

Estrogen Receptor (α and β) Expression in the Excurrent Ducts of the Adult Male Rat Reproductive Tract

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ABSTRACT: The understanding of estrogen's function in the male reproductive tract is limited, and estrogen receptor (ER) localization in the reproductive tract of the adult male rat has not been described. In the present study, ER α was localized by immunohistochemistry using ER21 antibody, which recognizes only ER α . Strongest immunoreactivity was seen in epithelia of ductuli efferentes and the initial segment of the epididymis. Nuclei of both ciliated and nonciliated cells were positive. The epithelium of the rete testis, and caput, corpus, and cauda epididymides stained less intensely for ER α . The vas deferens epithelium was ER α -negative. Stromal tissue in the excurrent ducts was also ER α -positive. Using 3H-estradiol autoradiography, specific binding of estradiol was seen in nuclei of ductuli efferentes. Estrogen

receptor α mRNA expression was greatly enhanced in ductuli efferentes compared to other regions of the male tract and was 3.5x greater than in the uterus. For comparison, the presence of ER β was determined using reverse transcription polymerase chain reaction (RT-PCR) amplification. Estrogen receptor β mRNA was expressed throughout the male tract and in the prostate. These results indicate that all organs in the male excurrent ductal system of the rat express ER α and are potential targets of estrogen. However, the ductuli efferentes are the site of the most intense ER α expression. The role of ER β remains to be determined, but its expression appears ubiquitous in the male tract.

Key words: Efferent ductules, immunohistochemistry.
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Dependence of the male reproductive tract on androgens for normal development and function is well known, but there are relatively few studies investigating the role of estrogen in male reproductive tissues (Peyre and Laporte, 1966; Meistrich et al, 1975; Brown and Amann, 1984). As a result, the function of estrogen in the male is not understood. However, exposure to estrogen or estrogen-like chemicals during fetal or perinatal development has been shown to induce major pathological effects in the head of the epididymis of adult males, both in human and experimental animals (McLachlan et al, 1975; Bibbo et al, 1977; Gill et al, 1977; Bern and Talamantes, 1981; Newbold et al, 1986). Recent studies indicate that even small doses of estrogen can have profound effects on growth and differentiation of the male reproductive system (vom Saal et al, 1997). Estrogens have been found in relatively high concentrations in rete testis fluid and semen (Claus et al, 1987, 1992; Eiler and Graves, 1977; Free and Jaffe, 1979), and estrogen recep-

tor (ER) has been reported in the male reproductive tract of several species (Danzo and Eller, 1979; Murphy et al, 1980; Younes and Pierrepont, 1981; Schleicher et al, 1984; Tekpetey and Amann, 1988; Toney and Danzo, 1988; West and Brenner, 1990; Iguchi et al, 1991; Goyal et al, 1997). In these reports, the distribution of ER varied widely within the male reproductive tract, depending upon several factors, including species and the method used to localize ER, i.e., binding assay autoradiography, or immunohistochemistry. In the adult rabbit, cytosol-specific estrogen binding is present throughout the epididymis but is highest in the caput (Toney and Danzo, 1988), whereas, in the ram, estradiol (E₂) binding is the highest in cauda epididymidis (Tekpetey and Amann, 1988). In the mouse, autoradiographic localization of 3H-E₂ was greatest in the epithelium and connective tissues of ductuli efferentes and the initial segment of the epididymis, with lesser binding in the distal tract (Schleicher et al, 1984). Using immunohistochemistry, ER was localized primarily in the epithelium of ductuli efferentes but was also found in the caput epididymis (Iguchi et al, 1991). However, in the goat and monkey, only the nonciliated cells of the efferent ductuli epithelium stained ER-positive (Goyal et al, 1997; West and Brenner, 1990).

The discovery of a second form of ER (ER β) has fur-

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ther complicated interpretation of earlier data. Autoradiography, which detects ER by localizing E₂ binding, would be expected to identify both ER and ER, while immunohistochemical studies may have detected

either form or both ER and ER. Estrogen receptor β differs from ER primarily in the N-terminus domain (Kuiper et al, 1996; Mosselman et al, 1996). Therefore, some antibodies against regions highly conserved in ER and ER may recognize both forms of ER, while other antibodies recognize only ER. Although ER was found in testis, prostate, and epididymis (Kuiper et al, 1996, 1997), its specific distribution and function in the male reproductive tract are unknown.

Recent concern over environmental estrogen exposure and male infertility (Sharpe and Skakkebaek, 1993), as well as the demonstration that increasing free-estradiol levels by as little as 0.11 pg/ml in male mouse fetuses during prenatal development led to large increases in adult prostate weight (vom Saal et al, 1997), has focused attention on the role of estrogen in the male and has further emphasized its potential role in the etiology of male reproductive tract diseases. Despite the importance of the rat as a research animal, ER distribution has not been described in this animal. Using immunohistochemistry, autoradiography, and mRNA analysis we found ER, protein and message, to be predominantly in the ductuli efferentes but present in the epithelium and connective tissue cells from the rete testis to the cauda epididymidis and absent in the epithelium of the vas deferens. Estrogen receptor β mRNA appears to be expressed throughout the excurrent ducts of the adult male reproductive tract.

Materials and Methods

Ovalbumin, aprotinin, leupeptin, dithiothreitol, β -mercaptoethanol, and Ponceau S stain were purchased from Sigma (St. Louis, Missouri). Acrylamide, sodium dodecyl sulfate (SDS), and other reagents used for SDS-PAGE (polyacrylamide gel electrophoresis), including molecular weight standards, were obtained from Bio-Rad (Richmond, California). Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia (Piscataway, New Jersey). Horseradish peroxidase (HRP)-cojugated goat antibody to rabbit IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, California). SuperSignal substrate was purchased from Pierce (Rockford, Illinois). The diaminobenzidine-HRP kit was purchased from Vector Laboratories (Burlingame, California). QuikHyb solution was purchased from Stratagene (La Jolla, California). Superscript reverse transcriptase was purchased from Gibco BRL (Gaithersburg, Maryland).

Preparation of ER-21

A peptide (ER₂₁) containing the first 21 amino acids of the rat and human estrogen receptor (MTMTLHTKASGMALLHQIQGN) (Greene et al, 1986) was synthesized in the Howard

Hughes Medical Institute at the University of Chicago and used without purification. Peptide ER₂₁ was coupled to ovalbumin with glutaraldehyde (Walter et al, 1980; Prins et al, 1991) at a ratio of 2 mg peptide to 5 mg ovalbumin. Male New Zealand white rabbits (5-6 weeks old) were injected at several subcutaneous sites with a mixture of peptide/ovalbumin conjugate (approximately 400 μ g of total peptide per animal) in an emulsion (600 μ l) of phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.4) and complete Freund's adjuvant (1:1, v/v). Animals were boosted approximately every 2 - 3 weeks in incomplete Freund's adjuvant. Beginning at 3 months, animals were boosted periodically with the ER₂₁-ovalbumin conjugate (200 μ g peptide per rabbit) in incomplete adjuvant. Blood was collected 7-10 days after each boost.

Peptide-specific antibodies were purified by peptide-affinity chromatography. The resin was prepared by coupling crude ER, peptide (4 mg) to cyanogen bromide-activated Sepharose 4B (2 ml) in 50 mM sodium phosphate, 150 mM NaCl, pH 6.5. Unreacted sites were blocked with 1 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4. Prior to purification, total rabbit immunoglobulin was first precipitated from serum with 40% saturated ammonium sulfate. The pellet was then dissolved in a volume of PBS equal to one-fourth of the serum volume and mixed with the resin in a small column on a rocking platform for 2 hours at room temperature. The column was drained, washed with 5 bed volumes of PBS, and bound immunoglobulins were eluted with 0.2 M glycine-HCl buffer, pH 2.8, into 1 M Tris (final pH 8). Typically, a 2-ml column could remove 90% of the peptide-specific antibodies in 25 ml of serum (-2 mg IgG per 10 ml serum).

Cell Extracts

Whole-cell extracts of MCF-7 and HeLa cells were prepared by releasing cells from monolayer cultures with cell dissociation solution (Sigma) and lysing pelleted cells with 4 volumes of detergent buffer (50 mM Tris, pH 7.4, 400 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.25% NP-40) containing a protease inhibitor cocktail (Landel et al, 1994). Clarified extract was obtained by centrifugation at 10,000 X g. For SDS-PAGE, extracts were mixed with an equal volume of sample buffer (2% SDS, 5% β -mercaptoethanol, 10% sucrose, 50 mM Tris, pH 6.8, and 0.005% bromophenol blue). All samples were heated at 90°C for 5 minutes prior to electrophoresis.

Western Blot Analyses

Proteins from cell extracts were separated by electrophoresis under reducing conditions in 10% polyacrylamide gels, essentially as described previously (Landel et al, 1994) except for the substitution of minigels for larger slab gels. Resolved proteins were then transferred electrophoretically to nitrocellulose (Schleicher and Schuell, Keene, New Hampshire) at room temperature for 1 hour at 200 V in a buffer containing 0.025 M glycine, 0.192 M Tris, and 10% methanol. Lanes containing molecular weight standards were cut out after staining with Ponceau S. Strips were treated with 3% Carnation nonfat dry milk in 50 mM Tris/saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.4) with 0.2% Tween 20, pH 7.4 for 1 hour, and then with purified ER₂₁ (1 μ g/ml) in the presence or absence of a 50-60-fold molar excess

(1 $\mu\text{g/ml}$) of ER21 peptide or with control immunoglobulin for 1 hour, followed by HRP-conjugated goat antibody to rabbit IgG (1:2,000) for 1 hour. All incubations were carried out at room temperature in TBS containing 1% dry milk and 0.2% Tween 20. After each incubation, blots were washed (4 X 5 minutes) with TBS containing 0.2% Tween 20. Immunoreactive bands were visualized by treatment with SuperSignal Substrate for 8 minutes according to the manufacturer's instructions. Moist blots were covered with a clear plastic sheet and exposed to Hyperfilm-MP (Amersham, Arlington Heights, Illinois) for 0.5-5 minutes.

Immunohistochemistry

In preliminary experiments, the only ER antibody to provide consistent results in the male rat tissues was a rabbit polyclonal antisera (ER21) developed against the 21 amino acid N-terminus of the rat/human ER. This antibody is specific for ER vs. ER because the N-terminus region of ER is not conserved and is considerably shorter in length than that in ER (Kuiper et al, 1996). Estrogen receptor-21 antibody does not cross-react with ER (Greene and Kuiper, unpublished data). Small samples of male reproductive tract organs, from the rete testis to the vas deferens, and rat uterus (an ER-positive control) were rapidly collected from 4 to 6-month-old Sprague-Dawley rats, immediately placed in 1 ml of cold (4°C) Hank's balanced salt solution (HBSS), and then irradiated in a microwave oven (600 Watts) for 6 seconds (Slayden et al, 1995). Tissues were then removed, rinsed in cold 0.05 M (PBS), pH 7.3 for 10 minutes, placed in Tissue Tek freezing compound (OCT), and then frozen in liquid propane. Frozen tissue sections (6 μm) were thaw-mounted onto microscope slides and once again microwaved for 2 seconds (Slayden et al, 1995).

Fixation and all subsequent steps were performed at 4°C. Tissues were fixed twice in picric acid-paraformaldehyde-polyvinylpyrrolidone (PVP) (0.2% picric acid, 2% paraformaldehyde, 1.5% PVP; 360,000 mol wt) for 10 minutes. Tissues were then rinsed twice (2 minutes each) with 85% ethanol + 1.5% PVP and PBS + 1.5% PVP and the residual formaldehyde was removed by two rinses (2 minutes each) in 0.05 M Na borohydride in PBS + 1.5% PVP. Following rinses (3X; 2 minutes each) with PBS + 1.5% PVP and PBS + 1.5% PVP + 0.1% gelatin, endogenous peroxidases were inhibited by incubation with 0.3% H₂O₂ in methanol, and the tissues were rinsed again with PBS + 1.5% PVP and PBS + 1.5% PVP + 0.1% gelatin as above. Tissues were then treated with 2% normal goat serum for 30 minutes at 4°C and then incubated overnight at 4°C in a moisture chamber with the ER21 antibody at a concentration of 3.6 $\mu\text{g/ml}$; control tissues were treated as above but were incubated with affinity-purified normal rabbit IgG (4 g/ml). Visualization of antibody binding was performed using the diaminobenzidine-HRP method. Prior to exposure to the biotinylated secondary antibody, nonspecific biotin and avidin binding were minimized by treating the tissues with blocking reagents (Vector Laboratories, Burlingame, California). Following three washes with PBS + 0.1% gelatin, each slide was exposed for 15 minutes at 4°C to 2% normal goat serum and 1 drop avidin D, washed once with PBS + 0.1% gelatin, and then exposed for 15 minutes to 2% normal goat serum and 1 drop of the biotin-blocking solution.

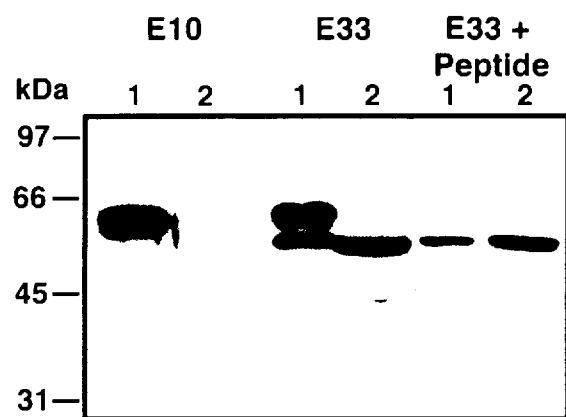


FIG. 1. Western blot analysis of whole-cell extracts from MCF-7 cells (lane 1) or HeLa cells (lane 2). Proteins were separated by sodium dodecyl sulfate-sodium acrylamide gel electrophoresis (SDS-PAGE) on a 10% minigel and transferred to nitrocellulose. E10 and E33 represent the results for affinity-purified estrogen receptor 21 (ER21) antibodies obtained from two different rabbits. Strips were incubated with affinity-purified ER21 (1 $\mu\text{g/ml}$) in the absence or presence (E33 + peptide) of a 60-fold molar excess of ER21 peptide (1 $\mu\text{g/ml}$). Immunoreactive bands were visualized with SuperSignal substrate, as described in Materials and Methods. The positions of molecular weight markers (kDa) are shown on the left. The immunoreactive estrogen receptor (ER) band is at 65 kDa.

Tissues were exposed for 45 minutes to biotinylated secondary antibody, washed 3X with PBS + 0.1% gelatin, and then incubated for 45 minutes at room temperature with an avidin-biotin complex prepared according to instructions included with the kit. Following three washes with PBS + 0.1% gelatin and three washes with 0.03 M Tris buffer (pH 7.6), matched control and ER-antibody slides were incubated with 0.05% 3,3'-diaminobenzidine and 0.003% H₂O₂ in 0.03 M Tris buffer. The reaction was monitored microscopically and was stopped when a brown positive-reaction product was observed on the ER-antibody-containing slide. The reaction was terminated by three washes in 0.03 M Tris buffer. Tissues were dehydrated in a graded series of ethanol and mounted with Permount. Both E10 and E33 antibody preparations, representing antibodies from two different rabbits, were tested. The E10 antibody (Fig. 1) was used in the illustrations displayed (Fig. 2). E33 gave equivalent nuclear staining but showed a greater cytoplasmic background staining, probably due to the unknown protein contamination seen in the western blot (Fig. 1).

Male reproductive tissues from three different rats (90 days of age) were processed for immunohistochemistry in separate trials to replicate the results. Staining reactions were observed with an Olympus Vanox Photomicroscope with planapochromatic lenses. Images were captured with a Sony Digital Photo Camera DKC-5000 using Adobe Photoshop and printed using the Kodak Digital Science 8650 dye-sub printer.

To verify that the polyclonal ER21 antibody bound specifically to rat ER in the immunohistochemical reaction, the antibody preparation (3.6 $\mu\text{g/ml}$) was incubated for 30 minutes at 4°C with the N-terminal peptide (4 $\mu\text{g/ml}$) against which the antibody was developed. The complex was applied to efferent ductule and epididymal tissues as the primary antibody.

Steroid Autoradiography

Ductuli efferentes were excised, placed into Ham's F-12 medium, and cut into 1-mm² pieces. The tissue was then placed in 1 ml of HBSS that contained 2% bovine serum albumin (BSA) and 10 nM [³H]-estradiol (3H-E₂; 130-170 Ci/mmol, New England Nuclear, Boston, Massachusetts) at 37° C. Simultaneous competition studies were performed by adding unlabeled E₂ to the wells containing the tissue and 3H-E₂ so that the final concentration of the cold hormone was a 1,000-fold excess. Tissues were incubated with 3H-E, for 2 hours, washed with constant stirring in HBSS that contained 2% BSA, and prepared for autoradiography as previously reported (Rogers, 1973; Cooke et al, 1991).

RNA Isolation

Ductuli efferentes and regions of the epididymis (initial segment, caput/corpus, and cauda) and vas deferens were isolated in ice-cold Ringer's solution and placed in microcentrifuge tubes. All tissues were frozen rapidly in liquid nitrogen. Total RNA was isolated by the guanidinium isothiocyanate/phenol chloroform method (Sambrook et al, 1989).

Northern Analysis

Total RNA (10 µg) was separated on a 1.5% agarose gel and blotted to nylon membranes by capillary transfer. Membranes were hybridized in QuikHyb solution with ³²P-labeled cDNA probes made from isolated inserts of cloned rat ER cDNA. Northern blots were checked for loading variations by a final hybridization with 28S rRNA cDNA probe. Relative quantification was determined using a PDI scanning densitometer and Discovery software.

Reverse Transcriptase-Polymerase Chain Reaction

Reaction

Total RNA was extracted from each tissue and 1 µg was reverse-transcribed to single-stranded complementary DNA (cDNA) using the 3' RACE protocol and Superscript reverse transcriptase (Gibco BRL, Gaithersburg, Maryland). Polymerase chain reaction amplification of ER and ER were carried out on equal quantities of cDNA products (2 µl) using a PTC-150 minicycler and "Touchdown" PCR conditions (MJ Research Inc., Waltham, Massachusetts). Polymerase chain reaction products were run out on a 3% Nusieve 3:1 agarose gel and visualized by ethidium bromide staining under ultraviolet (UV) light. Complete reverse transcriptase-polymerase chain reaction (RT-PCR) amplifications were carried out three times to confirm the presence of ER and ER for all tissues tested.

Primers

Rat ER primers were chosen using Primer Designer (Scientific and Educational Software, State Line, Pennsylvania) and numbered according to GENBANK notation (the left primer being 1176L [GCCT-FATTGGATGCTGAACC] and the right primer being 1804R [TTGTAGAGATGCTCCATGCC]). Rat ER primers, numbered according to GENBANK notation, were provided by Dennis Lubahn, University of Missouri, and were made to span exon 9 of rat ER. Location of the primers were: left primer, 1689L (AACAGGGCATGGAACATCTGCT), and right

primer, 1893R (TCCGCCTCAGGCCTGGCCATCA). As ER and ER sequences are very similar, great care was taken in designing the primers so that the important polymerase priming site, the 3' end of the oligonucleotide primers, was sufficiently dissimilar between the two genes to prevent 3'-end hybridization to the ER cDNA. The ER left primer was made from a region of the gene that is dissimilar from ER with only one out of six of the 3'-most nucleotides matching the ER sequence. The ER right primer was made from a region that has high similarity to ER. However, the three nucleotides at the 3' end are dissimilar. Polymerase chain reaction runs using one ER primer pair with one ER primer did not yield any amplification product.

Results

Western Blot Analysis

Affinity-purified ER21 antibodies (E10 and E33), obtained from two different rabbits, were used to analyze proteins in whole-cell extract from MCF-7 (ER-positive) and HeLa (ER-negative) cells (Fig. 1). Using the E10 preparation, a single immunoreactive ER band was observed at 65 kDa in the MCF-7 cell extract. No immunoreactive bands were observed in the HeLa extract. Using the E33 preparation, the same 65 kDa ER band was observed in MCF-7 cell extract, but there was also a nonspecific band at 60 kDa in both cell extracts. In the presence of a 60-fold molar excess of ER21 peptide, the 65-kDa band was absent leaving only the nonspecific band. Thus, the ER21 antibody appears to be specific for full-length ERα (Greene et al, 1986). Both antibody preparations resulted in identical patterns of nuclear staining in the male reproductive system with immunohistochemistry. However, the E33 preparations gave increased background cytoplasmic staining.

Immunohistochemistry

Positive immunostaining for ERα was observed in nuclei of both epithelial and stromal components (connective tissue) of the excurrent ducts of the male rat reproductive tract (Fig. 2). The strongest reaction was observed in the cells of the ductuli efferentes (Fig. 2B). Both ciliated and nonciliated epithelial cells exhibited very strong immunostaining (Fig. 2B,C), as did the smooth muscle surrounding the ductules and blood vessels in the connective tissue stroma (Fig. 2I). Fibroblasts (Fig. 2I) and other connective tissue cells possessed immunoreactivity of varying intensities. Nuclei of endothelial cells lining the blood vasculature were also strongly positive for ER immunostaining (Fig. 2I).

In the initial segment of the epididymis, moderately strong immunoreactivity was seen in all epithelial cell types (Fig. 2E) as well as in the peritubular smooth muscle. In the caput, corpus, and caudal epididymides (Fig. 2F,G,H), the intensity of nuclear staining was greatly reduced.

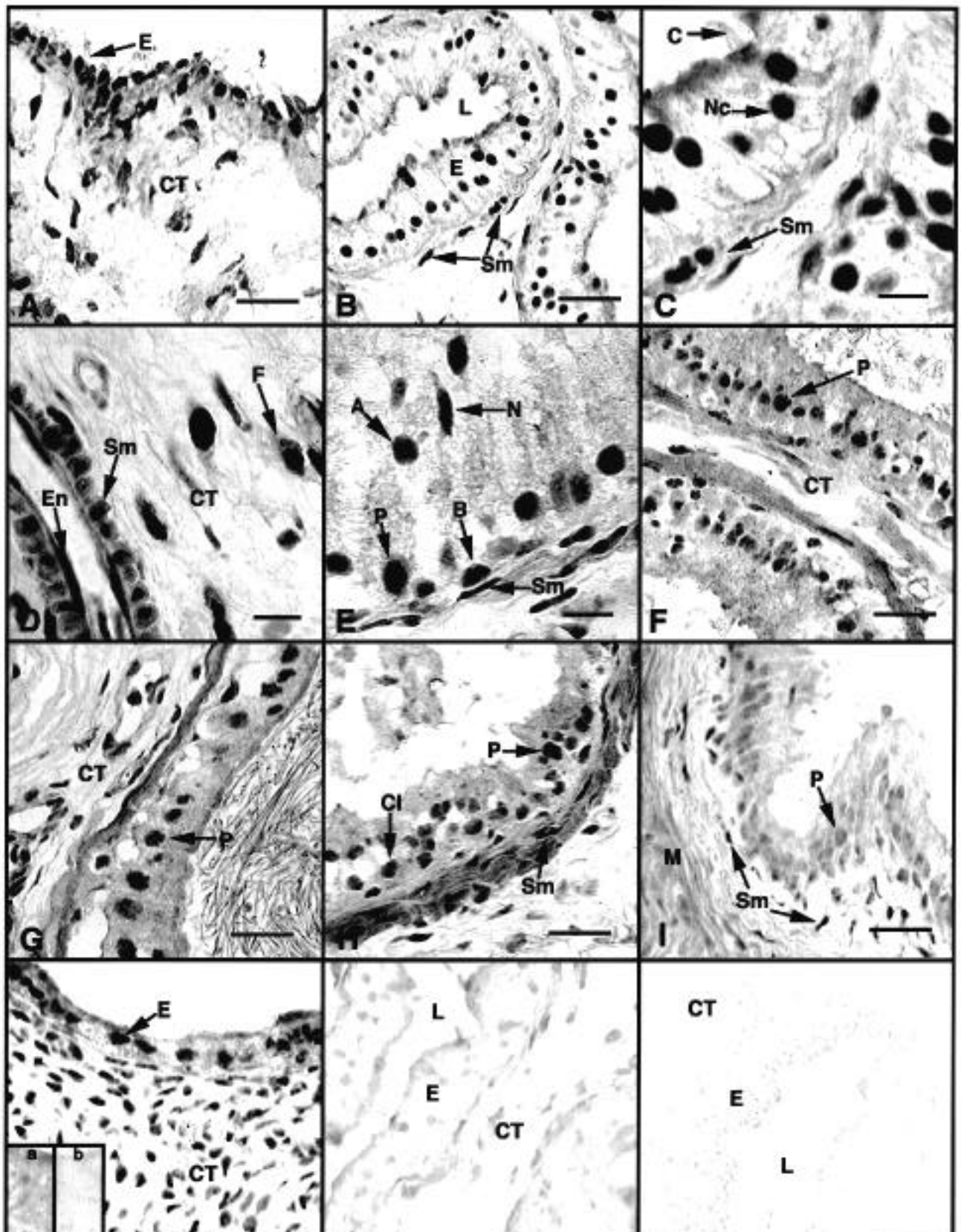


FIG. 2. Immunohistochemical localization of the estrogen receptor in the male rat reproductive tract (A-I) using the ER21 antibody. Control sections are seen in J-L. (A) Rete testis. Nuclei of epithelial cells (E) of the rete are positive as well as some cells in the underlying connective tissue (CT). Bar = 25 μ m. (B) Ductuli efferentes. Nuclei of epithelial (E) cells lining the lumen (L) and smooth muscle cells (Sm) show strong immunoreactivity.



FIG. 3. Autoradiogram of 1H-E, binding in the ductuli efferentes of the rat. (A) Strong nuclear 3H-E binding is present in the epithelial cells (Nu), and weaker binding is noted in the smooth muscle cells (Sm) lining the epithelium. Bar - 5 μ m. (B) The 3H-E, was competed with an excess of nonradioactive E, which abolishes the specific binding of 1H-E, seen in Figure 3A. Nu, nucleus of an epithelial cell; Sm, smooth muscle cell.

duced in epithelial cells compared to that seen in the ductuli efferentes and initial segment. Vas deferens epithelium was negative or only minimally stained. The peritubular smooth muscle cells were also immunopositive along the length of the epididymis, except in the vas deferens. In the vas deferens, ER immunoreactivity was seen in smooth muscle cells of the subepithelial connective tissue but not in the outer muscularis (Fig. 21).

Control Tissues

Rat uterus was used as an ER-positive control tissue and incubated with an ER21 antibody titer identical to that used for the male tissue. Both uterine epithelium and stroma were ER-positive (Fig. 2J), as previously reported (Gee et al, 1990). When normal rabbit IgG (Fig. 2J[a]) was used in place of ER21, only a minimal level of nonspecific staining was detected. This slight immunoreactivity was completely abolished when ER21 antibody was preincubated with the peptide against which the ER21 antibody was developed (Fig. 2J[b]). Similarly, male tissues incubated with normal rabbit IgG instead of ER21 antibody displayed no specific immunoreactivity (Fig.

2K). Preincubation of the primary antibody with the peptide eliminated all immunostaining in the male reproductive tissues in both the epithelium and stroma. The efferent ductule tissue is shown in Figure 2L.

Steroid Autoradiography

To determine whether the ER immunostaining in the male reproductive system represents a functional receptor capable of binding estrogen, we performed 1H-E2 autoradiography. Epithelial cells of the ductuli efferentes showed strong nuclear localization of the radioactive steroid (Fig. 3A). However, the stromal cell nuclei contained fewer silver grains than the epithelial cells. An excess of unlabeled E, abolished this localization (Fig. 3B).

RNA and RTPCR Analysis

Segments of rat reproductive tract (ductuli efferentes, initial segment, caput/corpus, cauda, and vas) were subjected to northern analysis for ER α . Control tissues included were uterus (strongly positive) and skeletal muscle (greatly reduced levels of ER). As seen in Figure 4, high levels of ER α mRNA were detected in the ductuli efferentes and

Bar = 25 μ m. (C) Ductuli efferentes at higher magnification. Nuclei of both ciliated and nonciliated (Nc) cells are intensely stained. Cilia (C) are noted projecting into the lumen. A strong immunostaining for estrogen receptor was also found in the smooth muscle cells (Sm). Bar = 10 μ m. (D) Connective tissue area of the ductuli efferentes. Nuclear estrogen receptor-positive cells associated with the small arteriole include the endothelium (En) and the surrounding smooth muscle (Sm). The connective tissue (CT) contains numerous estrogen receptor-positive cells including fibroblast-like cells (F). Bar = 10 μ m. (E) Initial segment epididymis. All epithelial cells of this region are estrogen receptor-positive but appear to show slightly less immunoreactivity than seen in the ductuli efferentes. Nuclei of basal (B), principal (P), apical (A), and narrow (N) cells are stained. Beneath the epithelium, the thin nuclei of smooth muscle cells (Sm) are also positive. Bar = 10 μ m. (F) Caput epididymis. Nuclei of the principal (P) epithelial cells are slightly positive, as well as the smooth muscle cells in the connective tissue (CT). Bar = 25 μ m. (G) Corpus epididymis. Nuclei of the principal (P) epithelial cells and cells within the connective tissue (CT) show slight immunoreactivity to estrogen receptor. Bar = 25 μ m. (H) Cauda epididymis. Nuclei of the principal (P) and clear cells (C) are slightly positive, whereas the smooth muscle cells (Sm) appear to be strongly positive. Bar = 25 μ m. (I) Vas deferens. Principal cell nuclei (P) lining the lumen of the vas show little more than background staining. However, the smooth muscle cells (Sm) just beneath the epithelium are positive for estrogen receptor, while the muscularis (M) appears negative. Bar = 25 μ m. (J) Adult rat uterus. The uterine tissue was treated simultaneously with the male reproductive tissues, and estrogen receptor-positive nuclei are noted in the epithelium (E) and connective tissue (CT). The small insets are controls for the uterus. (a) Tissues were incubated with an affinity-purified normal rabbit IgG instead of the primary antisera. (b) The antibody preparation was incubated first with the N-terminal peptide against which the antibody was developed. Bar = 25 μ m. (K) Control slide of the ductuli efferentes. The tissues were incubated with an affinity-purified normal rabbit IgG instead of the primary antisera. Only a slight background staining is noted in the nucleus and cytoplasm of the epithelial cells (E) and cells of the connective tissue (CT). L, lumen of the ductule. Bar = 25 μ m. (L) Control slide of the ductuli efferentes. The antibody preparation was incubated first with the N-terminal peptide of the rat estrogen receptor, against which the antibody was developed. No staining is noted in nuclei of the epithelium (E) or in the connective tissue (CT). L, lumen of the ductule. Bar = 25 μ m.

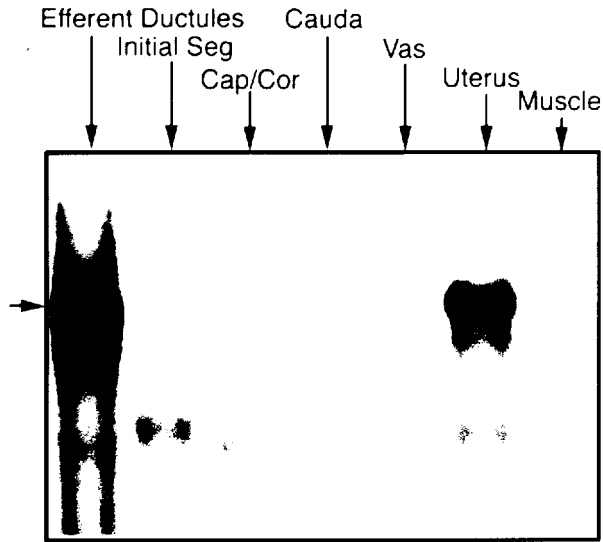


FIG. 4. Northern analysis of estrogen receptor (ER α) mRNA expression of total RNA (10 μ g each) isolated from the tissues indicated above each lane. The arrow indicates the position of the ER α mRNA.

uterus. However, levels of ER α were greatly reduced in all other tissues; the next strongest message was found in the initial segment, which was comparable to that found in skeletal muscle. In the figure shown, message in the caput/corpus was negligible. Normalization of densitometric data to 28S rRNA levels on the northern blot indicated that the ductuli efferentes expressed 3.5-fold more ER α mRNA than uterus per unit of total RNA. With the ductuli efferentes expression set at 100%, the relative expressions of ER α mRNA in the other tissues was 0.92% (initial segment), 1.0% (caput/corpus), 0.76% (cauda), 1.0% (vas), 28.9% (uterus), and 0.92% (skeletal muscle).

For comparison, the presence of ERP and ER α mRNA in the different organs was determined by Touchdown PCR on the same cDNA using either ER α or ERP primers (Fig. 5). Although the PCR results are not quantitative, they show that both ER α and ERP mRNA are present throughout the male excurrent ducts and in the control tissues used, the prostate and uterus.

Discussion

This study has determined that excurrent ducts of the adult male rat reproductive tract express estrogen receptor. In one region, the ductuli efferentes, the magnitude of ER α mRNA expression exceeds that in female reproductive organs such as the uterus. Estrogen receptor α was localized by immunohistochemistry in the epithelium of rete testis, ductuli efferentes, and the initial segment and caput, corpus, and cauda epididymides of the adult male rat but was absent or greatly reduced in the vas deferens epithelium. The ductuli efferentes showed the most intense immunoreactivity and contained nearly 3.5 \times as much ER α mRNA per milligram of total RNA as did the rat uterus. Smooth muscle cells were ER α -positive throughout the tract, although ER α staining was limited to the subepithelial layer of smooth muscle in the vas deferens. Steroid autoradiography demonstrated that the ER located in the ductuli efferentes was functional and capable of binding estrogen. Immunohistochemical results for ER α indicated more intense staining in ductuli efferentes and the initial segment of the epididymis but dramatically less staining in the remaining tissues. Estro

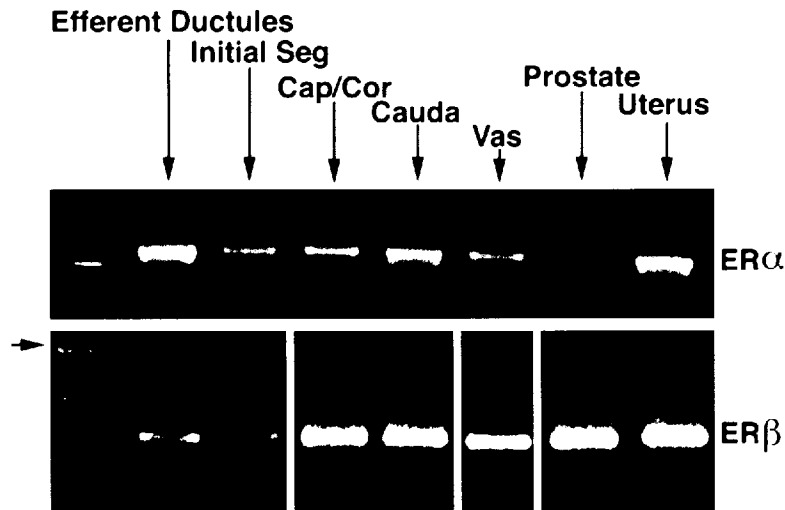


FIG. 5. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of estrogen receptor α (ER α) and ERP using Touchdown PCR conditions. Lane 1 in both (top) and (bottom) panels is a 100-bp ladder with the 500-bp band showing as the brightest band. The top panel shows amplification of a 628-bp ER α cDNA band from RNA isolated from the tissue sources as indicated above each lane. The lower panel shows the results of using the same cDNA as used in the top panel but with ERP primers to amplify a 204-bp ER β cDNA band. The arrow on the left of the bottom panel shows the position of the 500-bp size marker.

gen receptor mRNA in ductuli efferentes was also exceedingly high, but the initial segment and remaining tissues expressed greatly reduced amounts of ER message. Because of the recent discovery of an ER (Kuiper et al, 1996, 1997; Mosselman et al, 1996), we decided also to look for the presence of ER mRNA using RT-PCR (currently the only reliable method to specifically detect its presence). Estrogen receptor β was detected in all of the different regions of the epididymis, and in the ductuli efferentes, vas deferens, and prostate. This data is consistent with the recent report that ER can be detected by PCR in testis, epididymis, and prostate (Kuiper et al, 1997). The data presented here is the first demonstration of ER in all regions of the epididymis and in the ductuli efferentes. These findings indicate that ER needs to be taken into account in future studies of ER function.

This is the first study to localize ER in the excurrent ducts of the male rat using an antibody (ER21) directed against the N-terminal region of rat ER. Estrogen receptor 21 does not cross-react with ER (Greene and Kuiper, unpublished data). Using this antibody, ER showed nuclear localization in epithelia of both ductuli efferentes and epididymis, similar to that in the adult mouse (Iguchi et al, 1991). Also similar to the mouse, the epithelium of the vas deferens was negative, but the surrounding stromal cells were positive. However, in contrast to the mouse, rat connective tissue in the ductuli efferentes and caput epididymidis was positive for ER, whereas these regions were negative in the mouse (Iguchi et al, 1991). The strong staining of ductuli efferentes epithelium in the rat is consistent with data collected from other species (Schleicher et al, 1984, 1989; West and Brenner, 1990; Goyal et al, 1997). However, in the goat (Goyal et al, 1997) and monkey (West and Brenner, 1990), ER was found exclusively in the epithelium of the ductuli efferentes. Furthermore, in the goat and monkey, ER was observed in a single cell type, the nonciliated cell. In the rat, the ciliated cell nucleus appeared as strongly ER-positive as did the nonciliated cells of the efferent ductules. Estrogen receptor has been reported in the epididymis of several species, including the mouse (Iguchi et al, 1991), rabbit (Danzo and Eller, 1979; Toney and Danzo, 1988), ram (Tekpetey and Amann, 1988), dog (Younes and Pierrepoint, 1981), and human (Murphy et al, 1980; Palacios et al, 1993).

In the rat, nuclei of peritubular fibroblasts, vascular smooth muscle cells, and endothelial cells possessed similar immunoreactivity to ER. Unlike the epithelium, however, immunoreactivity in smooth muscle did not appear to diminish in the distal tubules. In fact, the only significant ER immunoreactivity seen in the wall of the vas deferens was the thin smooth muscle layer of the submucosa. In the mouse epididymis, binding of ^3H -estradiol by peritubular smooth muscle cell nuclei was re-

duced compared to the epithelium and to a lesser extent than in fibroblasts (Schleicher et al, 1984). In the rat, moderate to strong ER immunostaining of muscle layers was observed, which is similar to staining in the female reproductive tract (Slayden et al, 1995). The presence of ER in smooth muscle cells is especially important because in other male reproductive organs they are a major target of estrogen during development, which changes the expression of ER during adulthood (Prins and Birch, 1997). Also, similar to the female, endothelial cells of the blood vascular system exhibited ER α staining (Leiberman et al, 1990; Whitaker et al, 1994; Farhat et al, 1996). Therefore, future studies that compare stromal and epithelium localizations of the two receptors will be important in deciphering their respective functions.

One difficulty of using ligand-binding data and molecular analysis alone is the lack of cellular localization of ER. With steroid autoradiography, localization of E $_2$ binding to specific cell types is possible, but this method does not differentiate between ER and ER binding. Therefore, the combination of methods used in the present study support the hypothesis that ER is distributed extensively along the length of the male reproductive tract. However, the distribution may depend upon the region and the type of receptor. Estrogen receptor is most abundant in the ductuli efferentes.

The major function of the ductuli efferentes, the region containing the highest concentration of ER, is the reabsorption of fluid, ions, and other solutes (Ilio and Hess, 1994). These ductules are unique in that they reabsorb over 90% of the luminal fluid in the rat (Jones and Jurd, 1987; Clulow et al, 1994). Past studies have suggested that substances other than androgens alone are needed to maintain normal function in the head of the epididymis, including the ductuli efferentes (Fawcett and Hoffer, 1979; Hermo and Morales, 1984; Douglass et al, 1991), but only recently have studies recognized estrogen as one of those potential factors. The first paper to describe an ER knockout mouse (ERKO) showed that animals can survive without ER. However, ERKO males were infertile (Lubahn et al, 1993). Interestingly, the seminiferous tubules, rete testis (Eddy et al, 1996), and ductuli efferentes (Lubahn, unpublished data) were dilated in the ERKO males, which suggests that loss of a functional ER results in diminished fluid reabsorption in the adult male. Since sperm pass rapidly through the ductuli efferentes (English and Dym, 1981) and must become concentrated before entering the epididymis, it is possible that the reabsorptive function of the ductules is linked to sperm output. One mechanism by which this link could be established is the recent discovery that testicular germ cells and sperm contain active P450 aromatase and are capable of converting androgens to estrogens (Nitta et al, 1993; Janulis et al, 1996a,b). Thus, an additional source of es

trogen present in rete testis fluid (Free and Jaffe, 1979) may be the germ cells, which could then modulate epithelial functions as sperm transit the ductuli efferentes. Whether such a physiological mechanism is plausible remains to be determined.

The epididymis also reabsorbs fluid, but its main function appears to be the secretion of proteins that interact with sperm during storage (Robaire and Hermo, 1989; Cornwall and Hann, 1995). It is not clear what role estrogen might have in the epididymal epithelium. The apical and narrow cells of the initial segment have been identified as ER-positive in mouse and human (Palacios et al, 1993; Echeverria et al, 1994) and are positive in the rat, but their functions appear to be related to ion transport, rather than to protein secretion (Adamali and Hermo, 1996).

In conclusion, ER protein and mRNA predominate in the ductuli efferentes and head of the epididymis. The ductuli efferentes have a uniquely high concentration of ER α mRNA that is substantially greater than even that expressed in the uterus. The presence of ER in both the epithelium and connective tissues suggests that estrogen could play an important role in the regulation of the excurrent ductal system. Based upon the overwhelming presence of ER in the head of the epididymis and the abnormal changes in this region in the ERKO mouse, the emphasis of future studies will be to determine the potential relationship between estrogen and fluid reabsorption in the male reproductive tract. However, the presence of ER in efferent ductules and epididymis raises the possibility that multiple functions will be assigned to ERs in the male reproductive tract. An understanding of the functions of ER α and ER in the adult will help to uncover the mechanisms of environmental toxicity due to perinatal exposures to estrogenic compounds such as diethylstilbestrol, which produces abnormalities in the head of the epididymis in both humans and rodents (McLachlan et al, 1975; Gill et al, 1979).

Note Added in Proof-A recent publication by Fisher et al also reported localization of ER in the adult male rat reproductive tract using a different antibody. They report ER staining only in Leydig cells and epithelial cells of rete testis and efferent ductules. Fisher JS, Millar MR, Majdic G, Saunders PTK, Fraser HM, Sharpe RM. Immunolocalisation of estrogen receptor within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *J Endocrinol* 1997;153:485-495.

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