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Transcription and Translation of Estrogen Receptor-β in the Male Reproductive Tract of Estrogen Receptor-α Knock-Out and Wild-Type Mice*

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ABSTRACT

Estrogen receptor-α (ERα) has been identified in the male reproductive tract, but the role of estrogen in the male has not been well characterized. *In vivo* mutations in ERα genes have demonstrated the necessity for ERα-mediated action in male fertility. We asked whether both ERβ messenger RNA and protein were present in the male reproductive tract of wild-type and ERα knock-out (ERα KO) mice, and whether ERβ could compensate for the lack of ERα in infertile male ERα KO mice. Immunohistochemical localization with both N- and C-terminal anti-ERβ antibodies demonstrated that ERβ is present in the Leydig cells of the testes and in the epithelium of both the efferent ductules and the initial segment of the epididymis. RT-PCR amplification was used to confirm ERβ transcription in these tissues. In conclusion, we observed that ERβ messenger RNA and protein continue to be expressed in the Leydig cells, elongated spermatids, efferent ductules, and the initial segment of the epididymides of ERα KO mice, but the presence of ERβ is not able to compensate for the absence of ERα in male reproductive function. (*Endocrinology* **139:** 2982–2987, 1998)

RECENT concern over environmental estrogens and their potential for inducing pathological effects within the male and female reproductive systems has resulted in a resurgent interest in the normal physiological actions of estrogens within the reproductive system. Although the multifaceted roles of estrogens within the female reproductive tract have been well studied and characterized, the role of estrogens within the male reproductive tract remains unclear.

In men, mutations in either the aromatase enzyme or the estrogen receptor-α (ERα) gene result in infertility problems (1–3), and targeted disruption of the ERα gene causes sterility in both male and female mice (4, 5). Fluid reabsorption in the efferent ductules and, to a lesser extent, in the initial segment of the epididymis was recently discovered to be under estrogen regulation (6). These findings suggest an absolute prerequisite for estrogen/ERα in normal male reproductive function.

Histopathological examination of testes from young ERα knock-out (ERα KO) mice reveals that there is normal development of the seminiferous tubules. As the animals reach puberty, however, the seminiferous epithelium begins to exfoliate, and marked amounts of fluid accumulate within the seminiferous tubules, rete testis, and efferent ductules (6, 7). The efferent ductules become severely ectatic (dilated), and metaplasia of the lining epithelium subsequently develops as the epithelium degenerates from simple columnar to simple cuboidal epithelium. The dilation and metaplasia of the efferent ductules are consistent with their inability to reabsorb the rete testis fluid.

Past work has demonstrated that ERα is present within the male reproductive tract. Specifically, ERα has been localized to Leydig cells and Leydig cell precursors (8). In addition, ERα immunostaining is observed in the rete testis, the epithelium of the efferent ductules, and sporadically within the epididymis (9–11).

A novel ER (ERβ) has been localized to the testis, ovary, prostate, hypothalamus, bone, and various other internal organs by messenger RNA (mRNA) analyses (12–16). The specific physiological actions of ERβ and its functional interaction with ERα have not yet been resolved, although *in vitro* functional heterodimerization of ERα and ERβ have been reported (17–19). Recently, using a rabbit polyclonal antibody generated against an 18-amino acid stretch within exon 5 of rat ERβ, ERβ was immunohistochemically localized to the Sertoli cells within the rat seminiferous epithelium (20). To more fully understand ERβ and its potential role in male fertility, we used RT-PCR amplification and immunohistochemistry to localize ERβ in Leydig cells, elongated spermatids, efferent ductules, and the initial segment of the epididymis of wild-type (WT) and ERα KO mice. Neither method revealed any qualitative differences in expression of ERβ in ERα KO *vs.* WT tissue.

Materials and Methods

Sample collection and immunohistochemical techniques

ERα KO and WT sibling male mice in a mixed C57BL/6J/129 background were anesthetized with carbon dioxide and killed by cervical

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dislocation. Institutional animal care protocols were followed. Five WT and ERα KO 100- to 110-day-old male mice were used for immunohistochemistry and RT-PCR. The testes, efferent ductules, and epididymides were fixed for histology in Bouin's solution for 12 h. The tissue was then dehydrated in increasing concentrations of ethanol and embedded in paraffin. Sections of 5–6 μm were cut and mounted on poly-L-lysine-coated microscope slides. The tissue was rehydrated in decreasing concentrations of ethanol. The slides were then incubated in 0.05 M glycine-HCl and 0.1 M Tris-buffered saline (TBS; pH 3.5) and heated in a microwave oven at high temperature for 5 min. After washing in 0.1 M TBS, endogenous peroxidase activity was blocked by placing the slides in a 1:10 dilution of 30% hydrogen peroxide-methanol for 30 min. The slides were washed with 0.1 M TBS and incubated in normal goat serum for 30 min to block any nonspecific binding.

The two primary epitope-specific rabbit polyclonal antibodies against ERβ were obtained from Affinity BioReagents (Golden, CO). The N-terminal antibody was generated against a peptide from amino acids 55–70 of the rat ERβ sequence (AEPQKSPWCEARSLEH), and the C-terminal antibody was generated against a peptide from the last 19 amino acids of the rat ERβ sequence (CSSTEDSKNKESSQNLQSQ). The region of rat ERβ from which the N-terminal antibody was generated has the same amino acid sequence as mouse ERβ. There are only three amino acid differences within the C-terminal peptide between mouse and rat that still permit cross-reactivity of the antibody in murine tissues. Western blots and gel mobility shift assays were performed for the both the N- and C-terminal ERβ antibodies (Jurutka, P. W., and M. R. Haussler, manuscript in preparation). Using transfected COS-7 monkey kidney epithelial cells, the C-terminal ERβ antibody detected the protein via Western blots in transfected extracts, but not in untransfected extracts. Gel mobility shift assay revealed that C- and N-terminal ERβ antibody specifically shifted an ERβ-containing complex; preimmune sera and an ERα monoclonal antibody were not able to shift the ERβ-containing complex.

The N-terminal antibody was used at a dilution of 1:500, and the C-terminal antibody was used at a dilution of 1:50. These dilutions were chosen based on multiple preliminary trials in which dilutions spanning 1:50 to 1:1000 for both antibodies were used. The respective antibodies were placed on the tissue and incubated overnight at 4 C in a humidified chamber. Unbound primary antibody was washed off the tissue with 0.1 M TBS buffer (pH 7.4). The tissue was incubated with antirabbit IgG secondary antibody (Vectostain kit, Vector Laboratories, Burlingame, CA) for 30 min. The secondary antibody was washed off the tissue with 0.1 M TBS buffer (pH 7.4). Avidin and biotin from the Vectostain kit were mixed and incubated on the tissues for 30 min. Peroxidase was detected by a mixture of 3,3′-diaminobenzidine (DAB; Dako Corp., Carpinteria, CA) and 0.03% hydrogen peroxide. The slides were counterstained for 1 min with Gill's hematoxylin, dehydrated, and coverslipped with Permount (Fisher, Fairlawn, NJ). Photomicrographs were digitized using a Nikon microscope (Nikon Corp., Melville, NY) attached to a Sony ccd iris RGB camera (Sony, Tokyo, Japan). Images were digitalized using Image I software (NIH, Bethesda, MD); they were compiled using Adobe Photoshop 3.0 for Macintosh and printed with a Mitsubishi Codotonic dye sublimation printer (Mitsubishi, Tokyo Japan).

Leydig cell purification

Mouse Leydig cells from the testes of groups of 10 ERα KO and WT mice were dispersed enzymatically with collagenase and dispase (21). Seminiferous tubule elements were removed by filtration through two layers of 100-μm pore size nylon mesh, and the preparation was further purified by centrifugal elutriation at 2000 rpm and a flow rate of 16 ml/min to eliminate sperm and other germ cells. The final fraction of the purified Leydig cells was obtained after centrifugation through Percoll, collecting cells with a buoyant density of 1.070 g/ml or greater. Assessment of purity was performed by histochemical staining for 3β-hydroxysteroid dehydrogenase (an enzyme that is specific to the Leydig cells), which was typically 95% or greater.

Total RNA isolation

Testes, epididymides, and purified Leydig cells from ERα KO and WT sibling mice were rapidly frozen in liquid nitrogen and then stored at −80 C. RNA was isolated using guanidine thiocyanate and phenol/chloroform extraction (Tri-Reagent, Sigma Chemical Co., St. Louis, MO). RNA was reconstituted in 50 μl diethylpyrocarbonate-treated water and then stored at −80 C. The quality of the RNA was checked by agarose gel electrophoresis and quantitated spectrophotometrically.

RT-PCR amplification protocol

One microgram of total isolated RNA was reverse transcribed to complementary DNA. RT-PCR amplification was carried out using the Titan one-tube RT-PCR system kit (Boehringer Mannheim, Indianapolis, IN). Each reaction tube contained (final concentrations) 0.2 mM deoxy-NTPs (Promega), 5 mM dithiothreitol (Boehringer Mannheim), 5 U RNasin (Promega), 1 × RT-PCR buffer with 1.5 mM Mg^{2+} mix (Boehringer Mannheim), and enzyme mix, AMV, and Expand High Fidelity PCR-system (Boehringer Mannheim). The RT reaction was carried out at 42 C for 30 min. Touchdown PCR, which spanned from 68–50 C for 40 cycles, was used (22). A second generation of PCR was performed that had a predwell at 94 C for 1 min, followed by 15 cycles of 94 C for 30 sec, 58 C for 30 sec, and 72 C for 1 min, and ending with a postdwell at 72 C for 5 min. A heminested reverse primer in both the N- and C-terminal regions was used in combination with the same forward primer used in the first generation. The amplified DNA was fractionated electrophoretically on a 2% agarose gel, then stained with ethidium bromide and visualized under UV light. The gels were digitized using a Mitsubishi image capture system.

Primers

Primers for both the N- and C-terminal regions of ERβ primers were designed based on the mouse ERβ sequence (17, 23). ERβ gene intron/exon splice sites and exon numbers were predicted based on the conserved gene structures of ERα and other steroid receptors (24–26). The splice sites between exons 2 and 3 as well as those between exons 8 and 9 were confirmed by PCR amplification of complementary DNA and genomic DNA and by sequencing (data not shown). The location and sequence of the primers used are listed in Table 1.

Results

Immunohistochemical localization of ERβ

Positive immunohistochemical staining for ERβ was present within the Leydig cells of testes of 110-day-old WT and ERα KO sibling mice (Fig. 1, A and C). Within these cells, immunoreactive staining was detected within the cytoplasmic, perinuclear, and nuclear regions. The Leydig cells in the ERα KO appeared to be hypertrophied, which is compatible with the 2-fold increase in testosterone that has been previously reported (5). Using the N-terminal antibodies, staining was also present in the elongated spermatids (Fig. 1I) in WT,

TABLE 1. The table indicates primers used for RT-PCR amplification, including their exon location, sequence, and the generation in which they were used

Name of primer	Location of primer	Sequence of primer	PCR generation(s)
mER-β16forward	Exon 2 of mER-β	5′-CATTCTACAGTCCTGCTGTGATGA-3′	1st and 2nd
mER-β246reverse	Exon 3 of mER-β	5′-GGGTCTCTCTGTTTACAGGCAA-3′	2nd
mER-β264reverse	Exon 3 of mER-β	5′-CGCCAAGCTTCCTCTTCAGGGT-3′	1st
mER-β1285forward	Exon 9 of mER-β	5′-AACAAGGGCATGGAACATCTGCT-3′	1st and 2nd
mER-β1471reverse	Exon 9 of mER-β	5′-CGTCACTGTGACTGGAGGTTCTG-3′	2nd
mER-β1488reverse	Exon 9 of mER-β	5′-TCCGCCTCCAGCCTGGCCGTCA-3′	1st

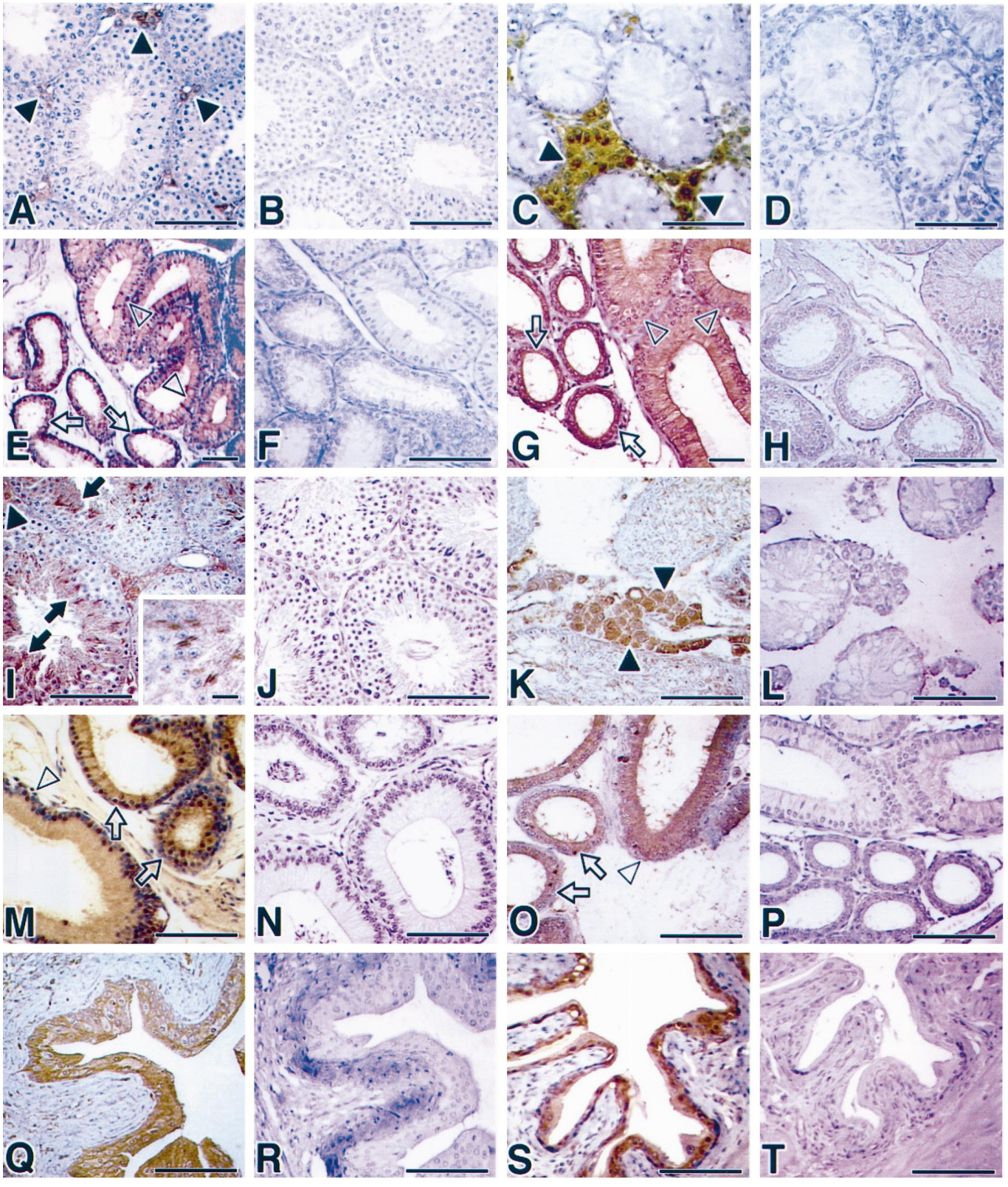

FIG. 1. Immunohistochemical staining of ERβ in WT and ERα KO tissues using both N- and C-terminal ERβ antibodies from Affinity BioReagents. A–H, The C-terminal ERβ antibody was used; I–T, the N-terminal ERβ antibody was used. Photomicrographs on the *left* (A, B, E, F, I, J, M, N, Q, and R) are from WT mice. Photomicrographs on the *right* (C, D, G, H, K, L, O, P, S, and T) are from ERα KO mice. A and C, ERβ staining is present in the Leydig cells (*solid arrowhead*) of both WT (A) and ERα KO (C) testes, respectively. B and D, When the C-terminal peptide is previously incubated with the C-terminal ERβ antibody, no staining is present in the Leydig cells in serial sections of WT and ERα KO testes, respectively. E and G, The efferent ductules (*open arrow*) and initial segment of the epididymis (*open arrowhead*) of WT (E) and ERα

but not ERα, KO mice. Within the efferent ductules, moderate amounts of immunoreactive staining for ERβ were present in the cytoplasm of the nonciliated and ciliated epithelial cells (Fig. 1, E, G, M, and O). Staining was also present in the cytoplasm of the pseudostratified simple columnar epithelial lining of the epididymis in both WT (Fig. 1, E and M) and ERα KO (Fig. 1, G and O). In the WT efferent ductules and initial segment of the epididymis, the epithelial cells of the efferent ductules and initial segment had more nuclear staining than the ERα KO tissues. The immunoreactive staining was judged to be specific based on the lack of significant DAB staining after incubations of the primary antibody with competing amounts of N- or C-terminal peptides (Fig. 1, B, D, F, H, J, L, N, and P). Additionally, DAB staining was not detected in sections that were incubated with rabbit preimmune serum alone or in sections that were not exposed to the primary antibody (data not shown). The urinary bladder, in which copious ERβ mRNA and protein expression have been identified (13, 14, 20), was used as a positive control tissue. The transitional epithelium of the urinary bladder was more intensely stained than the submucosa or muscularis externa using the N-terminal antibody in WT and ERα KO males (Fig. 1, Q and S). This staining was not present in peptide-competed sections (Fig. 1, R and T). Similar staining of the transitional epithelium of WT and ERα KO urinary bladders was seen using the C-terminal antibody (data not shown).

RT-PCR localization for ERβ mRNA

To avoid potential artifacts from genomic DNA contamination, RT-PCR amplification of ERβ across intron/exon boundaries was performed. RT-PCR amplification of ERβ in the testes and epididymides of ERα KO and WT sibling mice revealed the expected PCR product of approximately 230 bp for the N-terminal primers (Fig. 2A, lanes 1–4). The C-terminal primers resulted in the expected approximately 186-bp band in the WT testes, ERα KO testes, WT epididymis, and ERα KO epididymis (Fig. 2B, lanes 1–4). Signal intensities were not measured, but no qualitative differences could be detected in the band intensities between WT and ERα KO. No amplification was observed in samples that lacked RNA (Fig. 2, A and B, lane 7). No amplified product was present in RNA samples that were RT-PCR amplified without AMV reverse transcriptase (data not shown). Additionally, to further eliminate the possibility of contaminating DNA, the samples were pretreated with ribonuclease-free deoxyribonuclease before the RT enzyme was added. To verify the identity of the amplified PCR product, the bands were sequenced and confirmed to be mouse ERβ.

To further sublocalize the transcription of ERβ within the testis, WT and ERα KO Leydig cells were purified, and total RNA was isolated for subsequent RT-PCR. The RT-PCR conditions were the same as those used for the testis and epididymides. Primers from both the N- and C-terminal regions were used as before. As demonstrated in Fig. 2A, lanes 5 and 6, the expected 230-bp band (using the N-terminal primers) was present in both the WT and ERα KO Leydig cells. Using primers in the C-terminal region, an approximately 186-bp band product was detected in both the WT and ERα KO Leydig cells (Fig. 2B, lanes 5 and 6). This is consistent with the immunohistochemical data, which localized ERβ to the Leydig cells of the testes. These bands were excised, sequenced, and verified to be mouse ERβ.

Discussion

The somewhat surprising presence of both cytoplasmic and nuclear staining for ERβ protein within the Leydig cells and efferent ductular and initial segment epithelial lining raises intriguing questions about what ERβ is doing in these cells and where ERβ may be acting within these cells. Recently, using the C-terminal ERβ antibody from Affinity BioReagents, both nuclear and cytoplasmic stainings were also observed in specific neurons within the brain (27). Cells within the lateral septum, CA1 and CA2, positively stained for ERβ within the perikarya and in the cell processes.

It has been demonstrated using immunohistochemical and/or immunogold staining that ERα can be present in both the cytoplasm and nucleus of human mammary carcinoma cells, rabbit endometrial epithelial cells, and MCF-7 cells (28–30). Different fixation techniques may also play a role in the apparent subcellular immunohistochemical staining of ER (31).

A recent study using a rabbit antirat ERβ antibody generated from exon 5 of the rat demonstrated positive staining in Sertoli cell nuclei (20). However, in contrast with the present data in the mouse, these researchers stated that although there was interstitial staining, it appeared to be nonspecific. Thus, they could not determine whether the Leydig cells or peritubular myoid cells were positive for ERβ. Using the N- and C-terminal ERβ antibodies that were generated from exons 2 and 9, respectively, we found Leydig cell staining in both WT and ERα KO mouse testes, which was confirmed by RT-PCR analysis.

One potential reason for these different findings may be species variation between mouse and rat in the cellular localization of ERβ, as has been observed in ERα immunolocalization in rat and marmoset monkey male reproductive tracts (10). Neonatal Leydig cells from both rat and marmoset were immunopositive for ERα. Adult rat Leydig cells were strongly positive for ERα, whereas adult marmoset Leydig cells were only weakly positive for ERα. Additionally, ERα

KO (G) mice stain positive for ERβ. F and H, C-Terminal ERβ peptide competition resulted in no staining for ERβ in serial tissues run in parallel to those incubated with the primary antibody. I and K, The N-terminal ERβ antibody verified that ERβ is present in the Leydig cells (*solid arrowhead*) of both WT (I) and ERα KO (K) animals. Additionally, in the WT testes (I), the elongated spermatids and spermatozoa (*solid arrow*) stain positively with the N-terminal antibody, but no staining is seen in the sections incubated with the C-terminal antibody. The high magnification *inset* in I shows the staining of the elongated spermatids. J and L, No staining is present in previously N-terminal ERβ peptide-competed serial sections. M and O, The N-terminal antibody demonstrated positive ERβ staining in the efferent ductules (*open arrow*) and initial segment of the epididymis (*open arrowhead*) of WT (M) and ERα KO (O) mice. N and P, N-Terminal peptide competition resulted in no staining in the efferent ductules and epididymis of WT and ERα KO mice, respectively. Q and S, Using the N-terminal ERβ antibody, positive staining was present in the transitional epithelial cells of the urinary bladder in both WT (Q) and ERα KO (S) mice. R and T, No staining was present in urinary bladder serial sections of WT (R) and ERα KO (T) mice that were incubated with N-terminal peptide-competed ERβ antibody. Magnification: E and G, *bar* = 90 μm; I (*inset*), *bar* = 15 μm; A and the remaining photomicrographs, *bar* = 100 μm.

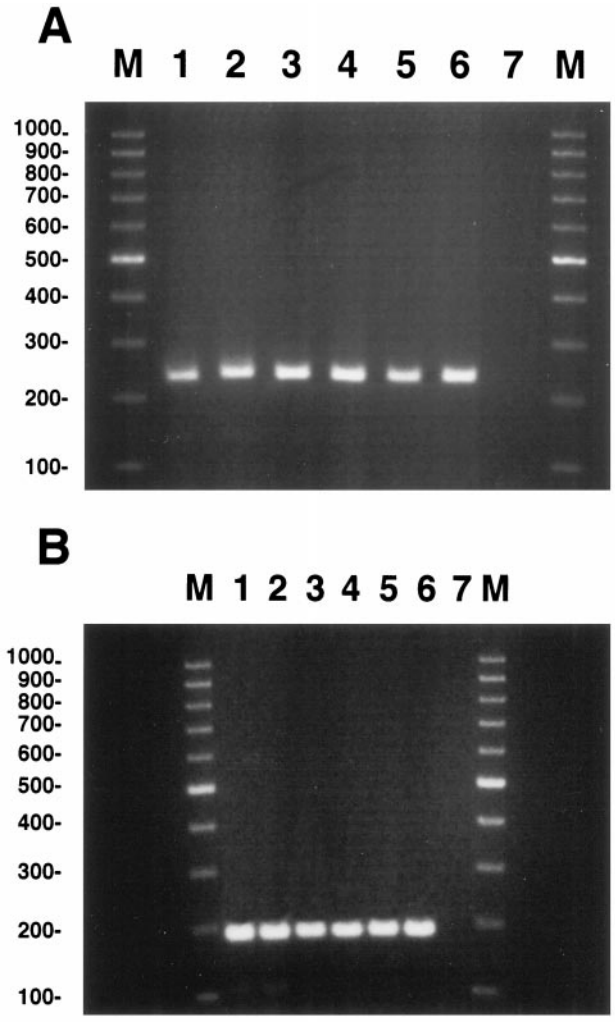

FIG. 2. RT-PCR amplification for ERβ using primers generated based on mouse N-terminal (A) and C-terminal (B) sequences. The marker (M) is a 100-bp ladder. A and B: Lane 1, WT testis; lane 2, ERα KO testis; lane 3, WT epididymis; lane 4, ERα KO epididymis; lane 5, WT Leydig cells; lane 6, ERα KO Leydig cells; lane 7, no RNA negative control. A, Using primers generated from the N-terminal region of mouse ERβ, the expected 230-bp band is present in lanes 1–6 but not in the negative control lane (7). B, Using primers generated from the C-terminal region of mouse ERβ, the expected 186-bp band is present in lanes 1–6 but not in the negative control lane (7).

was immunolocalized to rat rete testis and efferent ductules, but in the marmoset only the efferent ducts were positive.

Similar to the rat (20), the transitional epithelium of the urinary bladder in the mouse stained positively for ERβ. In the rat, the muscularis externa of the urinary bladder also stained strongly positive for ERβ (20). However, when we used both the N- and C-terminal ERβ antibodies, little or no staining of the muscularis externa was detected in the mouse urinary bladder.

The elongated spermatids demonstrated ERβ staining with the N-terminal ERβ antibody, but not the C-terminal antibody. There are several possible reasons for this difference in antibody binding. The first is that there may be novel alternate spliced forms of ERβ within the testes in addition to those previously identified in the ovary, pituitary, and various other tissues (23, 32–34).

Alternatively, this difference in spermatid staining using the N- *vs.* the C-terminal ERβ antibodies may be due to differences in affinities of the N- and C-terminal ERβ antibodies. This affinity difference may exist because of sequence variation within the peptide antigen between rat and mouse. This possible difference in affinities combined with potentially lower concentrations of ERβ in the elongated spermatids may also account for the staining differences. Finally, the N-terminal ERβ spermatid staining may simply be the result of binding to another unrelated protein with a common epitope. Further developmental studies are underway to confirm the presence of ERβ protein in elongated spermatids.

No qualitative difference was detected in either ERβ mRNA or protein concentration in ERα KO *vs.* WT tissues. No difference was detected using ribonuclease protection assay for ERβ mRNA expression in WT *vs.* ERα KO mice (35). This suggests that ERβ alone cannot maintain normal male reproductive function. As it has been shown that ERβ preferentially heterodimerizes with ERα (17–19), the possibility thus exists that ERβ may not exert significant physiological action without ERα.

Although ERβ concentrations are similar in both genotypes, there seems to be a difference in the subcellular localization of ERβ in the efferent ductules and epididymis of WT *vs.* ERα KO mice. In ERα KO animals, cytoplasmic staining is present, but nuclear staining is scant to absent, whereas in the epithelium of the WT efferent ductules and epididymis, ERβ staining is present in the nuclei and cytoplasm. This differential subcellular localization is not observed in the testes and urinary bladder. We do not have an explanation for this finding. It is interesting to speculate that perhaps ERα may be needed, either directly or indirectly, for ERβ localization or retention in the nucleus in certain cell types.

Estrogen is produced in the male reproductive tract and may exert local effects in the male reproductive system (36). In the prepubescent animal, the Sertoli cells are the main source of estrogen via aromatase conversion of testosterone (37). As the animal matures, the Leydig cells and spermatozoa become the main sources of estrogen within the testes (38, 39). As the sperm traverse the excurrent duct system, there is a decrease in their P450 aromatase activity (40). This suggests that the estrogens synthesized by the sperm act within the efferent ductules and/or the epididymis.

It has been demonstrated that prenatal exposure to estrogen in mice results in Leydig cell tumors and/or adenoma formation (41, 42). As most tumors are derived from premature or primordial cells, estrogen may potentially prevent differentiation of the progenitor Leydig cells and/or result in the Leydig cells regressing back to a dedifferentiated state, which would make them more susceptible to uncontrolled division and subsequent neoplastic transformation. This work, performed on normal and neoplastic Leydig cells, suggests that estrogen may act in an autocrine and/or paracrine manner via ERα and/or ERβ within the Leydig cells.

In this report we have positively identified, via immunohistochemistry and RT-PCR amplification, ERβ in some of the same male reproductive tissues and cells as ERα. The exact function and regulators of ERβ need to be further determined, although it is now clear from *in vitro* studies that ERβ may regulate specific genes differently from ERα (43). Based on our

findings, it does not appear that ERα regulates ERβ concentrations in the murine male reproductive tract. As ERβ has a proclivity to heterodimerize with ERα, additional work needs to be performed to resolve the roles of ERα and ERβ in conjunction with one another and the individual functions of the receptors. Potentially, each receptor may play different roles at various stages of life and in various tissues. Taken together, the immunohistochemical and RT-PCR amplification data indicate that there is qualitatively no difference in the mRNA and protein expression of ERβ in WT *vs.* ERα KO mice. Thus, ERβ alone does not appear to be capable of maintaining normal reproductive function in ERα KO mice.

Note Added in Proof

While this manuscript was in press, an additional reference was published which immunohistochemically localized ERβ to rat fetal and adult Leydig cells and to various rat testicular germ cells including intermediate and B-type spermatogonia, pachytene spermatocytes between stages III and XII, and the cytoplasm of secondary, dividing spermatocytes (stage XIV). [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. J Endocrinol 156:R13–R17].

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