Expression of estrogen receptor-α and -β mRNAs in the male reproductive system of the rat as revealed by in situ hybridization

C N Mowa and T Iwanaga
Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku, Sapporo 060–0818, Japan

(Requests for offprints should be addressed to C N Mowa, Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18-Nishi 9, Kita-ku, Sapporo 060–0818, Japan; Email: Chishimba@lycos.com)

ABSTRACT
We mapped the cellular expression of estrogen receptor (ER) α and ERβ mRNAs in the male reproductive system of the rat during development and adulthood by in situ hybridization. The expression patterns of ERα mRNA in the gonad, efferent duct and initial segment of the epididymis during the perinatal period were essentially similar to those of the adult: ERα mRNA signals were expressed most intensely in the epithelia of the efferent ducts and initial segment of the epididymis, and in the interstitial cells of the testis from the prenatal period to adulthood. However, ERα mRNA signals in the primordial epididymis and vas deferens during the prenatal period were confined to the outermost cellular layer of the ducts, whereas thereafter they were only expressed weakly in the epithelium and stroma of the epididymis and moderately in the muscle layer of the vas deferens. ERβ signals were expressed intensely (1) in primordial germ and Sertoli cells only during the prenatal period, (2) in arterial walls in the adult testis, and (3) in the epithelium of the sex accessory glands from the perinatal period to adulthood. This report is the first to describe the cellular distribution of ER mRNA in the male reproductive organs during the perinatal period, and complements and confirms earlier data on its distribution in the adult. The broad expression of ERs in male reproductive organs suggests roles for estrogen in regulating tissue development and reproductive events.

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INTRODUCTION
One of the primary roles of estrogen is to induce the growth and function of reproductive and neuronal cells that are related to sexual development and behavior (Galand et al. 1971). Produced mainly in the ovary and adrenal gland in the female, estrogen exerts a strong influence over reproductive events (Freeman 1988). Although the presence of estrogen in the male gonad has been well documented for more than 50 years (see review by Carreau et al. 1999), it is only recently that studies on the exact role of estrogen in male reproduction and fertility have been undertaken (Meistrich et al. 1975, Couse & Korach 1999, Hess 2000, Lee et al. 2000). Aromatase, a microsomal enzymatic complex which irreversibly converts androgens into estrogen, has been demonstrated in testicular cells including Sertoli and Leydig cells and, more recently, in germ cells (Janulis et al. 1998, Carreau et al. 1999). Even though the overall production of estrogen by the testis, which is the major source of estrogen in the male, is much less than that produced by the ovaries, the concentration of estrogen in the rete testis and caput epididymis fluids has been reported to be higher than serum estradiol in the female (Hess et al. 1997b). Consequently, the influence of estrogen on male sexuality is expected to be profound (vom Saal 1980, Byskov & Hoyer 1988).

Estrogenic effects on target tissues are principally mediated by estrogen receptors α (ERα) and β (ERβ), members of the steroid/thyroid hormone receptor superfamily, which regulate gene transcription through estrogen-responsive elements (Koike et al. 1987, Kuiper et al. 1996). Investigators have demonstrated the presence of ER using
histological procedures as described below. The older rats were anesthetized by intraperitoneal injection with pentobarbital sodium solution (Nembutal, Abbot, Chicago, IL, USA), 0·5 ml/kg body weight, whereas the neonates were anaesthetized with ether vapor. The animals were treated in accordance with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare, UK.

Probes

Proven ERα and ERβ probes, complementary to 301–346 base pairs of ERα cDNA (Koike et al. 1987) and 45–90 base pairs of ERβ cDNA (Kuiper et al. 1996) respectively, were utilized in this study (Mowa & Iwanaga 2000). The oligonucleotides were labeled with 35S-dATP, using terminal deoxyribo-nucleotidyl transerase (Promega, Madison, WI, USA) at a specific activity of 0·5 × 109 d.p.m./µg DNA.

In situ hybridization

The in situ hybridization procedure was performed as previously described (Mowa & Iwanaga 2000). Briefly, 20 µm cryostat sections were mounted on glass slides, fixed in 4% paraformaldehyde and then acetylated with 0·25% acetic anhydride in 0·1 M triethanolamine-HCl (pH 8·0). The prepared sections were prehybridized for 2 h in a buffer containing 50% formamide, 0·1 M Tris–HCl (7·5), 4 × SSC (1 × SSC = 150 mM NaCl and 15 mM sodium citrate), 0·02% Ficoll, 0·02% polyvinylpyrrolidone, 0·02% bovine serum albumin, 0·6 M NaCl, 0·25% sodium dodecyl sulfate (SDS), 200 µg/ml tRNA, 1 mM EDTA and 10% dextran sodium sulfate. Hybridization was performed at 42 °C for 10 h. The slides were washed in 2 × SSC containing 0·1% sarkosyl (Nacalai Tesque, Kyoto, Japan) and twice at 55 °C in 0·1 × SSC containing 0·1% sarkosyl. The sections were exposed to Hyperfilm-β max (Amersham International, Amersham, Bucks, UK), dipped in Kodak NTB2 nuclear track emulsion and exposed.

The specificity of the in situ hybridization was confirmed by the disappearance of signals when an excess dose of corresponding cold oligonucleotides was added to the hybridization fluid. Consistent ER mRNA signals above background levels were considered positive and scored as strong, moderate or weak.

MATERIALS AND METHODS

Animals

Normal immature and adult Wistar male rats, from fetal day 14 to 90 days after birth, were killed for...
RESULTS

Testis

Initial signals of ERα mRNA in the primordial gonad were diffusely expressed in the mesenchyme by fetal day 14 (Fig. 1A). By fetal day 17 its signals aggregated largely to the interstitial cells, identified as precursor Leydig cells (Fig. 1B). Scattered round cells within the developing seminiferous tubules, presumed to be gonocytes, expressed detectable signals for ERα mRNA from the late prenatal to the late neonatal periods (Fig. 1B,C). By adulthood, testicular ERα mRNA signals were exclusively confined to the Leydig cells (Fig. 1D).

Like ERα mRNA expression, initial signals of ERβ mRNA in the primordial gonad appeared by fetal day 14 and were diffusely distributed. By fetal day 17, intense signals were localized to the cells found within and along the basal lining of the seminiferous tubules, identified as gonocytes and precursor Sertoli cells respectively (Fig. 1E). The interstitial cells during the prenatal period
possessed only weak signals of ERβ mRNA, in contrast to the intense signals located within the seminiferous tubules (Fig. 1E). The signal intensity of ERβ mRNA in the germ and Sertoli cells gradually decreased with development, and the signals were barely detectable in adulthood. Furthermore, the signals in Leydig cells progressively weakened with age, becoming undetectable after birth. However, in adulthood, distinct signals of ERβ mRNA were localized to the walls of arteries located between seminiferous tubules (Fig. 1F).

Reproductive tract

By fetal day 17, distinct and intense signals of ERα mRNA appeared in the epithelium of the mesonephric tubules, which gives rise to the efferent ducts and, presumably, the initial segment of the epididymis (Fig. 2A). In contrast, ERα mRNA expression in the mesonephric ducts, from which the epididymis and vas deferens arise, were limited to the outermost cell layer of the ducts at the onset of their appearance (fetal day 14) (Fig. 2D). ERα mRNA expression in the primordial epididymis and vas deferens gradually spread inwards towards the centrally located epithelium during the perinatal period (Fig. 2E–F). The epithelium in the mesonephric ducts lacked signals of ERα mRNA (Fig. 2D,F). In the adult rat, the expression pattern and intensity of ERα mRNA in the efferent duct and initial segment of the epididymis remained unchanged (Fig. 2B,C), whereas the stroma and epithelia in the rest of the epididymis (Fig. 2G) and smooth muscle layer in the vas deferens (Fig. 2H) expressed weak to moderate signals for ERα mRNA.

ERβ mRNA signals in the mesonephric tubules ducts were weak during the perinatal period and were localized to the stroma but were not in the epithelia. This expression pattern remained unchanged after the differentiation of these structures into the efferent duct, initial segment of the epididymis, epididymis and vas deferens. In the adult rat, ERβ mRNA expression was diffusely localized to the stroma and muscle layer of the efferent ducts, epididymis (Fig. 3B,C) and vas deferens.

Sex accessory glands and urethra

By fetal day 17, distinct and intense signals of ERα mRNA were detectable in the subepithelial stroma of the glandular structures adjacent to the developing bladder (Fig. 2I) and of the urethra (Fig. 2K) respectively. The glandular structures were identified as the primordia of the prostate and seminal vesicular glands, which develop precociously. Following parturition and differentiation, ERα mRNA signals persisted in the stroma and, in some glands, they also extended to the smooth muscle layers of the excretory ducts, but were never localized to the glandular and ductal epithelium. This expression pattern remained unchanged in adulthood. In contrast, the initial signals of ERβ mRNA, recognizable by postnatal day 8, in the sex accessory glands were localized diffusely in the stroma and epithelia of the glands. Similar to ERα mRNA, ERβ mRNA signals in the urethra were localized to the muscle layer and became detectable by fetal day 17 (Fig. 3A). Thereafter, they were observed mainly in the epithelial cells of the seminal vesicles (Fig. 3D), prostate (Fig. 3E), and urethra, bulbo-urethral (Fig. 3F) and urethral glands (Fig. 3G).

A summary of the differential expression patterns of ERα and ERβ mRNAs in the cells of the male reproductive organ of the rat as revealed by in situ hybridization is shown in Table 1 and Fig. 4.

DISCUSSION

ER mRNA expression in the testis

In rodents, spermatogenesis commences soon after the appearance of the seminiferous tubules, around fetal day 15 (Rugh 1988). The undifferentiated gonocytes proliferate and increase in cell size up to fetal days 16–17, and thereafter remain in a quiescent state until after birth (Rugh 1988). The intense prenatal expression of ERβ mRNA in the undifferentiated gonocytes and Sertoli cells, which coincides with the onset of their proliferation, suggests that estrogen may play a role in regulating early germ cell production via ERβ. In support of this idea is the finding that estrogen stimulates proliferation of gonocytes isolated from the testes of postnatal day 3 rats (Li et al. 1997). Similarly, the onset of ERα mRNA expression in interstitial cells of the testis correlates with the beginning of androgen production and the presence of aromatase in these cells (Ojeda & Urbanski 1988). Thus, estrogen may also be involved in regulating steroidogenesis during perinatal development via ERα. Furthermore, the demonstration of aromatase activity and ER mRNAs in both the spermatozoa and the Leydig cells (Levallet et al. 1998) indicates the existence of local estrogen autocrine systems in the testis. Finally, the findings that intratesticular administration of estrogen inhibits testosterone production by Leydig cells and that the initial response of Leydig cells to luteal hormone treatment in the adult rat results in increased secretion of testosterone, followed by a significant
Figure 2. Cellular localization of ERα mRNA in the reproductive tract and sex accessory glands of the rat during development and adulthood. (A) Intense ERα mRNA signals are localized to the epithelia in primordial efferent duct (ed) and initial segment of the epididymis (epd) at fetal day 17. (B–C) In the adult, signals of ERα mRNA are confined to the epithelia (ep) of the efferent duct (B, short straight part of the efferent duct) and initial segment of the epididymis, located at the borders with the efferent duct (C, epithelium type – columnar). (D) Detectable signals of ERα mRNA are first seen at fetal day 14 in the outermost cellular layer (*) of the primordial epididymis and vas deferens, as revealed by the bright (i) and dark-field (ii) images (ep, epithelia; s, stroma). (E, F) At fetal day 17, ERα mRNA signal intensity increases significantly in the serosa (*) of the primordial epididymis (E) and vas deferens (F) and significant signals spread to the stroma (s) (ep, epithelia). (G, H) In the adult, ERα mRNA signals are localized to the epithelia (ep) and stroma (s) of the epididymis (G), and to the smooth muscle cells (m) of the vas deferens (sp, spermatozoa) (H, i, ii). (I) In the sex accessory glands, the first signals of ERα mRNA are observed at fetal day 17 in the stroma (s), not in the epithelium (ep). (J) At postnatal day 24, strong signals of ERα mRNA are detected in the lamina propria (lp) and muscle layer (m) of the sex accessory glands. (K) Signals of ERα mRNA in the primordial urethra are limited to the smooth muscle (m) (fetal day 17). Bar=100 µm (A, C–E, H, J, K), 200 µm (B, F, G, I).
increase in estrogen production, confirms the above conclusion (Saez 1994). It is noteworthy that the differential expression pattern of ER subtypes in the testis is analogous to that seen in the ovary: ERβ is localized to the granulosa cells (oocyte-nurturing cells, i.e. Sertoli cells), whereas ERα is expressed in the theca/interstitial complex (steroid-producing cells, i.e. Leydig cells) (Mowa & Iwanaga 2000).

The differential cellular expression of ERα and ERβ mRNA in the testis, as revealed in this study, is generally consistent with previous immunohistochemical and in situ hybridization studies using rodents, with minor differences (Saunders et al. 1997, 1998, Fisher et al. 1997). In accordance with the results of the present study, the expression of ERβ mRNA was demonstrated in the total RNA of a 14-day postconception mouse testis by ribonuclease protection assay (RPA) (Jefferson et al. 2000). The signal intensities of ERβ mRNA and protein, and ERα mRNA, significantly decreased after postnatal days 5 and 19 respectively (Jefferson et al. 2000). In their earlier study, Saunders and colleagues (1997) failed to detect any significant immunoreactivity of ERβ in the adult testis, while a different research group (Fisher et al. 1998) localized ERα largely in the interstitial cells from the fetal period through to adulthood in rats, in agreement with our observation. Furthermore, similar to the present data, no ERβ immunoreactivity was found in the germ cells of adult testis by Pelletier et al. (2000). On the contrary, some immunohistochemical and in situ hybridization studies have reported expression of ERβ protein and mRNA in the differentiated germ cells of the adult testis (Saunders et al. 1998, van Pelt et al. 1999) and ERα protein in the Sertoli cells, round spermatocytes and developing spermatids of adult rats (Pelletier et al. 2000). Although the cause of this
discrepancy is unclear, the idea that ERβ plays no role in gametogenesis during adulthood is supported by analyses of ERβ-knockout mice, which are fertile and show no apparent morphological and functional abnormalities in the testis (Couse & Korach 1999).

**ER mRNA expression in the efferent duct, epididymis and vas deferens**

The role of estrogen and the expression of its receptors in the efferent duct have been elaborately investigated in the past largely by immunohistochemistry, steroid autoradiography and RT-PCR, notably by the laboratories of Hess (Cooke et al. 1991, Hess et al. 1997a,b, 2000, Hess 2000, Lee et al. 2000). However, data on the cellular distribution of ER mRNA in the rat from efferent duct to vas deferens, particularly in development, are lacking. This study is the first to describe the cellular expression of ER mRNA in the efferent duct, epididymis and vas deferens during development, and extends our knowledge of ER mRNA expression in the adult rat.

Clulow et al. (1994) have shown that the efferent duct concentrates spermatozoa by re-absorbing more than 90% of the fluid released from the testis, a process essential for sperm maturation and storage and, subsequently, crucial for optimal male fertility. Among the factors involved in this process are solutes such as Na⁺ and Cl⁻ in luminal fluids, and the water channel protein, aquaporin-1 (AQP-1), expressed in the epithelium (Clulow et al. 1994, Fisher et al. 1998). In the efferent ducts of the adult, estrogen is thought to regulate fluid re-absorption, in part, by modulating the expression of AQP-1 (Fisher et al. 1998). Thus, loss of ERα function in the ERα-knockout mice leads to low sperm count and marked structural alterations such as decrease in epithelia height, number of cilia, height of microvillus brush border and density of lysosome and endocytotic organelles (Hess et al. 2000). Consequently, these changes interfere with the resorptive functions of the efferent duct (Couse & Korach 1999, Hess 2000, Lee et al. 2000). It is interesting to note that AQP-1 signals, like those of ERα reported here, are intensely expressed in the efferent duct from the prenatal period through adulthood (Fisher et al. 1998). Furthermore, in perinatal rats treated with diethylstilbestrol, AQP-1 immunoreactivity was abolished by postnatal day 18, accompanied by severe dilatation of efferent ducts, suggesting impaired function of fluid re-absorption (Fisher et al. 1999). These

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**Table 1.** Chronological order of appearance and changing patterns of ERα and ERβ mRNA expression in the reproductive organs of the male rat.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Age (days)</th>
<th>ERα</th>
<th>ERβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germ cells</td>
<td>19f, 7p, adult</td>
<td>+/−, +, −</td>
<td>++++, ++, +/−</td>
</tr>
<tr>
<td>Sertoli cells</td>
<td>19f, adult</td>
<td>−, ~</td>
<td>+, +/−</td>
</tr>
<tr>
<td>Leydig cells</td>
<td>17f, 7p, adult</td>
<td>++++, +, ++</td>
<td>+, +/−, ~</td>
</tr>
<tr>
<td>Efferent duct Epithelium</td>
<td>17f, ~</td>
<td>++++, ~</td>
<td>−, ~</td>
</tr>
<tr>
<td>Stroma</td>
<td>17f, adult</td>
<td>+, +/−</td>
<td>+/−, −</td>
</tr>
<tr>
<td>Epididymis Epithelium</td>
<td>14f, adult</td>
<td>−, +</td>
<td>−</td>
</tr>
<tr>
<td>Stroma</td>
<td>14f, 19f, adult</td>
<td>+++, ++++, +</td>
<td>+/-, +/-, +</td>
</tr>
<tr>
<td>Vas deferens Epithelium</td>
<td>14f, ~</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Stroma</td>
<td>14f, 19f, adult</td>
<td>++, ++++, ++</td>
<td>+/-, +/-, +/−</td>
</tr>
<tr>
<td>Sex accessory glands Epithelium</td>
<td>17f, 8p, adult</td>
<td>−, ~</td>
<td>−, +, +++</td>
</tr>
<tr>
<td>Stroma</td>
<td>17f, 8p, adult</td>
<td>++++, 11, ~</td>
<td>−, +/−, −</td>
</tr>
</tbody>
</table>

f=fetal; p=postnatal.

*ER signal strength: +, weak; ++, moderate; ++++, strong; +/−, inconsistent signals;
~ signal unchanged thereafter.

The column under Age indicates when significant ER mRNA signals become detectable; the middle and last figures (where applicable) indicate the age at which ER mRNA signal strength sharply increases and plateaus respectively.
observations, taken together with the present findings, indicate that provision for fluid re-absorption is established long before the onset of sexual maturation. Thus, estrogen may play a role in both the development and function of the efferent duct during the perinatal period. ERα and ERβ have been localized in the developing and mature epididymis and ductus deferens of rodents by previous studies using immunohistochemistry, RT-PCR, RPA and steroid autoradiography, but not by in situ hybridization (Cooke et al. 1991, Greco et al. 1992, Hess et al. 1997a, Jefferson et al. 2000). The data of these studies are largely consistent with the present data. Studies by steroid autoradiography, which detects cells expressing both ER subtypes, demonstrated that fetal mesenchyme accumulates labeled estrogen (Cooke et al. 1991). Following their differentiation into stroma, fibroblasts and smooth muscle cells, the signal pattern changed, with stroma and fibroblasts retaining the signals until adulthood, whereas the smooth muscle cells had weak or no signals (Cooke et al. 1991). The signal pattern of fetal mesenchyme described above may correspond to the pronounced expression of ERα mRNA in the primordial epididymis and vas deferens, as observed in the present study, whereas the stromal signals seen in the adult may correspond to the expression of both ER mRNA subtypes in the stroma of the same structures.

ERα mRNA expression in the epididymis, originally detected in the outermost cell layer of the duct and later in the epithelia and stroma, suggests roles for estrogen in its development and function. The epididymis is a negligible site of sperm concentration in comparison to the efferent duct (Clulow et al. 1994). It is, instead, a site of sperm storage and maturity characterized by accumulation of high concentrations of organic compounds (Clulow et al. 1994). Hence, the presence of ERα mRNA signals in the epididymis implies that...
estrogen modulates secretion of factors that promote the maturity and viability of spermatozoa. This conclusion is consistent with data for ERα-knockout mice, whose spermatozoa lack motility and the ability to fertilize oocytes in vitro (Couse & Korach 1999). Like the epididymis, the initial signals of ERα expression in the vas deferens were localized to the outermost cell layer of the duct. The similarity in the expression patterns between the two ducts may reflect their common ontogenic origin (Rugh 1988). In the adult, the presence of ERα mRNA in the muscle layer of the vas deferens, in accordance with previous immunohistochemical studies, confirms an earlier proposal that estrogen may regulate contraction of the vas deferens, essential for transporting the spermatozoa. Interestingly, our previous study revealed significant expression of ERα mRNA in the muscle coat of the rat oviduct (Mowa & Iwanaga 2000), and physiological studies have demonstrated that estrogen administration accelerates transportation of the oocyte in the oviduct (Harper 1988), possibly via ER expressed in the oviductal muscle coat.

ER mRNA expression in the sex accessory glands

Prins et al. (1997, 1998) have eloquently described in detail the pattern of ERα and ERβ mRNAs/proteins expression in the prostate gland of the rat from birth to adulthood using immunohistochemistry, in situ hybridization and RT-PCR. They, with others, have demonstrated that ERβ mRNA signals are intense in the epithelium of the prostate gland and that this intensity increases with development and age, in contrast to the situation in the stroma where intensity decreases with age (Prins et al. 1998, Pelletier et al. 2000). The present study confirms these earlier findings and provides new data on the expression of ERα and ERβ mRNAs in the primordia of the sex accessory glands, in general, during the prenatal period. However, our data are inconsistent with the findings of Jefferson et al. (2000) who, using RPA, were only able to detect low and varying levels of ERβ mRNA in the seminal vesicle and prostate glands of CD-1 mice between postnatal days 1–26. The cause of the discrepancy is unclear. It is, however, interesting to note that a different study group, which also used mice, found signals in the prostate and bulbourethral glands during development (Cooke et al. 1991).

The seminal vesicles and prostate glands in rodents develop into large structures by fetal day 18 and increase significantly in size after birth (Rugh 1988). The detection of intense ERα signals in the developing accessory glands suggests the involvement of ERα in the growth of these glands. The presence of ERβ in the epithelia of the glands after the neonatal period suggests that the regulatory role of estrogen in their growth and/or secretion is mediated by ERβ. Indeed, ERβ-knockout mice show growth impairment in the secretory portion of the accessory glands, manifested as hyperproliferation which is absent in ERα-deficient mice (Gustafsson 1999). On the other hand, ERα expression in the stroma and later in the excretory ducts of these glands indicates that estrogen may modulate the development of these structures and, following maturation, transportation of their secretory products via ERα.

Pheromones, which are vital for social and sexual intercourse, are released via the urine in adult rats (Sasaki et al. 1999). Other than the preputial glands, the source of pheromones in the reproductive organs is unclear. Since pheromone production is partly influenced by estrogen as well as by testosterone, the intense expression of ERβ in the secretory portion of the urethral gland indicates that this gland may be another source of pheromones.

Our findings of ERα and ERβ mRNA expression in the male reproductive tract confirm observations of earlier steroid autoradiographic, in situ hybridization, RT-PCR and immunohistochemical studies, which demonstrated expression of ER in the efferent ducts, epididymis, sex accessory glands and urethra (Fisher et al. 1997, Hess et al. 1997a, Prins & Birch 1997, Saunders et al. 1997, Prins et al. 1998, Pelletier et al. 2000). Furthermore, this study provides the first report on the onset and expression pattern of ER mRNA subtypes in the developing efferent duct, epididymis, some accessory glands and urethra.

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