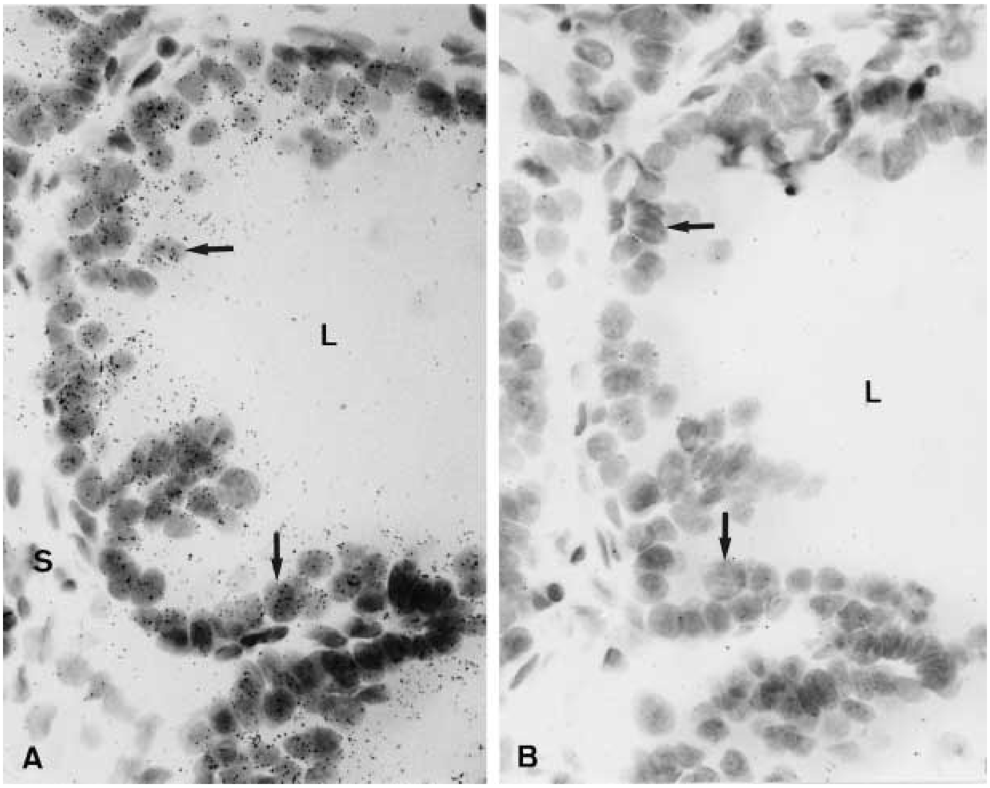

Figure 8 Rat prostate. (A) Localization of ERβ mRNA by *in situ* hybridization in rat prostate. Silver grains are overlying the epithelial cells (arrows) of a tubulo-alveolus. The stroma (S) is devoid of reaction. (B) Localization of ERα mRNA. No significant labelling can be detected in secretory epithelial cells or in stroma. L: lumen. × 620.

previous report indicating that the anterior pituitary expression of ERα is very high while that of ERβ is very low (Kuiper *et al.* 1997). In contrast, Mitchner *et al.* (1998) reported that by combining immunocytochemistry (to localize pituitary hormones and ERα) and *in situ* hybridization (to localize ER mRNAs) ERβ was present in gonadotrophs, lactotrophs, corticotrophs and folliculo-setellate cells at a lower level than ERα. Mitchner *et al.* (1998) also reported that 37% of the intermediate lobe cells were positive for the ERα protein. The finding that the vast majority of secretory cells (90%) in the anterior pituitary contain ERs agrees with previous studies indicating that the secretion of all the pituitary hormones can be directly modulated by oestradiol (Labrie *et al.* 1983).

The localization of AR in about 50% of secretory cells in the anterior lobe is in agreement with a report from Kimura *et al.* (1993) indicating that, in the human pituitary, most of the follicle-stimulating hormone (FSH) and luteinizing hormone cells and some growth hormone cells were immunopositive for AR. In the rat pituitary, AR immunoreactivity was found in nuclei of some secretory cells which were not identified (Sar *et al.* 1990). These receptors are likely to be involved in the direct action of androgens on gonadotrophin secretion, as observed in cultured anterior pituitary cells (Labrie *et al.* 1983).

Male reproductive organ

In the testis, ERα were found to be expressed in Leydig cells and germinal cells, while ERβ could only be detected in Sertoli cells. These results are in agreement with previous results indicating that the expression of ERα was predominant in adult rat testis (Kuiper *et al.* 1997). Paech *et al.* (1997) showed the absence of ERβ in the testis of wild-type and ERα knockout (ERKO) male mice. On the other hand, Saunders *et al.* (1998), using antibodies to a peptide in rat ERβ, found staining in nuclei of Leydig and Sertoli cells and pachytene spermatocytes. The discrepancy between the results from Saunders *et al.* (1998) and our results might be due to some cross-reactivity of the antibodies used by Saunders *et al.* (1998) with ERα or other protein(s), although appropriate controls were conducted by this group. Altogether, the results so far obtained on ER localization in testis suggest that oestrogens might play a role in the regulation of Leydig cell

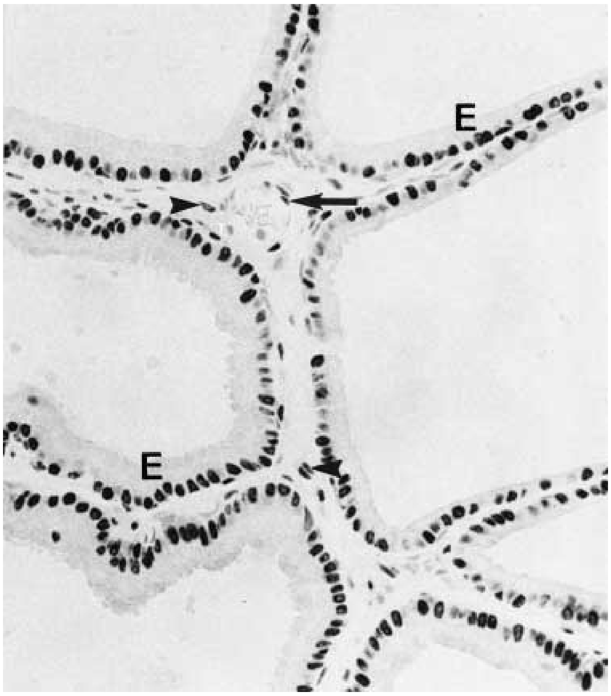

Figure 9 Immunostaining for AR localization in the rat prostate. Nuclear labelling is observed in the majority of epithelial secretory cells (E) of a tubulo-alveolus and in some stromal cells (arrowheads). Endothelial cells (arrow) of a blood vessel are also immunoreactive. L: lumen. × 300.

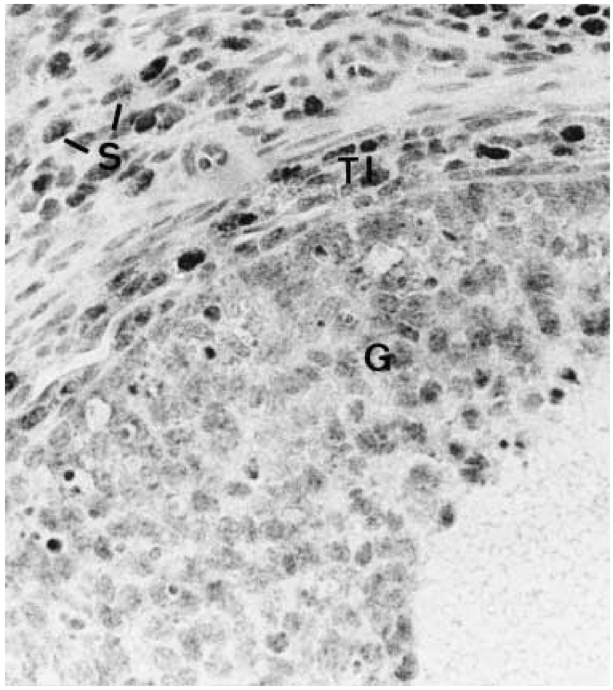

Figure 10 Immunolocalization of ERα in a rat ovary section. Nuclear staining is present in theca interna (TI) and stromal (S) cells. No specific labelling can be observed in the granulosa cells (G) of a growing follicle. × 590.

secretion and might have a direct influence on germ cell maturation. As previously reported by others (Sar *et al.* 1990), we have localized AR in nuclei of Sertoli cells. The presence of AR in Sertoli cells can be related to the role of androgens in the regulation of proteins such as androgen-binding protein which are secreted by the Sertoli cells (Wilson & Griswold 1979). Since Leydig cells, which produce testosterone, also contain ARs, it might be suggested that, in this cell type, androgens exert an intracrine or paracrine activity.

In the prostate, ERβ was found to be highly expressed in the epithelial secretory cells in tubulo-alveoli, while ERα was not detected either by *in situ* hybridization or immunocytochemistry. In the human prostate, Enmark *et al.* (1997) have also detected ERβ mRNA in epithelial secretory cells, the stroma being totally unlabelled. We have also recently reported that in the monkey ERβ mRNA is exclusively expressed in epithelial secretory cells (Pelletier *et al.* 1999). Using RT-PCR, Kuiper *et al.* (1997) have shown that, in the rat prostate, ERβ mRNA was highly expressed, while almost no ERα mRNA could be detected. The very low levels of ERα could explain our failure to detect any ERα expression. These previous findings and the present results could indicate that the ERβ protein is the predominant, if not the only, ER subtype present in the rat prostate. The role of oestrogens in prostate development and function is still unclear. Recently Krege *et al.* (1998) have reported that in 2- to 3-month-old mice lacking ERβ, the histology of the prostate appeared to be normal when compared with age-matched wild-type littermates. The observation that ERα is highly expressed and ERβ poorly expressed in the epididymis is in agreement with a previous report indicating that, in the rat epididymis, ERα mRNA was highly expressed, while the expression of ERβ mRNA was low (Kuiper *et al.* 1997).

In the prostate, nuclear staining for AR was found in epithelial cells of the tubulo-alveoli and stromal cells as well as endothelial cells in capillaries and larger blood vessels. Using immunocytochemistry, Sar *et al.* (1990) could detect AR only in the epithelial cells, the stroma being unstained. Recently, we have reported, in human prostate, the presence of AR in luminal cells of tubulo-alveoli and stromal cells as well as endothelial cells (El-Alfy *et al.* 1999). These results indicate that androgen cannot play a role only in the development and function of the epithelial and stromal cells but may also influence blood vessel development and function. Interestingly, Franck *et al.* (1998) have shown that, in the rat prostate, testosterone induces a rapid response of the vasculature that largely precedes growth of the glandular epithelium. In the epididymis and seminal vesicles, the localization of AR in

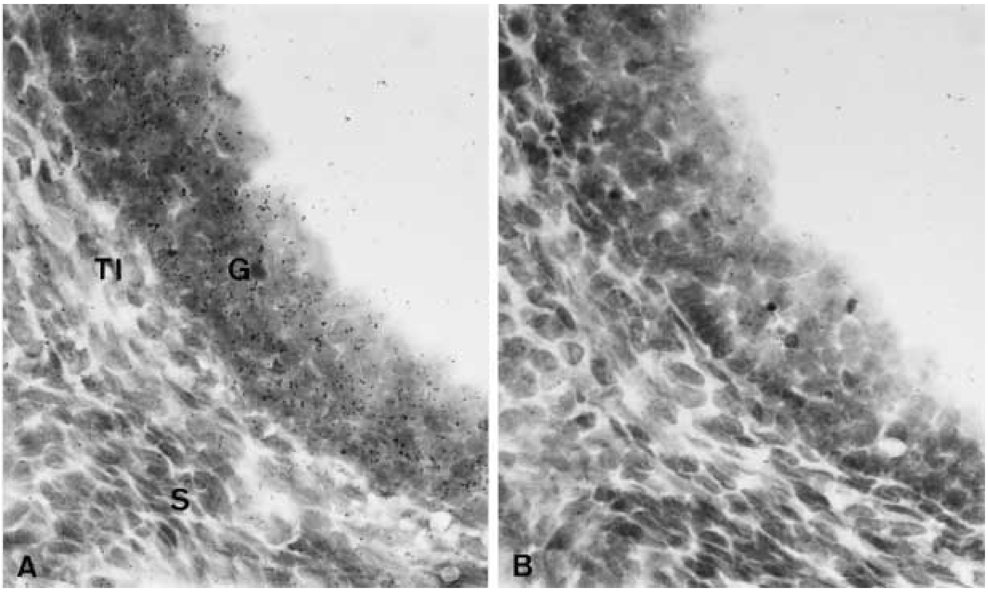

Figure 11 Rat ovary. (A) Hybridization with a labelled ERβ antisense probe. Granulosa cells (G) of a large follicle exhibit hybridization signal. The theca interna (TI) and stromal (S) cells are unlabelled. (B) Hybridization with a labelled ERβ sense probe did not generate any labelling. Only a few dispersed grains can be detected. × 620.

nuclei of epithelial and stromal cells is in agreement with a previous report from Sar *et al.* (1990).

Female reproductive organs

In the ovary, we observed that ERα was not expressed in the granulosa and corpora lutea cells but was detected in thecal cells, interstitial gland cells and germinal epithelium. As visualized by *in situ* hybridization and immunocytochemistry, ERβ was detected in granulosa cells of growing follicles. These results are similar to those recently reported by Fitzpatrick *et al.* (1999) and Sar & Welsch (1999) who used immunocytochemistry to detect the two ER subtypes. The precise role and mechanism of action of oestrogen in the rodent ovary is not well understood. Studies on female mice lacking aromatase, ERα or ERβ

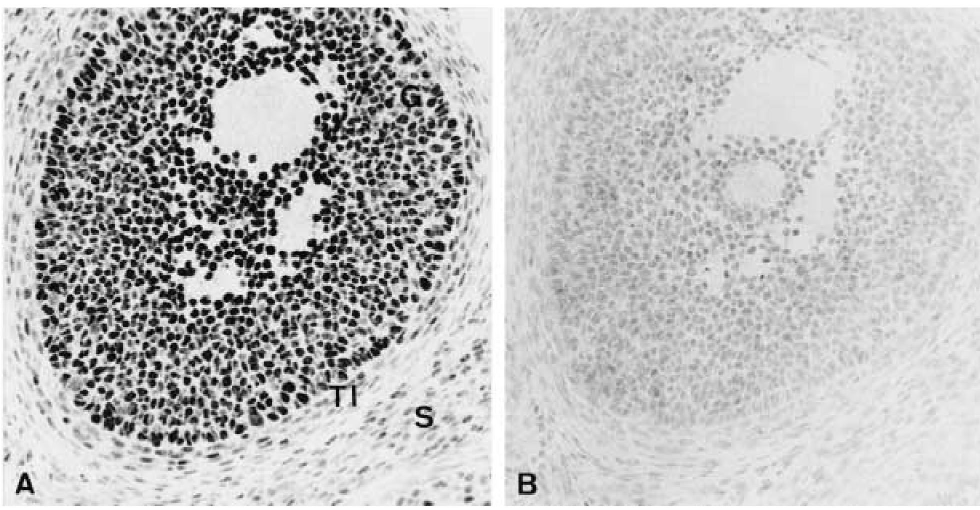

Figure 12 Rat ovary. (A) Immunolocalization of AR. Nuclei of granulosa cells (G) of a growing follicle are strongly stained, while weaker nuclear staining can be observed in theca interna (TI) and stromal cells (S). (B) Control section. Immunoabsorption of the antiserum with the antigen has completely prevented any staining. × 300.

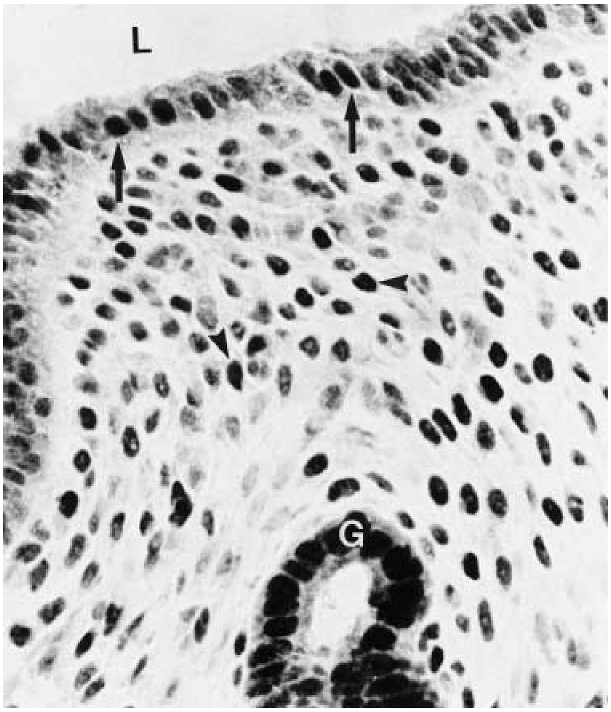

Figure 13 Immunolocalization of ERα in the uterus. Nuclear staining is present in the epithelial cells lining the lumen (arrows) as well as the glandular epithelial cells (G). Stromal cells (arrowheads) also exhibit nuclear staining. L: lumen. × 590.

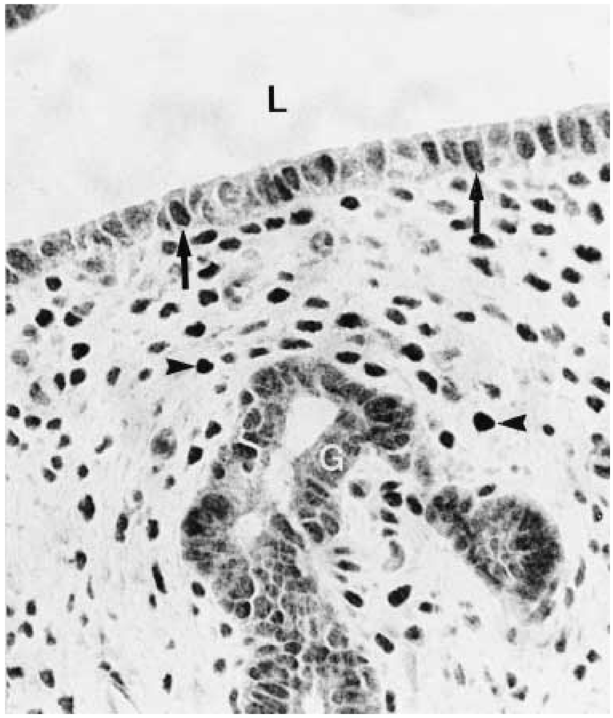

Figure 14 Immunolocalization of AR in the uterus. Nuclear labelling is observed in epithelial cells bordering the lumen (arrows) and glandular epithelial cells (G), Nuclei of stromal cells (arrowheads) are also stained. L: lumen. × 590.

seem to indicate that, for a normal function, the ovary requires the two known ERs (Lubahn *et al.* 1993, Fisher *et al.* 1998, Krege *et al.* 1998).

The present results indicating the presence of AR immunoreactivity in nuclei of granulosa cell in growing follicles and interstitial gland cells are in complete agreement with a previous report from Tetsuka *et al.* (1995) indicating that AR and its mRNA are highly expressed in the granulosa cells of rat ovaries. The finding that AR is located in granulosa cells is consistent with the postulate that thecal androgen is a paracrine modulator of granulosa cell function. It has already been shown that testosterone modulates FSH action in developing granulosa cells through the amplification of cyclic-mediated post-receptor signalling initiated by FSH (Hillier & de Zwart 1982).

In the oviduct and uterus, ERα but not ERβ was found to be expressed in luminal and glandular epithelium as well as in stromal and muscle cells. These findings are very similar to previous findings (Fitzpatrick *et al.* 1999, Sar & Welsch 1999) indicating that, by immunocytochemistry, ERα but not ERβ could be detected in the oviduct and uterus. Hiroi *et al.* (1999) have reported that, in the rat uterus, the nuclei of glandular and luminal epithelial cells were also immunostained with ERα antibodies and that only the nuclei of glandular epithelium cells were stained with anti-ERβ. Since RT-PCR analysis has shown low expression of ERβ mRNA in the rat uterus (Kuiper *et al.* 1997), it is possible that the approaches used in the present study were not sensitive enough to detect low amounts of ERβ at the cellular level. In mice lacking ERβ, the development of the uterus and oviduct appeared to be normal and these deficient mice, although they had reduced fertility, had normal pregnancy and delivery (Krege *et al.* 1998). These results then suggest that ERβ is not essential for the normal functions of the reproductive tract in the female mouse. It is, then, likely that ERα is the ER subtype involved in the mediation of the major effects of oestrogen on the uterus. In fact, in mice deficient in ERα (Lubahn *et al.* 1993), atrophy of the oviduct and uterus has been observed. Moreover, in these deficient animals, oestradiol administration had no effect on uterine weight while, in wild-type animals, it increased uterine weight and induced hyperhaemia in this organ.

The detection of AR in nuclei of epithelial cells as well as in the stromal and muscle cells in the oviduct and uterus agrees well with previous reports indicating similar localization in the human uterus (Kimura *et al.* 1993). In the rat, it has been shown, by *in situ* hybridization, that AR mRNA could be detected in the endometrium and endometrial glands as well as in the myometrium (Hirai *et al.* 1994). Although androgens have been shown to increase uterine weight in the rat (Armstrong & Papkoff 1976), very little is known about the role of androgens in the uterus. The presence of AR in uterine epithelial, stromal and myometrial cells suggests that androgens may exert a direct influence on the development and function of uterus.

The present findings clearly demonstrate a cell-specific localization of ERα, ERβ and AR in the reproductive tissues in the rat of both sexes. They contribute to establish the sites of action of androgens and oestrogens, thus leading to a better understanding of the role of these steroids in reproduction in both sexes. In the female, both ER subtypes appear to be involved in ovarian function while, in the uterus, ERα appears to be the predominant subtype. The presence of AR in granulosa and interstitial cells in ovaries and epithelial, stromal and muscle cells in the uterus strongly suggests that androgens can directly modulate the function of these organs. In the male, the differential localization of ERα and ERβ in the testis and prostate suggests that both ER subtypes are involved in the influence of oestrogens in male reproductive function. The presence of aromatase (Sharpe 1998) in the male genital tract suggests that locally produced oestrogens may play a role more important than that previously thought. In the testis, the localization of AR in the Leydig cells suggests an autocrine or intracrine activity of androgens while the localization of AR in Sertoli cell nuclei can be related to the androgen influence on protein secretion by these cells. The presence of AR in epithelial and stromal cells in the prostate confirms previous findings indicating an androgenic influence on both cellular components.

References

Armstrong DT & Papkoff H 1976 Stimulation of aromatization of exogenous and endogenous androgens in ovaries of hypophysectomized rats *in vivo* by follicle-stimulating hormone. *Endocrinology* **99** 987–991.

Byers M, Juiper GGJM, Gustafsson JA & Park-Sarge OK 1997 Estrogen receptor-b mRNA expression in rat ovary; down-regulation by gonadotropins. *Molecular Endocrinology* **11** 172–182.

Carson-Jurica MA, Schrader WT & O'Malley BW 1990 Steroid receptor family: structure and functions. *Endocrine Reviews* **11** 201–222.

Clark JH, Schrader WT & O'Malley BW 1992 Mechanisms of action of steroid hormones. In *Textbook of Endocrinology*, pp 35–90. Ed. J Wilson. Philadelphia: WB Saunders Company.

El-Alfy M, Luu-The V, Huang XF, Berger L, Labrie F & Pelletier G 1999 Localization of type 5 17β-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase and androgen receptor in the human prostate by *in situ* hybridization and immunocytochemistry. *Endocrinology* **140** 1481–1491.

Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M & Gustafsson JA 1997 Human estrogen receptor b-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism* **82** 4258–4265.

Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* **240** 889–895.

Fisher CR, Graves KH, Parlow AF & Simpson ER 1998 Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. *Proceedings of the National Academy of Sciences of the USA* **95** 6965–6970.

Fitzpatrick SL, Funkhouser JM, Sindoni DM, Stevis EP, Deecher DC, Bapat AR, Merchenthaler I & Frail DE 1999 Expression of estrogen receptor-β protein in rodent ovary. *Endocrinology* **140** 2581–2591.

Franck J, Lissbrant I, Damber JE & Berght A 1998 Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. *Endocrinology* **139** 451–456.

Givalois L, Li S & Pelletier G 1997 Age-related decrease in the hypothalamic CRH mRNA expression is reduced by dehydroepiandrosterone (DHEA) treatment in male and female rats. *Molecular Brain Research* **48** 107–114.

Hillier SG & de Zwart FA 1982 Evidence that granulosa cell aromatase induction/activation by FSH is an androgen receptor regulated process *in vivo*. *Endocrinology* **109** 1303–1305.

Hirai M, Hirata S, Osoda K, Hagibara K & Kato J 1994 Androgen receptor mRNA in the rat ovary and uterus. *Journal of Steroid Biochemistry and Molecular Biology* **49** 1–7.

Hiroi H, Inoue S, Watanabe T, Goto W, Orimo A, Momoeda M, Tsutsumi O, Taketani Y & Muramatsu M 1999 Differential immunolocalization of estrogen receptor α and β in rat ovary and uterus. *Journal of Molecular Endocrinology* **22** 37–44.

Kimura F, Mizokami A, Oconuma T, Sasano H & Nagura H 1993 Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *Journal of Histochemistry and Cytochemistry* **4** 671–678.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor β. *Proceedings of the National Academy of Sciences of the USA* **95** 15677–15682.

Kuiper GGJM, Enmark E, Puelto-Huiko M, Nilsson S & Gustafsson JA 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the USA* **93** 2925–2930.

Kuiper GJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138** 863–870.

Labrie F, Giguère V, Raymond V, Pelletier G, Veilleux R, Côté J & Antakly T 1983 Anterior pituitary cells in culture: a precise assay system for hypothalamic and peripheral hormones. In *Advances in Cellular Neurobiology*, pp 381–406. Eds S Federoff & L Hertz. New York: Academic Press.

Laflamme N, Nappi RE, Drolet G, Labrie C & Rivest S 1998 Expression and neuropeptidergic characterization of estrogen receptors (ERα and ERβ) throughout the rat brain: anatomical evidence of distinct roles of each subtype. *Journal of Neurobiology* **36** 357–378.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS & Smithies O 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences of the USA* **90** 11162–11166.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Shulz G, Umesono K, Blumberg B, Kastner P, Manuel M, Chambon P & Evans RM 1995 The nuclear receptor superfamily: the second decade. *Cell* **83** 835–839.

Mitchner NA, Garlick C & Ben-Jonathan N 1998 Cellular distribution and gene regulation of estrogen receptors α and β in the rat pituitary gland. *Endocrinology* **139** 3976–3983.

Paech K, Webb P, Kuiper GGJM, Wilson S, Gustafsson J-A, Kushner PJ & Scunlan TS 1997 Differential ligand activation of ER-α and ER-β at API sites. *Science* **277** 1508–1510.

Pelletier G, Dupont E, Simard J, Luu-The V, Bélanger A & Labrie F 1992 Ontogeny and subcellular localization of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the human and rat adrenal, ovary and testis. *Journal of Steroid Biochemistry and Molecular Biology* **43** 451–467.

Pelletier G, Luu-The V, Charbonneau A & Labrie F 1999 Cellular localization of estrogen receptor beta (ER-β) mRNA in cynomolgus monkey reproductive organs. *Biology of Reproduction* **61** 1249–1255.

Ruizeveld de Winter JA, Trapman J, Vermey M, Mulder E, Zegers ND & Van der Kwast TH 1991 Androgen receptor expression in human tissues: an immunohistochemical study. *Journal of Histochemistry and Cytochemistry* **39** 927–936.

Sar M & Welsch F 1999 Differential expression of estrogen receptor-β and estrogen receptor-α in the rat ovary. *Endocrinology* **140** 963–971.

Sar M, Lubahn DB, French FS & Wilson EM 1990 Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology* **127** 3180–3186.

Saunders PTK, Fisher JS, Sharpe RM & Millar MR 1998 Expression of oestrogen receptor beta (ERβ) occurs in multiple cell types, including some germ cells, in the rat testis. *Journal of Endocrinology* **156** R13–R17.

Sharpe RM 1998 The roles of oestrogen in the male. *Trends in Endocrinology and Metabolism* **9** 371–378.

Tacha DE & Chen T 1994 Modified antigen retrieval procedure: calibration technique for microwave ovens. *Journal of Histotechnology* **17** 365–366.

Tetsuka M, Whitelaw PF, Bremmer WJ, Millar MR, Smyth CD & Hillier JG 1995 Developmental regulation of androgen in rat ovary. *Journal of Endocrinology* **145** 535–543.

Wilson RM & Griswold MD 1979 Secreted proteins from rat Sertoli cells. *Experimental Cell Research* **123** 127–135.

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