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Tissue Distribution and Quantitative Analysis of Estrogen Receptor-α (ERα) and Estrogen Receptor-β (ERβ) Messenger Ribonucleic Acid in the Wild-Type and ERα-Knockout Mouse

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ABSTRACT

Until recently, only a single type of estrogen receptor (ER) was thought to exist and mediate the genomic effects of the hormone 17β-estradiol in mammalian tissues. However, the cloning of a gene encoding a second type of ER, termed ERβ, from the mouse, rat, and human has prompted a reevaluation of the estrogen signaling system. Based on *in vitro* studies, the ERβ protein binds estradiol with an affinity similar to that of the classical ER (now referred to as ERα) and is able to mediate the effects of estradiol in transfected mammalian cell lines. Essential to further investigations of the possible physiological roles of ERβ, and its possible interactions with ERα, are data on the tissue distribution of the two ER types. Herein, we have described the optimization and use of an RNase protection assay able to detect and distinguish messenger RNA (mRNA) transcripts from both the ERα and ERβ genes in the mouse. Because this assay is directly quantitative, a comparison of the levels of expression within various tissues was possible. In addition, the effect of disruption of the ERα gene on the expression of the ERβ gene was also investigated using the ERα-knockout (ERKO) mouse. Transcripts encoding ERα were detected in all the wild-type tissues assayed from both sexes. In the female reproductive tract, the highest expression of ERβ mRNA was observed in the ovary and showed great variation among individual animals; detectable levels were observed in the uterus and oviduct, whereas mammary tissue was negative. In the male reproductive tract, significant expression of ERβ was seen in the prostate and epididymis, whereas the testes were negative. In other tissues of both sexes, the hypothalamus and lung were clearly positive for both ERα and ERβ mRNA. The ERKO mice demonstrated slightly reduced levels of ERβ mRNA in the ovary, prostate, and epididymis. These data, in combination with the several described phenotypes in both sexes of the ERKO mouse, suggest that the biological functions of the ERβ protein may be dependent on the presence of ERα in certain cell types and tissues. Further characterization of the physiological phenotypes in the ERKO mice may elucidate possible ERβ specific actions. (*Endocrinology* **138:** 4613–4621, 1997)

TARGET TISSUES for estrogen have been traditionally defined as those possessing the classical estrogen receptor protein (now referred to as ERα) as detected by high affinity binding assays with radiolabeled-estradiol as well as the demonstration of a measurable physiological response upon exposure to the hormone. Mammalian tissues known to possess detectable levels of ERα include the tissues of the female and male reproductive tracts, the female mammary gland, bone, the cardiovascular system, and regions of the brain. The estrogen/ERα signaling system is known to play critical roles in the female, especially in the normal functions of the reproductive tract, the development of secondary sex characteristics, and in normal reproductive behavior. Recent descriptions of definitive phenotypes in the male mouse after targeted disruption of the ERα gene (1, 2) as well as in a human male that is homozygous for a natural mutation of the ERα gene and subsequently estrogen resistant (3) have now indicated critical roles for the estrogen signaling system in the male. Furthermore, both natural and synthetic estrogens, presumably acting through the ERα protein, have been implicated in the initiation and maintenance of neoplastic events, especially in the tissues of the uterus, ovary, and mammary gland (4).

Until recently, ERα was thought to be the only form of nuclear receptor able to bind estradiol and ultimately mediate its hormonal effects in the normal physiological processes of the mammal. This is in contrast to other members of the nuclear receptor superfamily in which multiple forms are known to exist, such as for the thyroid, retinoic acid, and progesterone receptors (5). However, the recent descriptions of a gene encoding a second type of estrogen receptor, termed ERβ, in the rat (6), mouse (7), and human (8) has prompted a reexamination of the estrogen signaling system. The ERβ protein is smaller than the ERα but possesses the modular structure of distinct functional domains (A–F) characteristic of the members of the superfamily of nuclear receptors. When compared with ERα, the protein sequence of the mouse ERβ demonstrates considerable homology in the DNA and ligand binding domains (6–8). Relative binding studies on *in vitro* translated protein have shown that the ERβ

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is able to bind 17β-estradiol with an affinity similar to that of ERα (8, 9). Transactivation studies using an estrogen responsive reporter construct transfected into the mammalian cell lines CHO, Hela, and Cos-1 have shown that the ERβ is able to mediate the effects of 17β-estradiol in a dose-dependent manner, although levels of induction were slightly lower than those obtained with ERα (6–8). Furthermore, these same studies demonstrated that the estrogen stimulated transactivation was specific to estradiol (6) and could be significantly reduced by the addition of the known ERα antagonists, hydroxy-tamoxifen (6, 7), ICI-182780, raloxifene (7), and ICI-164384 (8).

Knowledge of the distribution of ERβ in various tissues is limited at this time. In the rat, the highest levels of ERβ messenger RNA (mRNA) as detected by *in situ* hybridization were reported in the granulosa cells of primary, secondary, and mature follicles of the ovary as well as in the prostate epithelium (6). A recent report also demonstrated the use of *in situ* hybridization to detect ERβ mRNA in several regions of the anterior hypothalamus of the female rat (10). In the human, ERβ transcripts were detected by Northern blot analysis in the testis, ovary, and thymus (8). In the mouse, ERβ transcripts were not detected in the liver, heart, kidney, skeletal muscle, thymus, spleen, and brain when assayed by Northern blot, indicating that these tissues are either negative for expression or that ERβ mRNA levels exist below the level of detection using this technique (7). However, a more thorough study of the tissue distribution of ERβ is essential to continued investigations of its functions and importance to the whole estrogen signaling system. Herein, we describe an RNase protection assay (RPA) designed to detect and distinguish mRNA transcripts from both the ERα and ERβ genes in the mouse. This assay is directly quantitative, and therefore a comparison of the expression levels of the ERα and ERβ mRNAs within various tissues was possible. In the adult mouse, the highest level of ERβ mRNA was observed in the ovary of the female, the prostate and epididymis of the male, followed by the hypothalamus and lung in both sexes. In addition, a possible role for ERα in the regulation of the ERβ gene would be difficult to study in the wild-type (WT) mouse based on the known biological properties of each. Therefore, we have employed the transgenic ERα-knockout (ERKO) mouse, previously described as homozygous for a disruption of the ERα gene (11–13), to investigate the effect of the lack of functional ERα on the expression pattern of the ERβ gene. ERKO mice demonstrated slightly decreased levels of ERβ mRNA in the ovary, epididymis and prostate, whereas no altered expression levels or patterns of ERβ appeared in the other tissues of either sex when compared with WT litter mates.

Materials and Methods

RNA isolation

All procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All animals used were between the ages of 3 and 8 months. In cases of ovariectomy, females were used 14 days post surgery. After euthanasia, tissues were removed quickly and snap frozen in liquid nitrogen, followed by storage at −70 C until processing. All ovaries were trimmed of oviduct and capsule before freezing to reduce the level of cross-contamination by these surrounding tissues. All tissues were from individual animals with the exception of the oviduct, which was pooled from several animals of the same genotype and of similar age. Total RNA was isolated using TRIZOL reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol. Concentrations of the final preparations were calculated from an A_{260} reading using a Beckman DU-7 spectrophotometer. An aliquot of all RNA preparations was then analyzed on a 1% agarose gel to ensure integrity before further analysis.

Cloning of the ERβ complementary DNA (cDNA) probe template

A 262-bp cDNA fragment of the mouse ERβ gene was amplified from WT ovary RNA by RT-PCR. All RT-PCR reagents were purchased from Perkin-Elmer (Norwalk, CT) and all reactions carried out in a GeneAmp 9600 Thermal Cycler (Perkin-Elmer). The RT reaction was prepared according to the manufacturer's protocol using random hexamers, 0.5 μg of total WT ovary RNA, and scaled up to 50 μl per reaction. PCR was then carried out using the following primers specific for ERβ (bp numbers refer to the rat ERβ sequence (6), GCG accession no. U57439): forward (bp +454; 5′ TTCCCGGCAGCACCAGTAACC 3′) and reverse (bp +695; 5′ TCCCTCTGTTTGCGTTGACTAG 3′). The PCR reaction consisted of 5 μl of the cDNA preparation in a 25 μl reaction including 100 pmol of each primer, deoxy (d)-NTPS at 0.2 mM each, Invitrogen Optimized PCR buffer I (San Diego, CA) at 1× concentration, and 1.5 U UlTma DNA polymerase (Perkin-Elmer), a thermostable DNA polymerase with proofreading capability. Thermal cycling conditions consisted of an initial 95 C/2 min followed by 35 cycles at 95 C/30 sec, 58 C/45 sec, 72 C/30 sec; followed by a final incubation of 72 C/7 min. The amplified ERβ cDNA fragment was then cloned into the *Srf*I site of the pCR-Script SK(+) phagemid (Stratagene Cloning Systems, La Jolla, CA) according to the manufacturer's protocol such that transcription with T3 RNA polymerase would generate the antisense strand.

RNase protection assay

Sense and antisense riboprobes were generated from linearized templates using the Maxiscript kit (Ambion, Austin, TX), the appropriate RNA polymerase (T3 or T7), and the incorporation of [^{32}P]-CTP (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. The mouse ERα antisense riboprobe was 490 nucleotides (nt) in full length and produced a specific protected fragment of 366 nt as previously described (14). The mouse ERβ antisense riboprobe was generated from the cloned cDNA fragment described previously and was 318 nt in full length and generated a protected fragment of 262 nt. An antisense riboprobe specific for mouse cyclophilin, used to equate loading among lanes, was generated from the template pTRI-Cyclophilin (Ambion) at a full-length of 165 nt and produced a protected fragment of 103 nt.

For all RPA reactions 5×10^4 cpm of each probe, sample RNA, and yeast transfer tRNA (for a final total RNA equal to 25 μg) were mixed and ethanol precipitated at −70 C for 3 h to overnight. The resulting pellets were then processed through the RPA using the Hybspeed RPA kit (Ambion) according to the manufacturer's protocol. Final analysis of protected fragments was carried out by electrophoresis on a 1.5-mm thick, 6% bis-acrylamide/8.3 M urea/1× TBE gel (National Diagnostics, Atlanta, GA) that was then fixed, dried, and exposed to a phosphorimager screen followed by exposure to x-ray film. All RPA results were analyzed with the aid of a PhosphorImager Storm 860 and accompanying ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Results

Optimization of the RPA for ERα and ERβ transcripts

The RPA is a sensitive and effective assay for the detection of specific mRNA transcripts within a total RNA sample. Because one or more radiolabeled riboprobes is present in great excess during the time allowed for hybridization, the RPA allows for direct quantification of the levels of target mRNA species. An additional advantage of the RPA is the ability to simultaneously assay for the presence of several distinct transcripts in the same hybridization reaction by the

use of a cocktail of specific riboprobes. However, this is dependent upon the satisfaction of certain requirements, these being 1) the final protected probe fragments must be sufficiently different in size to allow for resolution by gel electrophoresis, and 2) the riboprobes must not cross-hybridize and thereby cause spurious background bands as well as limit the amount of riboprobe available for hybridization to the target mRNA.

The ER antisense riboprobes used in this study were specific for coding regions in the mRNA of each ER type as shown in Fig. 1. Radiolabeled sense riboprobes of the same respective sequences resulted in no protected fragments when incubated with WT ovary RNA or yeast transfer RNA (data not shown), indicating the specificity of each of the antisense riboprobes for their respective mRNAs. To determine if the designed antisense riboprobes for the mouse ERα and ERβ transcripts, as well as the riboprobe for cyclophilin (used for normalization among samples) satisfied the requirements described above, the assay was optimized using WT mouse ovarian RNA as a target. The ovarian RNA used for optimization was isolated from tissue pooled from several adult WT mice (and therefore is not one of the the same preparations assayed and shown in Fig. 3) and was chosen because it was known to possess relatively high levels of both receptor transcripts. As shown in Fig. 2, the riboprobes for ERα and ERβ were effective in detecting their respective transcripts when used separately. Furthermore, when the two riboprobes were combined in the same hybridization reaction, there was no compromise in the ability of each to fully detect their respective transcripts. Also, because the two resulting protected fragments differ in size by slightly over 100 nt, they are easily resolved by electrophoresis in a denaturing 6% bis-acrylamide gel system.

Distribution of ERα and ERβ mRNA in the female reproductive tract and mammary gland

The distribution of the mRNAs encoding the two ER types in the reproductive tract and mammary gland of female WT and ERKO mice is shown in Fig. 3. The ERα transcript was present at significant levels in all four of the WT tissues analyzed, with the uterus showing the greatest concentration. The WT ovary possessed the highest level of ERβ mRNA among the assayed tissues of the female reproductive tract (Fig. 3). This is in agreement with previous reports for the rat (6). Among sexually mature WT female mice, the average ratio of ERβ to ERα mRNA was approximately 5:1 and demonstrated a wide range of 0.3–12 (Table 1). This is most likely related to the stages of folliculogenesis present at the time of collecting the tissue. The animals used in this particular analysis were not assessed for their specific stage in the ovarian cycle, and therefore this information is not available. The levels of ERβ mRNA in ovaries from adult ERKO females appeared to be slightly lower than that in the WT mice, and more importantly showed a much more narrow range compared with WT mice (Table 2).

Although ERα was the predominant transcript in the uterus and oviduct, the ERβ transcript was detectable in these tissues, at a level at or below 5% of that in the ovary. This was also true for tissues of the cervix and vagina of WT mice (data not shown). However, mammary glands from adult females were negative for ERβ expression. The ERKO

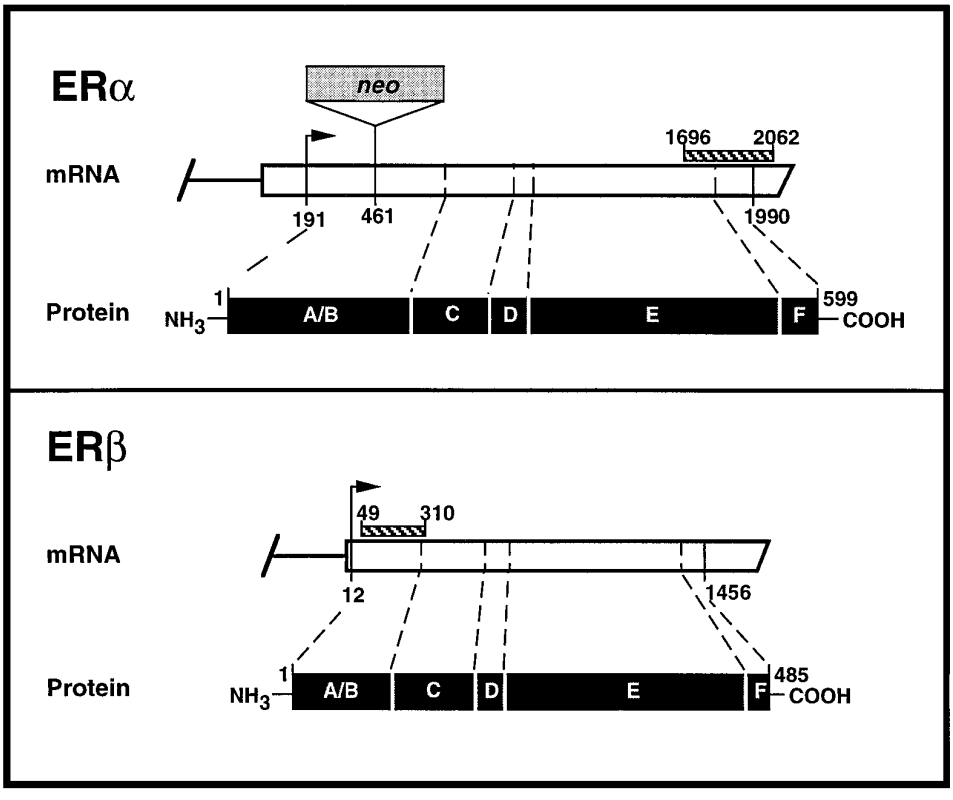

FIG. 1. Schematic drawing of encoding ERα and ERβ mRNA and proteins. The coding sequences for the mRNAs for each of the mouse estrogen receptors are shown as well as the approximate locations of those sequences encoding the specific modular domains of the receptor proteins. The antisense riboprobes for each mRNA are shown (*slashed bar*). The ERα antisense riboprobe was specific for bp 1696–2062, which encode a portion of the E and all of the F domains of the ERα protein. The ERβ antisense riboprobe was specific for bp 49–310, which encode approximately all of the of the A/B domain of the ERβ protein.

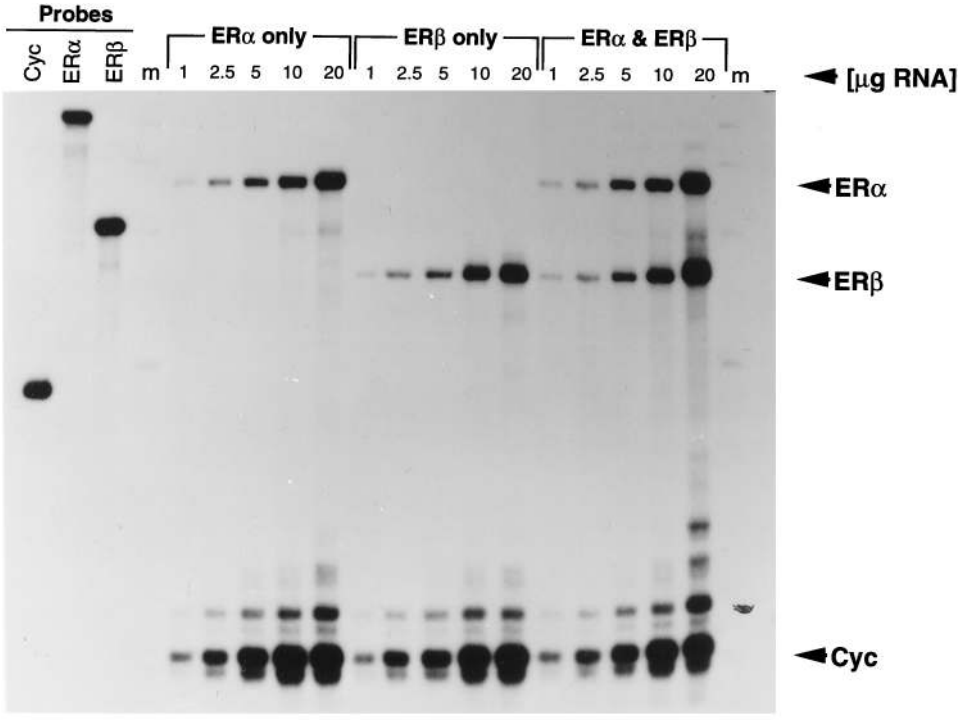

FIG. 2. Optimization of the RPA for detection of ERα and ERβ transcripts. Increasing amounts of WT ovarian RNA (isolated from tissue pooled from several animals) was hybridized with either the ERα riboprobe, ERβ riboprobe, or a cocktail of both riboprobes. The riboprobe specific for mouse cyclophilin (Cyc) mRNA was included in all reactions for normalization purposes. All antisense riboprobes were labeled by the incorporation of [^{32}P]-CTP as described in *Materials and Methods*. The full-length riboprobes are shown to the *left* and are as follows: ERα at 490 nt and protected a specific fragment of 366 nt; ERβ at 318 nt and protected a specific fragment of 262 nt; and Cyc at 165 nt and protected a specific fragment of 106 nt. Markers (m) are as follows: (nt) 500, 400, 300, and 200.

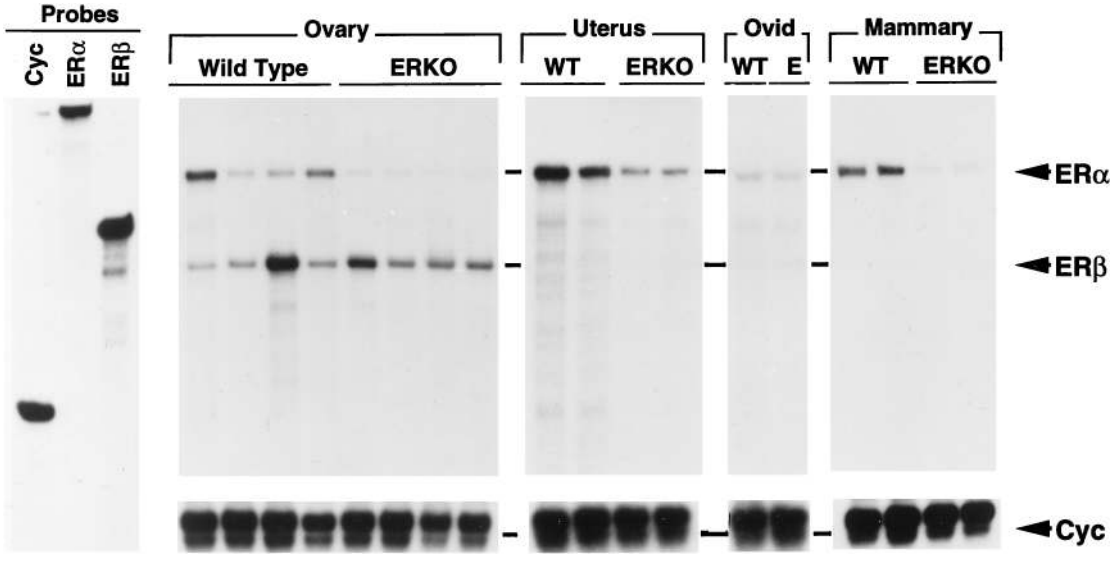

FIG. 3. Analysis of ERα and ERβ transcripts in the adult female reproductive tract and mammary gland by RPA. Total RNA was analyzed for the presence of the ER transcripts as described in *Materials and Methods*. Each lane represents the analysis of 10 μg RNA from individual females, except for the oviduct (Ovid) which was RNA from tissue pooled from several adult females of the same genotype. Each ERα genotype, WT or ERKO, was assayed in duplicate, except for the ovary, in which four females of each genotype are shown. Levels of the Cyc transcript were used for normalization between samples. All exposure times are equal for the panels showing the detected ER riboprobe fragments.

females showed no altered levels of ERβ expression in these tissues.

Although the ERKO mouse is homozygous for a targeted disruption of the ERα gene, tissues of the female reproductive tract as well as others to be presented below appear to possess detectable levels of ERα mRNA. It is important to note that the antisense riboprobe used in this study is specific for sequences located downstream from the site of the disrupting *neo* construct (see Fig. 1). The presence of ERα mRNA in the ERKO has been well described (12) and is due to transcriptional read through of the *neo* poly A signals, resulting in continuation to the termination signals innate to the ERα gene. However, the coding sequences of the resulting ERα transcripts in the ERKO mouse are disrupted by the presence of multiple premature stop codons within the disrupting construct, with the exception of a single splicing variant detectable only by RT-PCR and previously described (12). A similar phenomenom of transcriptional read-through and aberrant splicing has been reported in the transforming growth factor-α-targeted mice in which a comparable tar-

TABLE 1. Average ratio of ERβ to ERα mRNA in various tissues of the WT mouse

Tissue	ERβ/ERα in WT			
	n	Mean	(±)	Range
Reproductive tract				
Ovary	10	5.32	3.9	0.26–11.92
Epididymis	7	0.96	0.2	0.67–1.21
Prostate	3	0.95	0.3	0.77–1.25
Hypothalamus				
Female	7	0.48	0.1	0.38–0.74
Male	6	0.51	0.1	0.43–0.58
Lung				
Female	3	3.11	2.1	1.80–5.49
Female (ovariectomized)	5	2.92	0.5	1.94–3.74
Male	9	2.65	1.2	1.36–4.35

All values were calculated by the use of a PhosphorImager Storm 860 and accompanying ImageQuant software as described, in the *Materials and Methods* section. The (±) refers to the SD for each sample group.

TABLE 2. Average ratio of ERβ mRNA in ERKO tissues to ERβ mRNA in wild-type tissues of the mouse

Tissue	ERKO ERβ/Wild Type ERβ			
	n	Mean	(±)	Range
Reproductive tract				
Ovary	9	0.61	0.2	0.41–0.91
Epididymis	6	0.59	0.2	0.32–0.89
Prostate	2	0.72		0.66–0.78
Hypothalamus				
Female	8	1.01	0.5	0.61–1.95
Male	6	1.30	0.2	0.98–1.58
Lung				
Female	5	0.95	0.3	0.68–1.28
Female (ovariectomized)	4	0.99	0.2	0.83–1.23
Male	4	1.44	0.1	1.36–1.54

Shown is the average of those values calculated by dividing each individual value for ERβ mRNA in the ERKO samples by the average value for ERβ mRNA among the WT samples within the same tissue. Therefore, the data can be interpreted as such: >1.0, ERβ levels are higher in the ERKO samples; = 1.0, ERβ levels are equivalent between genotypes; <1.0, ERβ levels are higher in the WT samples. All values were calculated by the use of a PhosphorImager Storm 860 and accompanying ImageQuant software as described in the *Materials and Methods* section. The (±) refers to the SD for each sample group.

geting strategy was used (15). Despite the presence of these transcripts, the ERKO mice have been documented to be resistant to the actions of estradiol by several biochemical assays and have demonstrated many of the phenotypes expected to result from complete estrogen insensitivity (11–13, 16, 17).

Distribution of ERα and ERβ mRNAs in the male reproductive tract

The distribution of the mRNAs encoding the two ER types in the reproductive tract of WT and ERKO male mice is shown in Fig. 4. Once again, significant levels of ERα mRNA were detected in all the tissues analyzed. However, only the prostate and epididymis possessed detectable levels of ERβ mRNA, whereas the testes were negative. As shown in Table 1, the ERβ:ERα mRNA ratio in WT male mouse epididymis and prostate was approximately 1. However, in the ERKO males, the levels of ERβ appeared to have decreased in both the epididymis and prostate when compared with the WT (Table 2). The intermediate band that appears in the ERKO epididymis samples is due to DNA contamination of the RNA preparation and can be removed with pretreatment of the RNA samples with DNase I (data not shown).

Distribution of ERα and ERβ mRNAs in the pituitary and neural tissue

All assayed brain tissues of the female (Fig. 5) and male (Fig. 6) mouse expressed detectable levels of ERα mRNA, with the pituitary possessing the highest level in both sexes. There were no detectable levels of ERβ in the pituitary of mice of either sex. However, the hypothalamus did possess significant levels of ERβ mRNA in both the female and male, appearing to be the highest among the neural tissues assayed. As shown in Table 1, the levels of ERβ mRNA were consistent and approximately half that of ERα in the WT mice of both sexes. Detectable levels of ERβ mRNA also appeared in the cortex and olfactory bulb of each sex although at very low levels. As shown in Table 2, no effect of disruption of the ERα gene on ERβ levels was observed in the ERKO mice of either sex. Once again, the intermediate band that appears in several of the samples is due to DNA contamination of the RNA preparation and can be removed with pretreatment of the RNA samples with DNase I (data not shown).

As noted earlier, disrupted ERα mRNA is present in tissues of the ERKO mouse. Interestingly, expression of the ERα gene in the hypothalamus, olfactory bulb, and cortex of the ERKO mouse does not appear to be as attenuated when compared with levels in other ERKO tissues. Although the ERKO ERα mRNA is disrupted, these data indicate that transcription of the ERα gene in the tissues of the brain may be dependent on mechanisms that are not affected by the disrupting *neo* construct to the degree of those existing in the reproductive tract tissues.

Distribution of ERα and ERβ mRNAs in nonreproductive organ systems

Among the tissues of the nonreproductive organ systems that were assayed, the lung possessed the highest levels of ERβ mRNA in both sexes (Fig. 7). Although transcripts for each type of ER were present, ERβ mRNA was clearly predominant in the lung with an average ERβ:ERα ratio of 3.1 and 2.6 in WT females and males, respectively. In an attempt to determine if the ERβ gene may be regulated in the female lung by ovarian factors, mice of both WT and ERKO genotypes were ovariectomized for two weeks before tissue collection; however, no significant effect was seen (Table 1). The levels of ERβ in the lungs of ERKO mice did not appear to vary from that seen in the WT mice (Table 2).

In the tissues of the cardiovascular system, ERα mRNA was clearly detectable in the heart and aorta of female (Fig. 8) and male mice (data not shown). The heart of both sexes (male not shown) did possess slightly detectable levels of ERβ mRNA after extended exposure of the gels to x-ray film. The aorta of both sexes (male not shown) was clearly negative for ERβ expression when assayed by RPA. No genotypic effect on the ERβ levels in these tissues was observed in ERKO mice of either sex.

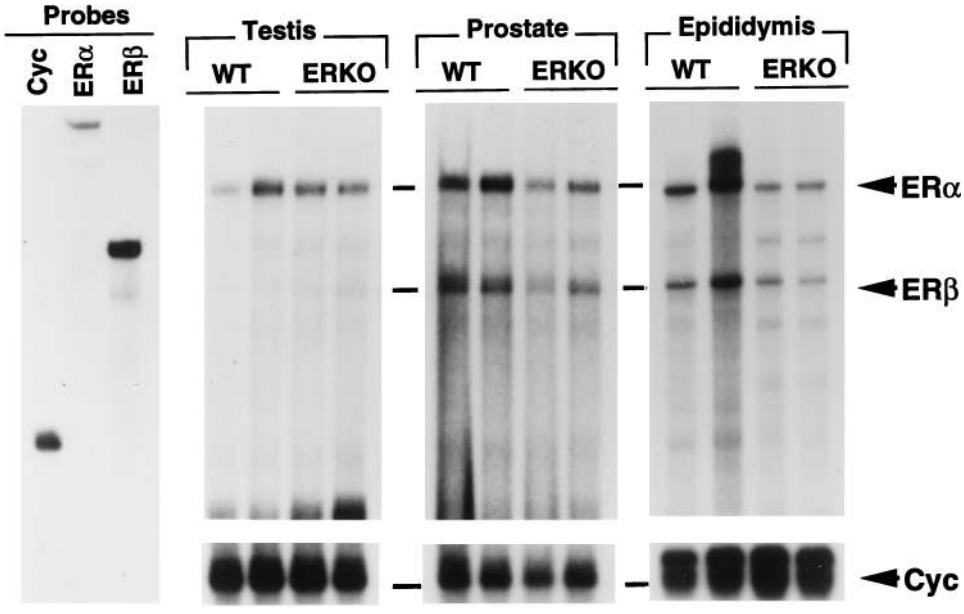

FIG. 4. Analysis of ERα and ERβ transcripts in the adult male reproductive tract by RPA. Total RNA was analyzed for the presence of the ER transcripts as described in *Materials and Methods*. Each lane represents the analysis of RNA from individual males at the following amounts: 15 μg for testis and epididymis, and 10 μg for prostate. Each ERα genotype, WT or ERKO, was assayed in duplicate. Levels of the Cyc transcript was used for normalization between samples. All exposure times are equal for the panels showing the detected ER riboprobe fragments.

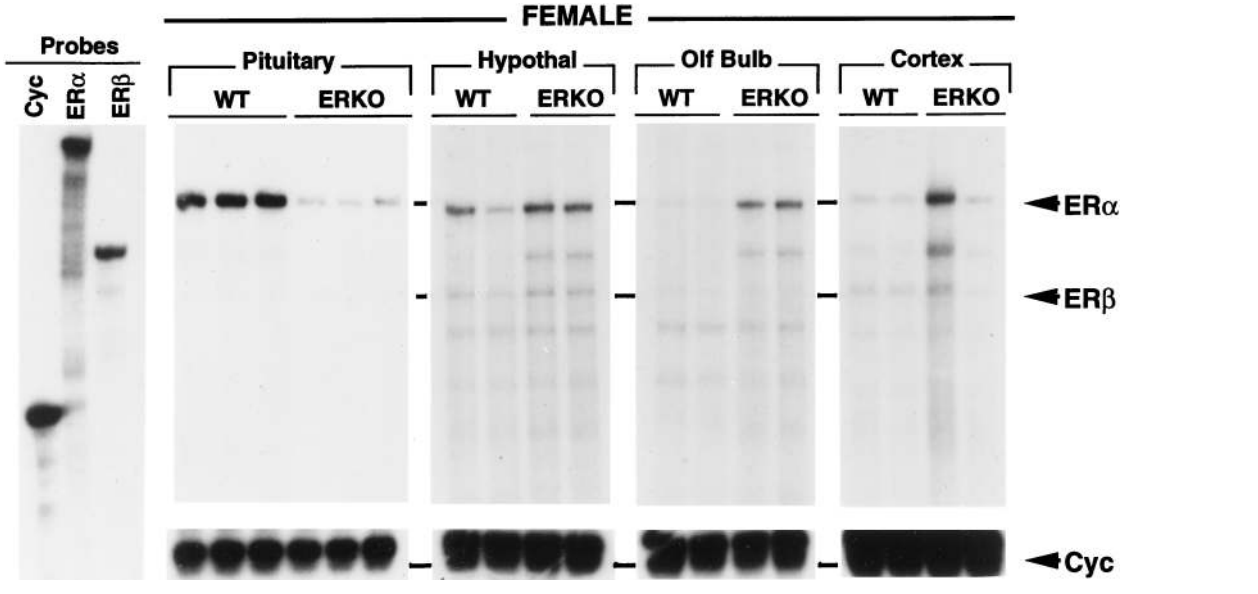

FIG. 5. Analysis of ERα and ERβ transcripts in the adult female pituitary and neural tissues by RPA. Total RNA was analyzed for the presence of the ER transcripts as described in *Materials and Methods*. Each lane represents the analysis of RNA from individual females at the following amounts: 4 μg for pituitary, 10 μg for hypothalamus (hypothal) and olfactory bulb (olf bulb), and 20 μg for cortex. Each ERα genotype, WT or ERKO, was assayed in duplicate except for pituitary, in which three individual animals of each genotype was assayed. Levels of the Cyc transcript was used for normalization between samples. All exposure times are equal for the panels showing the detected ER riboprobe fragments.

In both sexes, the liver possessed significant levels of ERα mRNA but only slightly detectable levels of ERβ mRNA (data not shown). Other tissues of both sexes that possessed undetectable levels of ERβ mRNA but were positive for ERα when assayed by RPA were kidney, spleen, skeletal muscle, and bone marrow (data not shown). Once again, no genotypic effect on the ERβ levels in these tissues was observed in ERKO mice of either sex.

Discussion

The cloning of a gene encoding a second type of estrogen receptor has introduced a new level of complexity to the estrogen signaling system in the mammal. In this report, we have described the design and use of a sensitive RPA to determine the distribution and level of expression of the ERβ gene, as well as allowed for comparison to that of the ERα gene in various tissues of the mouse. Transcripts encoding ERα were detected in all the tissues assayed of both sexes; however, several tissues, such as the mammary gland, kidney, and aorta, were negative for ERβ expression when assayed by the RPA. In the WT mouse, ERβ was the predominant species of ER encoding transcript in the ovary and lung. In the prostate and epididymis of the WT male, there appeared to be equal levels of the two transcripts, whereas in

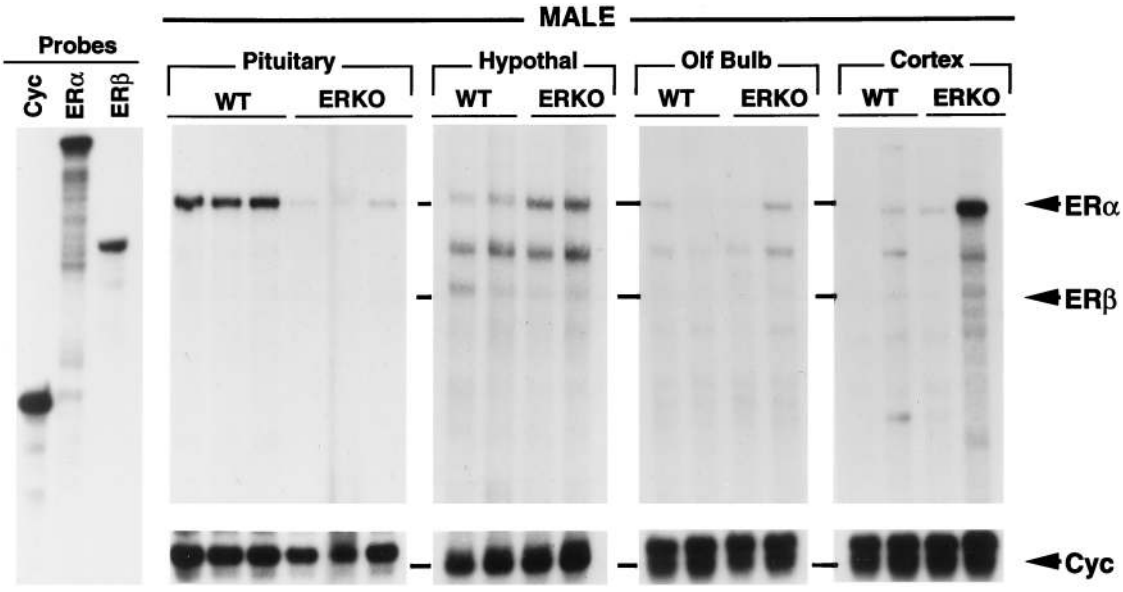

FIG. 6. Analysis of ERα and ERβ transcripts in the adult male pituitary and neural tissues by RPA. Total RNA was analyzed for the presence of the ER transcripts as described in *Materials and Methods*. Each lane represents the analysis of RNA from individual males at the following amounts: 4 μg for pituitary, 20 μg for hypothalamus (hypothal), and and 10 μg for olfactory bulb (olf bulb) and cortex. Each ERα genotype, WT or ERKO, was assayed in duplicate except for pituitary, in which three individual animals of each genotype was assayed. Levels of the Cyc transcript were used for normalization between samples. All exposure times are equal for the panels showing the detected ER riboprobe fragments.

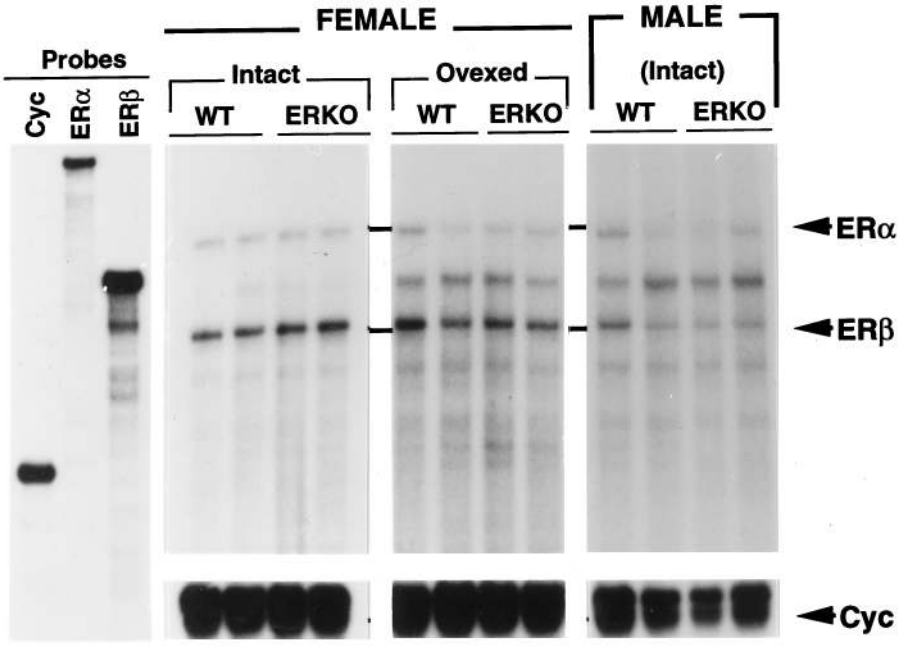

FIG. 7. Analysis of ERα and ERβ transcripts in intact female, ovariectomized female, and intact male lung by RPA. Total RNA was analyzed for the presence of the ER transcripts as described in *Materials and Methods*. Each lane represents the analysis of RNA from individual animals at 15 μg per lane. Each ERα genotype, WT or ERKO, was assayed in duplicate. Tissues from ovariectomized female mice were collected 14 days post surgery. Levels of the Cyc transcript were used for normalization between samples. All exposure times are equal for the panels showing the detected ER riboprobe fragments.

the hypothalamus of both sexes there was approximately twice the level of ERα mRNA. These data suggest that tissues such as the ovary, prostate, lung, and hypothalamus may be interesting targets to explore ERβ protein expression once antibodies or ligands of sufficient specificity and quality become available.

The initial descriptions of the ERKO female mouse reported the presence of residual high affinity binding in the uterus (11, 12, 16). However, a possible explanation for this binding was provided by the finding of a splice variant of the disrupted ERα mRNA that would result in a mutant form of ERα with an intact ligand binding domain (12). It has been suggested that this residual estrogen binding in the uteri of ERKO mice is due to ERβ (6). However, although very low levels of ERβ mRNA are present in WT and ERKO uteri as shown in this report, the high affinity binding factor in the ERKO uterus was previously shown to be recognized by the ERα antibody H222 (12), which has been reported to have no cross-reactivity with *in vitro* translated ERβ protein (9). Therefore, the residual estrogen binding activity found in the

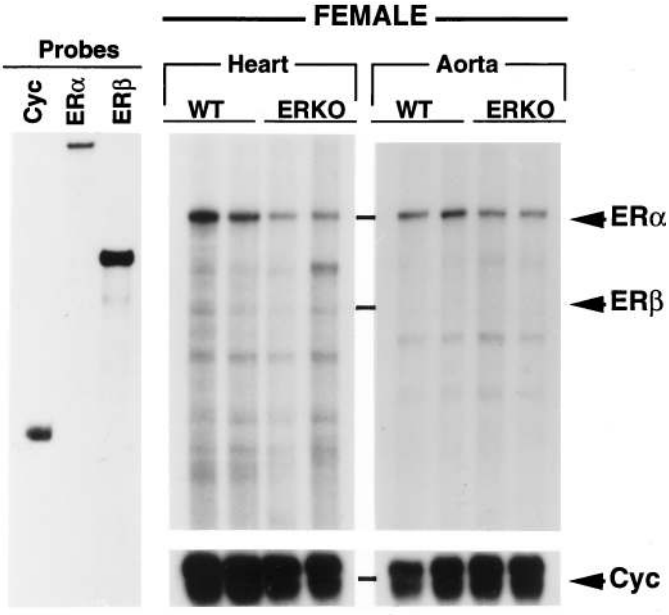

FIG. 8. Analysis of ERα and ERβ transcripts in female heart and aorta by RPA. Total RNA was analyzed for the presence of the ER transcripts as described in *Materials and Methods*. Each lane represents the analysis of RNA from individual animals at 15 μg per lane. Each ERα genotype, WT or ERKO, was assayed in duplicate. Levels of the Cyc transcript was used for normalization between samples. Exposure time for the heart was 2 days, whereas that for the aorta was 1 day.

uteri of ERKO mice is most likely not due to the presence of ERβ.

Significant ERβ expression is seen in the ovary of WT mice. This is in accordance with recent reports that have localized the presence of ERβ transcripts in the rat ovary to the granulosa cells of small preovulatory follicles (6, 18). Although the level of ERα transcripts in the WT ovary appeared relatively consistent, the level of ERβ mRNA showed a considerable range among sexually mature females (Table 1). The level of ERβ mRNA in the ovaries of adult ERKO mice appeared slightly lower on average and less variable when compared with WT (Table 2). However, the ovaries of adult ERKO females are acyclic and do not possess follicles in the most mature stages of folliculogenesis, resulting in altered proportions of the various cell types and stages compared with the WT ovary. Therefore, the apparent decrease in ERβ mRNA levels in the ERKO ovary may be due to a loss of certain cell types or stages or possibly to a direct regulatory effect on the ERβ gene.

It is interesting to note that the characteristic ovarian phenotype of multiple large, atretic follicles that eventually become hemorrhagic and cystic in the ERKO female mice (11, 16, 17) occurs in the presence of significant levels of ERβ expression. Because the ERKO ovary possesses primary and secondary follicles, it is possible that ERβ is essential to the early stages of folliculogenesis, and that these pathways have remained intact in the ERKO. However, the significant levels of ERβ mRNA in the ovary in combination with the pronounced phenotype that results from disruption of the ERα gene indicates a significant role for each type of the ER in the ovary. These data suggest a requirement for interaction between the two ER types for proper cell and gene specific regulation during the later stages of folliculogenesis.

In agreement with the results reported for the rat (6), the mouse prostate expressed significant levels of ERβ mRNA, whereas the testis was negative. The levels of the two ER transcripts in the WT mouse prostate and epididymis were high and relatively equal. In both of these tissues, the ERKO male demonstrated a decrease in the level of ERβ expression (Table 2). It is possible that this is either a reflection of specific ERα mediated down regulation of the ERβ gene, a decrease in mitotic activity among the cells of these tissues, or simply the loss of certain cell types in these tissues, and therefore warrants further investigation. Interestingly, the initial description of the human ERβ gene reported the prostate to be negative and the testis to be positive for ERβ mRNA when assayed by Northern blot techniques (8). This contrast with the present study suggests possible species specific expression patterns of the ERβ gene.

The absence of ERβ mRNA in the pituitary of both sexes, in contrast to the high levels of ERα mRNA, would suggest that ERα is the sole mediator of estrogen action in this tissue. This conclusion is supported by the finding that ERKO mice exhibit no estrogen regulated negative feedback on the expression of the gonadotropin genes in the pituitary (19). However, the WT and ERKO hypothalamus of both sexes possessed ERβ mRNA levels that were at least half of that for ERα. A recent report by Shughrue *et al.* (10) localized ERβ gene expression to several distinct regions of the hypothalamus in the adult female rat. Nonetheless, ERKO female mice appear to lack the effects of estrogen feedback in the hypothalamus (unpublished results), suggesting that ERα is essential to the regulatory roles of the hypothalamus as they relate to proper gonadal function. Furthermore, recent reports have described abnormal sexual behavior in the ERKO female (20, 21), including a lack of sexual receptivity, as well as in the ERKO male (2). Therefore, although the expression level of ERβ in the hypothalamus of ERKO mice appears normal, their abnormal behavioral phenotype indicates an important role for ERα in reproductive behavior.

The indications that estrogen may be a protective factor in the development of cardiovascular disease has been founded upon the observations that premenopausal women are at a much lower risk for this disease (22, 23). Therefore, the possibility of a role for ERβ in the tissues of the cardiovascular system is of great interest. However, in the mouse, whereas the heart of both sexes possessed just slightly detectable levels of ERβ, aorta from each sex demonstrated expression of ERα only when assayed by RPA. These data would suggest that the actions of estrogens on the cardiovascular system are most likely mediated in part by ERα. This is supported by reports of abnormal phenotypes in the cardiovascular tissues of ERKO mice, including compromised nitric oxide synthesis in the aorta (24) and a lack of estrogen-induced angiogenesis (25).

The biological significance of a second type of ER can be only speculated upon at this time. Two known isoforms of the progesterone receptor, PR_A and PR_B, are also known to exist and have been well described to form both homodimers and heterodimers, each complex having different gene regulatory effects (26–29). The ERβ protein possesses a short-

ened N-terminus when compared with ERα, similar to the relationship of PR_A to PR_B. It is interesting to note that no tissue analyzed in this study possessed ERβ as the sole species of ER transcript, whereas ERα mRNA was present at varying levels in all the tissues assayed and the sole form in several. However, because the assays herein were carried out on RNA extracted from whole tissue, possible differences in the localization of the two ER mRNAs within distinct cell types cannot be commented on from this analysis.

The lack of significant alterations in the expression levels or pattern of the ERβ gene in the ERKO mice would suggest that regulation of the ERβ gene may not be directly dependent on the actions of ERα. These data, in combination with the several described phenotypes present in both sexes of the ERKO mouse, suggest that some of the biological functions of the ERβ protein may be dependent on the presence of ERα. Recent studies with *in vitro* translated protein have shown that the formation of ERα/ERβ heterodimers preferentially occurs over the formation of homodimers (30), suggesting that a loss of functional ERα may also result in decreased ERβ activity. Also possible is that the functions of the ERβ are essential only during development, thereby allowing for the unpredicted successful generation of an ERα-knockout. Lastly, the ERβ may function to regulate the expression of genes and hormonal responses that have yet to be studied in the ERKO mouse. Because the ERβ gene appears to be expressed in a relatively normal pattern in the ERKO mouse, these animals should prove to be a valuable *in vivo* model to study the biological actions of this specific type of ER. Furthermore, the data presented here demonstrating expression of the ERβ gene in various tissues, and how it compares to that of the ERα gene, will be of significant importance in discerning the full roles of each receptor in the estrogen signaling system.

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