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Expression and Molecular Characterization of Estrogen Receptor Alpha Messenger RNA in Male Reproductive Organs of Adult Goats[1]

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ABSTRACT

The fact that male estrogen receptor alpha (ERα) knockout mice are infertile indicates a role for this receptor in male reproduction. Here, objectives were to evaluate ERα expression in male goat reproductive tissues at the transcriptional level using RNase protection assay (RPA) and in situ hybridization (ISH), and to clone a partial cDNA for caprine ERα using reverse transcription-polymerase chain reaction (RT-PCR). For RPA and ISH procedures, a radiolabeled antisense cRNA probe, generated in vitro from the ovine oER8 cDNA template, was employed. Evaluations were made on individual samples obtained from adult goats. Labeled cRNA sense probe was used as a negative control in ISH. A 530-base pair amplicon was generated by RT-PCR from efferent ductules (EDs), epididymis (EP), and testis, cloned from the ED and EP, and sequenced. The caprine ERα (cERα) cDNA displayed 81%–96% sequence identity with that of other species. A signal indicative of ERα mRNA was identified by both RT-PCR and RPA in all tissues, but was strongest in the ED. Compared with ED, ERα signal was sixfold lower in the EP, and 66-fold lower in the testis. Similarly, strong ERα expression was observed in ED epithelium, whereas little or no signal was detected in EP or testis by ISH. Thus, among different segments of the male reproductive tract and testis, the highest level of ERα mRNA expression was found in epithelium of the ED.

epididymis, estradiol receptor, male reproductive tract, steroid hormone receptors, testis

INTRODUCTION

Mammalian sperm take about 10–12 days to pass through the excurrent ducts, including the efferent ductules (EDs) and the head, body, and tail regions of the epididymis (EP) [1, 2]. During epididymal passage, developing sperm undergo a maturational process that enables them to acquire the abilities to move in a forward direction and fertilize ova [2, 3]. Sperm production and maturation are dependent upon testosterone, its 5α-reduced metabolite, dihydrotestosterone (DHT), or both [4]. In fact, the testosterone concentration required for sperm production in the testis and for sperm maturation in the EP is many-fold higher than that present in the blood [5, 6]. However, unlike testosterone, the role of estrogen in male reproduction remains unclear, although rete fluid within the testis, as well as that entering the ED and EP, contains a very high concentration of estrogen [7].

The estrogen receptor (ER) belongs to a superfamily of ligand-activated nuclear hormone receptors, including receptors for androgens, glucocorticoids, thyroid hormones, vitamin D_3, and retinoids [8]. Currently, two distinct ER subtypes, ERα and ERβ, have been characterized in various species [9]. Observations that mutant male mice lacking ERα [10] or both ERα and ERβ are infertile [11], whereas those lacking ERβ alone are fertile [12], indicate a role for ERα in male reproduction. Infertility observed in male aromatase-knockout mice further reinforces the significance of estrogen and its receptor system in male reproductive physiology [13, 14].

Because of the implied significance of ERs in male reproduction, a number of studies have described their localization at the protein and mRNA levels in male reproductive organs of various species, including rats [15–19], mice [20–25], monkeys [15, 26, 27], humans [28, 29], and roosters [30]. Collectively, these studies indicate that variable levels of ERα, ERβ, or both are present in most, if not all, male reproductive organs.

In an effort to understand the role of ER in male reproduction, we previously used immunohistochemistry to evaluate distribution, differentiation, and regulation of ERα in the testis and excurrent ducts of male goats [31–33]. Immunostaining for ERα protein, found only in the ED, was present at 1 wk of age and regulated by androgens. Objectives of the present study were to evaluate ERα expression in the testis and excurrent ducts at the transcriptional level using RNase protection assay (RPA) and in situ hybridization (ISH), and to clone a partial cDNA for caprine ERα (cERα) using the reverse transcriptase-polymerase chain reaction (RT-PCR). Goats were used as an experimental model because, morphologically, the reproductive tracts of male goats, rams, and bulls are similar [1, 34, 35]. Among these three species, male goats are preferred because they are nonseasonal breeders [36], inexpensive, and easier to handle for surgical procedures.

MATERIALS AND METHODS

Animals and Processing of Tissues

Ten mature male Nubian goats (five for RPA and RT-PCR and five for ISH), approximately 2–3 yr of age and with a satisfactory breeding soundness examination (scrotal circumference ≥26 cm, sperm abnormalities ≤20%), were used. Animals were kept in a covered shelter, allowed to walk freely, and were given hay and water ad libitum. A general management schedule for deworming, disease prevention, and hoof trimming was followed. Using general

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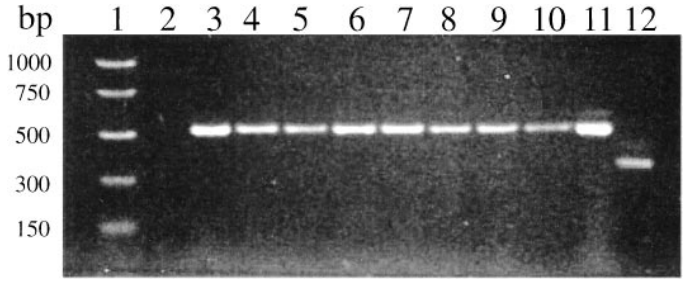

FIG. 1. Expression and distribution of ERα mRNA in adult male goat reproductive tissues detected by RT-PCR. Lane 1, DNA molecular weight markers; lane 2, negative control; lane 3, efferent ductules; lanes 4–9, epididymal regions I through VI, respectively; lane 10, testis; lane 11, sheep uterus (positive control); lane 12, mouse *rig/S15* mRNA (positive control for RT-PCR).

anesthesia and aseptic surgical techniques, both testes and their epididymides were removed. For RPA, tissue samples from five goats were collected from the testis, ED, and six regions of the epididymis (regions I–III comprised the proximal, middle, and distal regions of the head, respectively; regions IV–V, the proximal and distal regions of the body, respectively; and region VI, the tail, [31]). Tissues were snap-frozen in liquid nitrogen and stored at −70°C until processed. For ISH, testes and excurrent ducts from an additional five goats were fixed by vascular perfusion through the testicular artery with precooled 4% (w:v) paraformaldehyde (PAF) in PBS (pH 7.4). After vascular perfusion, 5-mm-thick sections of tissue each from the testis, ED, and regions I–VI of the EP were further fixed in PAF overnight at 4°C. After fixing, tissues were dehydrated in graded concentrations of ethanol, cleared in xylene, embedded in Paraplast-plus, cut at 5-μm sections, and mounted on slides coated with poly-L-lysine (Sigma Chemical Company, St. Louis, MO). All experimental and surgical procedures were approved by the Tuskegee University Animal Care and Use Committee.

RNA Isolation

Total RNA was isolated using TRIZOL reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer's protocol. The RNA concentrations were estimated at 260 nm using UV spectrophotometry. All RNA preparations was evaluated by denaturing agarose gel electrophoresis.

Reverse Transcriptase-Polymerase Chain Reaction

Primers for PCR were designed using a previously published sheep ERα sequence [37]. The sequence of sense and antisense oligonucleotide primers corresponded to nt 437–457 (5′-CTT CCC GCC GCT CAA CAG CGT-3′) and

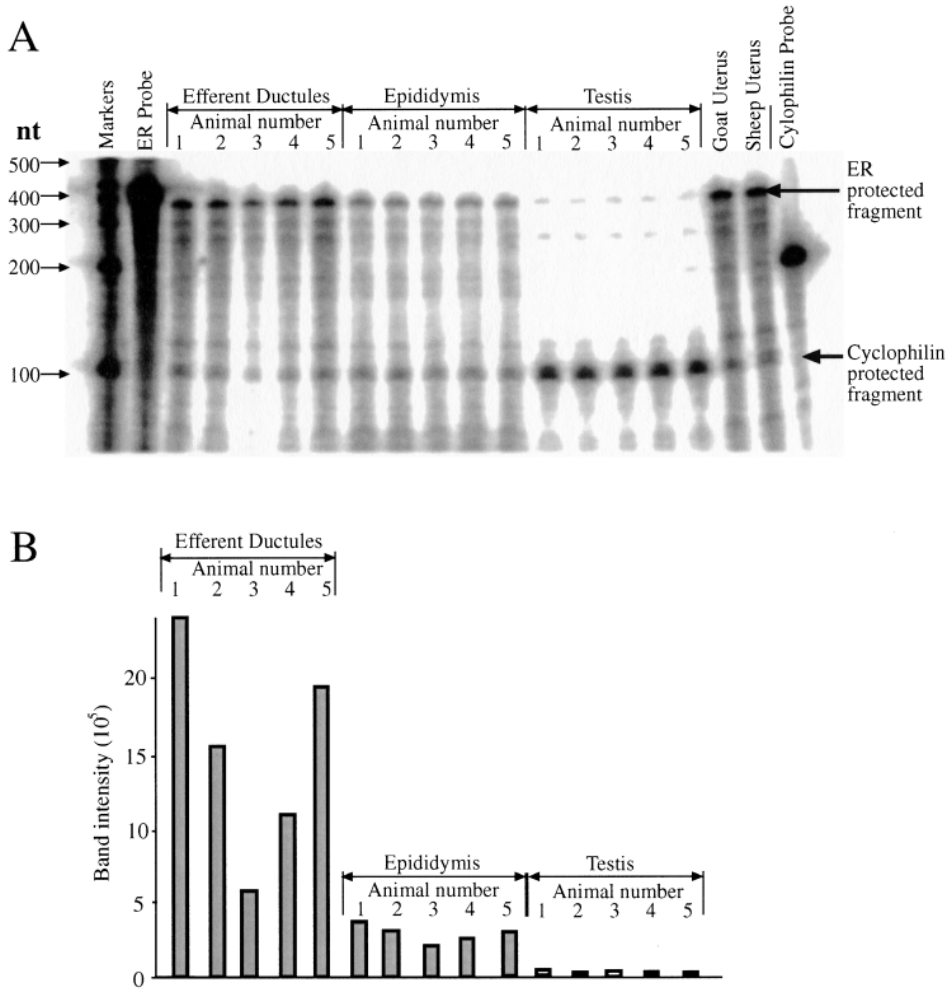

FIG. 2. **A**) RNase protection analysis of caprine ERα mRNA transcripts in efferent ductules (ED), epididymis (region III), and testis (T) of five adults goats (1–5). **B**) Histogram showing relative levels of caprine ERα mRNA in ED, EPIII, and T. Data are expressed in arbitrary units generated by phosphorimaging of the autoradiogram in **A**.

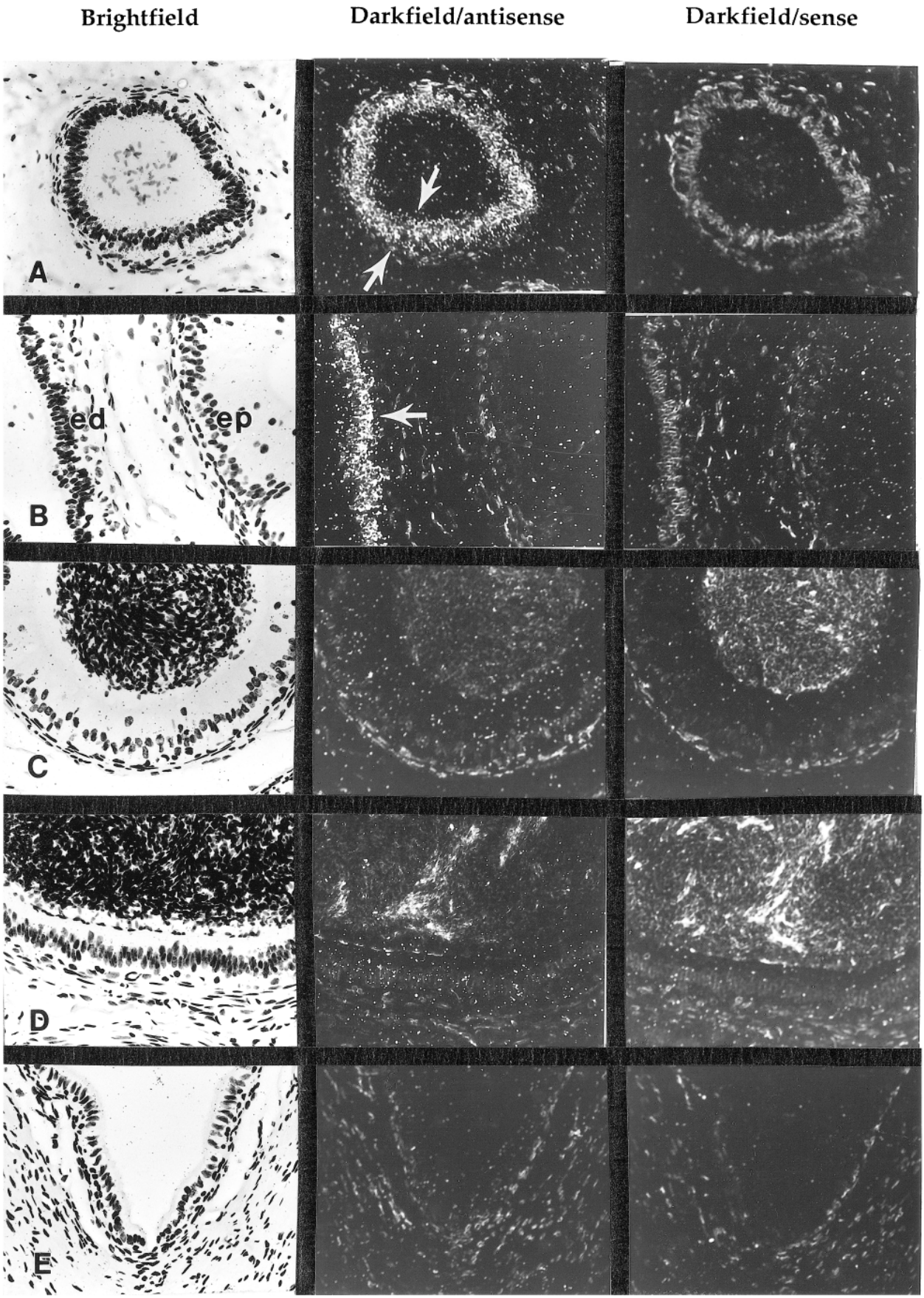

FIG. 3. **A**–**H**) In situ hybridization of ERα mRNA with a [^{35}S]cRNA antisense probe in reproductive organs of the adult male goat. Brightfield and darkfield photomicrographs of each organ were mounted together and enlarged to the same magnification. Adjacent sections incubated with antisense or sense probes were photographed in darkfield. **A**) A strong hybridization signal was found only in epithelial cells of the efferent ductules (arrows). **B**) Micrograph taken near the junction between the efferent ductules (ed) and region I of the epididymis (ep). Note strong signal confined to epithelial

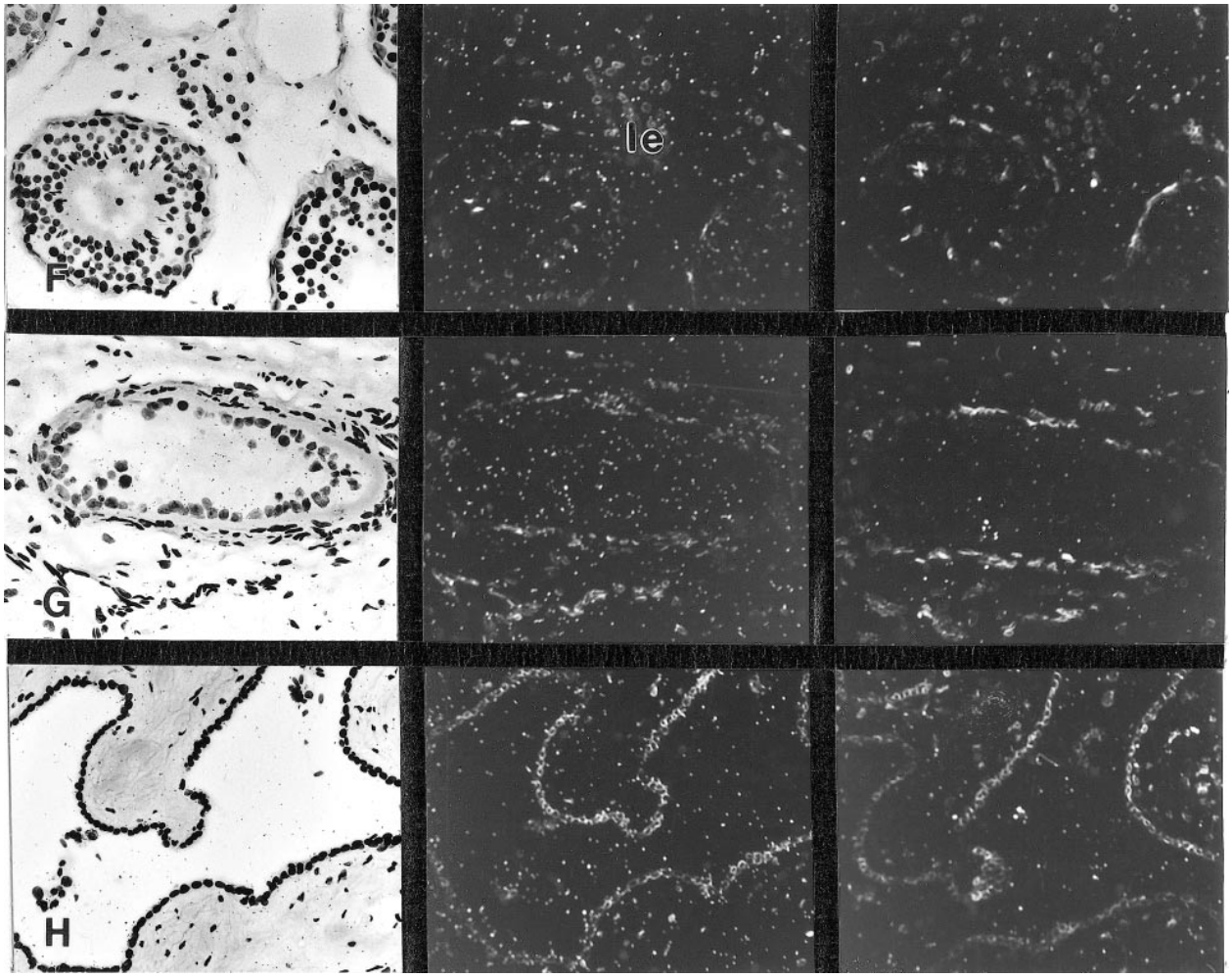

FIG. 3. Continued. cells of the ed (arrow). **C**–**H**) Signal slightly above background was observed infrequently, if at all, in other male reproductive organs, including region III (corresponds to the distal head near its junction with the body) of the epididymis (**C**), region VI (corresponds to the tail) of the epididymis **D**), ductus deferens (**E**), testis (**F**), tubulus rectus (**G**), and rete testis (**H**). Leydig cells, le. No specific hybridization signal was observed in sections incubated with a sense cRNA probe. ×185.

nt 947–967 (5′-TCT CTC TGG CGC TTG TGC TTC-3′), respectively, and the expected size of the PCR product was 530 base pairs (bp). Positive control primers for amplification of a conserved, constitutively expressed mRNA (*rig/S15*, ribosomal subunit protein), and a positive control RNA from mouse liver were used as control for PCR efficiency (Ambion Inc., Austin, TX). The expected product size for *rig/S15* gene was 361 bp. Two micrograms of total RNA from the testis, ED, and regions I–VI of the EP in the goat, and sheep uterus (positive control) were reverse transcribed with 100 units of Moloney murine leukemia virus (M-MLV) RT following instructions for the RETROscript kit (Ambion). An aliquot of 3 μl of RT product was used for PCR, which was performed on a Robocycler (Stratagene, La Jolla, CA) in a 50-μl reaction volume containing 1× *Taq* DNA reaction buffer, 1.5 mM $MgCl_2$, 0.125 mM of each dNTP, 0.5 μM of each primer, and 1 unit of Super *Taq* DNA polymerase (Ambion). The PCR conditions included an initial cycle at 95°C for 3 min; 30 cycles each at 95°C for 45 sec, 67°C for 1 min, and 72°C for 1 min; with a final extension step at 72°C for 10 min. The PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining and UV illumination.

Ribonuclease Protection Assay

Caprine ERα mRNA expression was quantified by RPA following instructions for the RPA II Kit (Ambion). Antisense ERα cRNA probe labeled with [α-^{32}P]UTP was generated by in vitro transcription (IVT, MAXIscript IVT Kit, Ambion) using T7 RNA polymerase and *Eco*RI linearized oER8 template [38, 39] (a gift from Dr. Nancy Ing, Texas A&M University). A full-length [α-^{32}P]UTP-labeled antisense cyclophilin cRNA probe was generated from the human pTRI-cyclophilin template (Ambion) and was used as an internal RNA loading reference to monitor RPA performance. Both probes were gel purified. Using total RNA from caprine ED, the cERα RPA was first optimized for RNA concentration and RNase dilution. Region III of the EP was included as a representative for the entire EP because preliminary trials did not reveal any measurable differences in ERα mRNA concentration among the six regions of the EP (data not shown). To avoid interassay variation, samples from all five animals were run simultaneously. Ten micrograms of total RNA from the testis, ED, and region III of the EP of each goat was hybridized in solution with fivefold molar excess of [α-^{32}P]UTP-labeled oER8 and cyclophilin antisense cRNA probes for 16 h at

42°C. RNA from sheep and goat uterus were included as positive controls. Hybridized probe and sample RNA were digested with a mixture of RNase A and T1, diluted 1:500, for 30 min at 37°C. Protected fragments, with expected sizes of 366 nt for cERα and 106 nt for cyclophilin, were precipitated from solution and resolved in a 5% polyacrylamide/8 M urea gel. The gels were dried and exposed to phosphorImager screens for 16 h at room temperature, and band signal intensity for protected fragments was quantified by ImageQuant software using a Molecular Dynamics PhosphorImager SI (Amersham-Pharmacia Biotech, Piscataway, NJ). Band densities of the protected fragments of ERα from individual animals were normalized to those of the cyclophilin mRNA and the results were expressed as arbitrary units. RPA data were subjected to ANOVA using general linear models procedures of the Statistical Analysis System [40]. Signal for cyclophilin was included in the analysis as a covariate to account for loading variation. Specific signal comparisons (relative units) were made among the testis, EP, and ED.

In Situ Hybridization

Paraplast sections were deparaffinized in xylene, rehydrated through a series of graded alcohols, refixed in fresh 4% PAF in 0.1 M PBS for 10 min, washed in 0.5× saline-sodium citrate for 10 min, and digested with proteinase K (20 μg/ml in 50 mM Tris, 5 mM EDTA pH 8.0). The procedure for ISH was performed as described previously [41]. Briefly, antisense and sense cRNA probes were labeled with [α-^{35}S]UTP (specific activity, approximately 1250 Ci/mmol; Amersham, Arlington Heights, IL) and produced by in vitro transcription from the *Eco*RI- and *Bam*HI-linearized oER8 cDNA template using a MaxiScript Kit (Ambion). Each slide contained two sequential sections that were hybridized for 16 h at 55°C in a humidified chamber with either antisense probe or the sense probe, in 50 μl hybridization buffer. Sections were digested with RNase A for 30 min. Autoradiography was performed using NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 in distilled water. Slides were exposed at 4°C for 12 wk, then developed at 15°C using D-19 developer (Eastman Kodak), and counterstained with mercury-free Harris modified hematoxylin with acetic acid (Fisher Scientific, Pittsburgh, PA). Both brightfield and darkfield images of sections were taken simultaneously to compare distribution of grains in different tissues of the same organ. Similarly, images from the test (antisense probe) and control (sense probe) sections were photographed simultaneously to permit objective comparisons between the two.

Caprine cDNA Sequencing and Analysis

The ERα RT-PCR amplicons from caprine ED and EP, and sheep uterus, were extracted from agarose gel using QIAquick spin columns (Qiagen, Chatsworth, CA). Samples were submitted to the Nucleic Acid Service Center at Auburn University for automated sequencing using a modification of the Sanger-dideoxy chain termination method [42] with an Applied Biosystems 373A (ABI Division, Perkin-Elmer, Boston, MA). The resulting sequence for the cERα was compared with that of other species using NCBI GenBank data entries and performing a BLAST-2 sequence comparison.

RESULTS

Identification of cERα mRNA by RT-PCR

A single 530-bp amplicon was identified by RT-PCR from all caprine reproductive organs examined, including the testis, ED, and all six regions of the EP, and sheep uterus (Fig. 1). As expected, the product size for the *rig/S15* housekeeping gene was 361 bp.

Quantitative Analysis of cERα mRNA by RPA

The protected fragment of cERα mRNA was detected in the testis, ED, EP, and uterus (Fig. 2A). Signal intensity for cERα was most intense for ED tissues and similar to that observed for the uterus (Fig. 2A). Consistently, results of statistical analyses indicated that mean signal intensity for cERα mRNA was lower for testicular than for excurrent duct tissues (testis $<$ ED and EP, $P < 0.001$), and higher in ED than in EP ($P < 0.001$). Mean signal intensity in the ED, measured from five animals, was approximately six times stronger than in the EP and 66 times stronger than in the testis. Individual observations for each tissue obtained from the five goats from which RPA data were generated are shown in Figure 2B.

Cellular Localization of cERα mRNA by In Situ Hybridization

ISH was used to determine which cells within the male goat reproductive organs express ERα mRNA. Among all organs examined, a clear signal was observed only in the ED. The signal was either negligible or absent in all regions of the EP, ductus deferens, and testis (Fig. 3). Within the ED, the signal was localized in epithelial cells. However, because of its diffuse distribution, it was not possible to discern differences in its intensity between ciliated and nonciliated cells of the epithelium. The lumen of the ED, smooth muscle cells surrounding the epithelium, or both, did not appear to contain silver grains above the background level.

Caprine ERα cDNA Sequence

Analysis of the cDNA sequence from RT-PCR products was performed to confirm ERα expression in male goat reproductive tissues. Utilizing primers from the ovine cDNA sequence reported by Madigou [37], a partial caprine cDNA sequence was generated from ED and EP, and submitted to GenBank (AF251192). The cERα cDNA showed 96% identity with the oER8 cDNA template used for RPA and ISH. Comparison of the cERα nucleotide sequence with that of other species in the GenBank database showed the following identities: 81%, mouse M38651; 81%, rat X61098; 82%, chicken X03805; 86%, horse AF124093; 88%, human M12674; and 93%, sheep Z49257. The partial cDNA sequence for the cERα translated into a 133 amino acid sequence. The sequence was rich in glycine, serine, tyrosine, and cysteine residues, which is typical for a class II zinc-binding motif [43].

DISCUSSION

This study describes the expression of ERα at the transcriptional level in the testis and excurrent ducts of the adult goat using RT-PCR, RPA, and ISH, as well as the cloning sequence and analysis of a partial cDNA for caprine ERα. Of all the male reproductive organs examined,

the strongest signal was found in the ED. The new caprine ERα cDNA showed 96% identity with the ovine partial ERα cDNA (oER8) used as probe in this study [38], and 93% identity with the full-length cDNA sequence for ovine ERα [37].

ERα has six functional domains, designated A through F [9]. The cDNA amplicon produced in this study from caprine tissues lies in domain C. This domain is highly conserved among species and within the superfamily of steriod/thyroid hormone nuclear receptors [9]. The C domain contains two zinc fingers that are responsible for binding the receptor to hormone-response elements. Consistently, a consensus region within the cERα cDNA was identified as a NucHorTF site, which has four cysteine residues that are responsible for tetrahedrally binding zinc and an N-terminal hydrophobic core. Sequence analysis of the caprine cDNA generated here confirmed detection of ERα mRNA expression in male goat reproductive tissues by RT-PCR.

Signal intensity for ERα mRNA in the ED, detected by RPA, was six times greater than that in the EP and 66 times greater than in the testis. These observations are similar to those reported for the rat [16]. Using Northern blotting and RT-PCR, these authors found ERα mRNA in the ED and the caput, corpus, and cauda regions of the rat EP, but its expression was stronger in the ED than in any region of the EP. Expression of ERα mRNA has also been detected by RT-PCR in the EP of mice [20] and monkeys [27], and in the testis of rats [17], mice [20], and monkeys [27]. None of these studies included ED tissues.

Similar to RPA, ISH also revealed a strong signal for ERα in caprine ED. In contrast, the testis, rete testis, ductus deferens, and all six regions of the EP displayed little or no signal above background. The results in the goat are essentially similar to those reported for the rooster, where a strong ISH signal was found in the ED and a weak signal in the EP [30].

An almost exclusive localization of ERα mRNA in the caprine ED epithelium, detected by ISH, was consistent with observations reported previously from our laboratory regarding nuclear immunolocalization of ERα protein in adult, male goats [31]. According to that study, among all male reproductive organs, including the testis, rete testis, ED, regions I–VI of the EP, and ductus deferens, ERα protein was immunolocalized only in the nonciliated cells of the ED. Similarly, ED was the only segment of the male reproductive tract that stained positively for ER protein in the monkey [15, 26]. However, there are immunocytochemical studies in mice [24, 25] and rats [16], and autoradiographic studies in mice [22, 23] in which ER protein staining was reported not only in the ED but also in the EP. Likewise, biochemical studies showed binding of [^{3}H]estradiol with cytosolic extracts, nuclear extracts, or both, of the epididymides in various species, including humans [44], rats [45], rabbits [46, 47], and rams [48]. Reasons for differences between studies may be species-related, procedural, or both. Regardless, it can be generally stated that ED, among all male reproductive organs, constitute a primary site of ERα expression at the level of both mRNA and protein.

Recently, another estrogen receptor, ERβ, has been cloned in a number of species, including rats [49], humans [50], cattle [51], and the goldfish [52]. Although our work on ERβ in male goats is still in progress, other studies have described its localization at the protein level, mRNA level, or both, in male reproductive organs of rats [16–19], mice [20, 21], monkeys [27], and humans [29]. These studies taken together indicate that ERβ is present in the testis, ED, EP, and prostate; but its expression is higher in the prostate gland than in other male reproductive organs and, compared with ERα, its expression was lower in the ED and EP.

Evidence of ER expression in the testis and excurrent ducts supports the idea that estrogens have a role in sperm production and maturation. Research data showing infertility in mutant male mice lacking ERα [10] or ERα and ERβ [11] lends credence to this idea. Additional studies indicating that aromatase-knockout mice developed infertility with age [13, 14] and that the rete testis fluid of several species contains a high concentration of estrogen [7, 53, 54] further underscore the potential role of estrogen and its receptor in male reproduction. Reasons for infertility in ERα knockout mice are not completely understood; however, it is hypothesized that one contributing factor is a disturbance in fluid absorption in the head of the EP, especially at the level of the ED [55, 56]. It is interesting that ED is known to absorb 90% or more of the rete testis fluid [57, 58], and also contain more ERα than any other male reproductive organ [16].

In conclusion, results of both RT-PCR and RPA analyses indicate that ERα expression occurs in the testis and throughout the length of the excurrent ducts in adult goats. Elevated ERα expression detected by ISH in ED epithelium can be interpreted to suggest that these cells are a primary estrogen target in the male reproductive tract. Generation of caprine ERα cDNA provides a tool that will allow us to perform additional studies on the role of the estrogen signaling system in epididymal and testicular development and function.

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