

Estrogen Receptors Are Present in the Epididymis of the Rooster

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ABSTRACT: Our recent discovery that testicular germ cells and epididymal sperm contain active P450 aromatase suggests that the reproductive tract may be a target for estrogen. Therefore, the objective of this study was to determine if estrogen receptors (ER) are present in the avian epididymis using immunocytochemistry, northern blot analysis, and in situ hybridization. Immunoperoxidase staining for ER was found principally in nuclei of nonciliated epithelial cells of proximal and distal efferent ductules and the epididymis duct. The ciliated cells also appeared to be slightly positive in the efferent

ductules than in epithelial cells of the epididymal duct or connective tissue cells. Strong specific hybridization signals for ER mRNA corresponded to the same areas exhibiting immunocytochemical localization. The presence of ER mRNA in the epididymis was confirmed by northern blot analysis, which showed a single band corresponding to approximately 7.8 kb, similar to that found in chicken oviduct. Based on these data, we suggest that the efferent ducts of the rooster are a primary target for estrogen and that estrogen may have a role in the regulation of avian epididymal function.

The avian epididymis, consisting of the efferent ducts (ductuli efferentes), connecting ducts (ductuli conjungentes), and the epididymis duct (ductus epididymidis), plays an important role in the resorption of luminal fluids from the testis (Clulow and Jones, 1988). Although sperm traverse this region of the male reproductive tract rapidly, these ducts resorb nearly 90% of the testicular plasma output before the sperm are stored for an extended period in the ductus deferens.

Androgens are considered the major hormones involved in the regulation of epididymal functions in mammals (Robaire and Hermo, 1988). However, there is considerable evidence that structure and function of the efferent ductules and the initial segment of the epididymis are also dependent on other luminal factors derived from the testis (Fawcett and Hoff, 1979; Douglass et al, 1991). The avian species contains homologous structures to the mammalian epididymis, i.e., a fluid-resorbing epithelium with ciliated cells (Tingari, 1972; Clulow and Jones, 1988); therefore, it is possible that some regions of the avian epididymis are less responsive to androgens and are regulated by other testicular factors.

One of these nonandrogen factors may be estrogen. In

some mammals, estrogen is found in higher concentrations in epididymal luminal fluid than in blood plasma (Free and Jaffe, 1979), which raises a question regarding the role of estrogen in the male reproductive tract. Our recent discovery that testicular germ cells and epididymal sperm in the rooster, bear, and mouse contain P450 aromatase (Nitta et al, 1993; Tsubota et al, 1997; Kwon et al, 1995; Janulis et al, 1996) suggests to us that the reproductive tract of several species may be a target for estrogen. Estrogen receptors (ER) have been localized in the male reproductive system of at least four mammalian species, with the highest labeling in the efferent ductules (Younes et al, 1979; Schleicher et al, 1984; Tekpetey and Amann, 1988; Toney and Danzo, 1988; West and Brenner, 1990; Cooke et al, 1991; Greco et al, 1992; Sata et al, 1994). However, the presence of ER in the avian male reproductive tract has not been determined. Therefore, the purpose of the present study was to determine if ER are present in the avian epididymis using immunocytochemistry, in situ hybridization, and northern blot analysis.

Materials and Methods

Animals

Mature, single-comb White Leghorn roosters were housed under a 15L:9D lighting schedule and provided feed and water ad libitum.

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Immunocytochemistry

The region containing the efferent ductules, connecting ducts, and epididymal duct was removed from 15 adult roosters. The tissue was placed in a drop of OCT medium (Tissue-Tek, Elkhart, Indiana), and frozen in isopentane (-196° C) immediately. Frozen tissues were sectioned at a 5 µm thickness in a cryostat microtome at -20° C. The protocol for immunocytochemistry was followed as previously described (Prins et al, 1991). Tissue sections were thaw-mounted on pre-cleaned glass slides and freeze-substituted for 2 days in anhydrous acetone containing calcium chloride at -80° C. The frozen sections were fixed in 0.2% picric acid, 2% paraformaldehyde, and 1.5% polyvinylpyrrolidone in 0.1 M phosphate-buffered saline (PBS) (pH 7.3) for 10 minutes at 4° C (Brenner et al, 1990). Sections were rinsed, treated with 2% goat serum (Vector, Burlingame, California) to reduce background staining and then incubated with a monoclonal anti-human estrogen receptor antibody (H-222) kindly provided by Dr. Geoffrey L. Greene, University of Chicago. Control sections were treated with rat immunoglobulin (Sigma, St. Louis, Missouri) in place of the primary antibody at the same concentration (5 µg/ml) as the primary antibody. Sections were then processed with a Vectastain® elite ABC kit (Vector, Burlingame, California), an avidin-biotin-peroxidase method. Peroxidase was visualized with a diaminobenzidine-H₂O₂ solution.

In Situ Hybridization

Fifteen adult roosters were anesthetized with sodium pentobarbital and then perfused through the ischiatic artery with 0.9% saline containing heparin until blood vessels were clear, followed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 20 minutes. The region containing efferent ductules, connecting ducts, and epididymal duct was removed and cut into thin cross-sections. Tissue slices were postfixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 3 hours at 4° C. Fixed tissues were dehydrated in a graded series of ethanol and embedded in paraffin. Histological sections (5 µm) were placed on pre-cleaned glass slides and dried in an oven at 37° C overnight. A 565-bp EcoRI-BspMI fragment of the plasmid P91023-B, coding for chicken ER sequence (kindly provided by Dr. Bert W. O'Malley, Baylor College of Medicine, Houston, Texas) was subcloned into the polylinker region of the transcription vector, pBluescriptHKS (Stratagene, La Jolla, California). The orientation of the cDNA insert in the sense or antisense direction with respect to the T7 promoter was confirmed by restriction mapping. After linearization with KpnI, the plasmid was transcribed by T7 polymerase using (α-³⁵S) uridine 5'-triphosphate (UTP) (Amersham Life Science, Arlington Heights, Illinois) to produce an antisense RNA probe using the MAXI script *in vitro* transcription kit (Ambion, Austin, Texas) according to the manufacturer's protocol. The plasmid DNA, which did not contain the cDNA insert for chicken ER, was linearized with AflIII and transcribed by T7 polymerase to make a cRNA probe for a negative control. In situ hybridization was carried out with a Sure Site®II system (Novagen, Madison, Wisconsin) according to the manufacturer's protocol. Briefly, tissue sections (5 µm) were rehydrated, deproteinized, acetylated, and hybridized

with the ³⁵S-labeled probes (1 × 10⁶ cpm/slide) at 50° C overnight. After hybridization, tissue sections were washed and then autoradiographed with NTB2 nuclear-track emulsion (Eastman Kodak, New Haven, Connecticut) for 1 week at 4° C. Slides were developed with Kodak D-19 (Eastman Kodak, New Haven, Connecticut) and counterstained with hematoxylin.

Northern Blot Analysis

Total RNA was isolated from the rooster epididymal tissue and chicken oviduct by the guanidinium isothiocyanate/phenol method as previously described (Bunick et al, 1994). Poly A+RNA was then prepared from isolated total RNA using the PolyATtract™ mRNA isolation kit (Promega, Madison, Wisconsin). Equal amounts of poly A+RNA (5 µg) from the epididymal tissues of the rooster and chicken oviduct were electrophoresed on a 1.5% agarose formaldehyde gel. The gel was blotted to a nylon membrane and the RNA was fixed to the membrane by baking at 80° C in a vacuum oven for 2 hours (Bunick et al, 1994). A 1.9-kb cDNA fragment for chicken ER was isolated from the plasmid vector (P91023-B) by restriction digestion and gel purification. The cDNA insert was labeled with (α-³²P) deoxycytidine 5'-triphosphate (dCTP) (Amersham Life Science, Arlington Heights, Illinois) using the Prime-a-GeneO labeling kit (Promega, Madison, Wisconsin). Hybridization was carried out in QuikHyb150 rapid-hybridization solution (Stratagene, La Jolla, California), according to the manufacturer's recommendations, at 65° C in a Robbins Scientific hybridization oven (Sunnyvale, California). The hybridized membrane was washed in 2X standard saline citrate (SSC; 1 X SSC; 0.3 M NaCl, and 0.03 M sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) for 30 minutes at room temperature, followed by 1 X SSC, 0.1% SDS for 30 minutes at 55° C. The membrane was then covered with a plastic wrap and exposed to a Kodak X-omat X-ray film (Eastman Kodak, New Haven, Connecticut) with an intensifying screen for 90 minutes at -80° C.

Results

Figure 1 presents the gross morphology of the rooster's rete testis, proximal efferent ductules, distal efferent ductules, and epididymal duct and the cellular morphology of a mucosal fold of the rooster proximal efferent ductule. In the rooster, unlike the mammal, the epididymis is a highly convoluted structure consisting of many mucosal folds.

Immunocytochemistry

Estrogen receptors were localized in the epididymis of the rooster by immunocytochemistry. Strong immunoreactivity was present in the nuclei of epithelial cells of proximal efferent ductules (Fig. 2A). Connective tissue also showed weak immunostaining (Fig. 2A). Control samples showed no specific nuclear staining when normal rat im

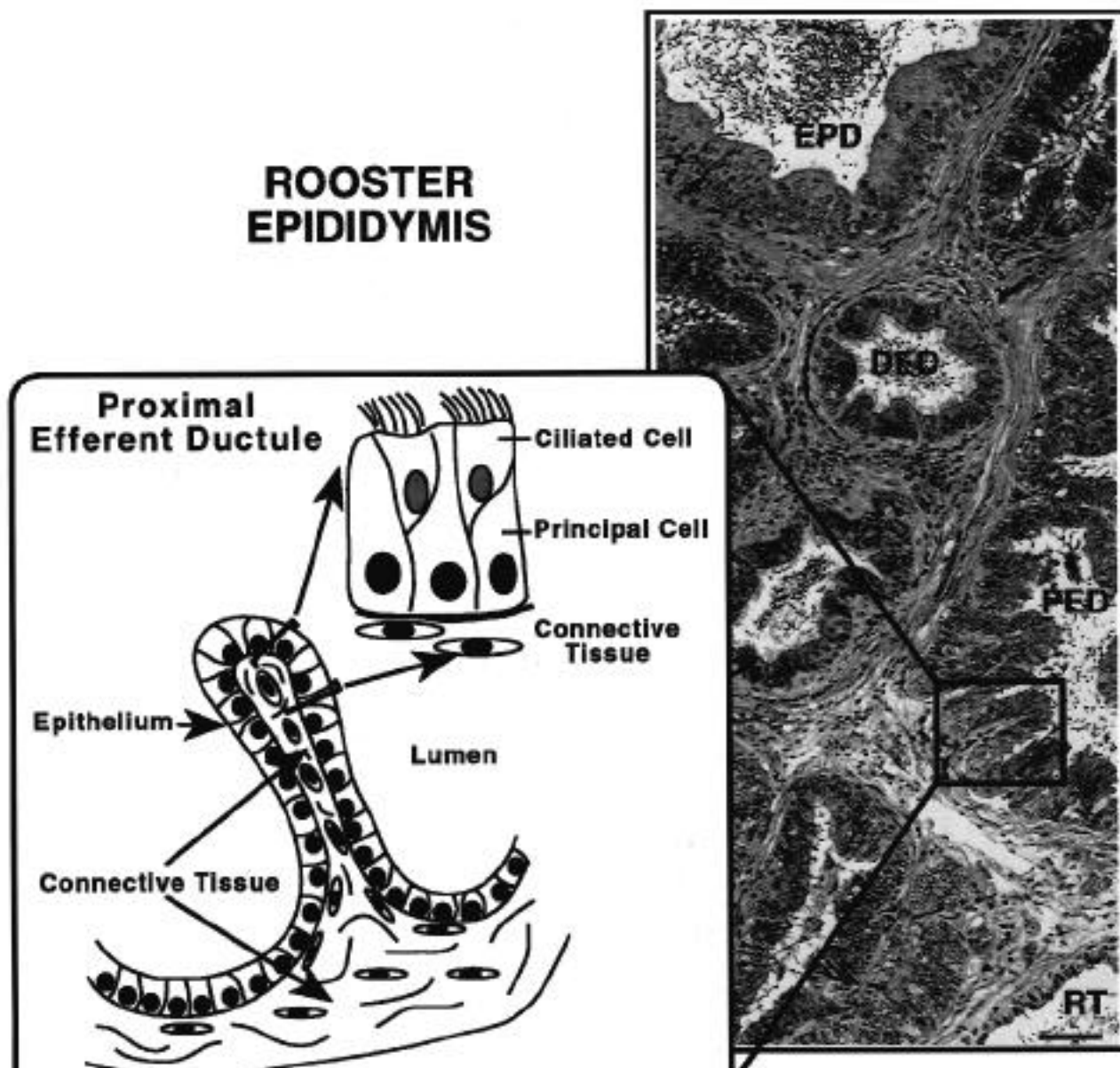


FIG. 1. Gross morphology of the rooster's rete testis (RT), proximal efferent ductules (PED), distal efferent ductules (DED), and epididymal duct (EPD) and the cellular morphology of a mucosal fold of the rooster efferent ductules. The epithelium (outer layer of cells) consists of ciliated and nonciliated (principal) cells. The inner core of tissue is connective tissue. The nuclei of ciliated cells are found in the apical region of the epithelium, whereas the nuclei of the nonciliated cells are located in the basal

munoglobulin was substituted for the primary antibody (Fig. 2B). Nuclei of epithelial cells in the rete testis showed very slight specific staining (Fig. 2C). In the proximal efferent ductules, specific immunostaining for ER was found in the nuclei of nonciliated cells. In these cells, nuclei are located typically at the base of the cell (Tingari, 1971). The ciliated cells appeared to be only slightly positive (Fig. 2D). Nuclei of nonciliated epithelial cells in distal efferent ductules were also stained intensely (Fig. 2E). In the epididymal duct, nuclear immunostaining

was moderately intense in the principal cell nuclei (Fig. 2F). The intensity of immunostaining for ER was more intense in epithelial cells of the efferent ductules than in connective tissue cells or epithelial cells of the epididymal duct (Fig. 2D-F).

In Situ Hybridization

Specific hybridization signals for ER mRNA were found primarily in epithelial cells of the proximal efferent ductules (Fig. 3A,B, low magnification; Fig.

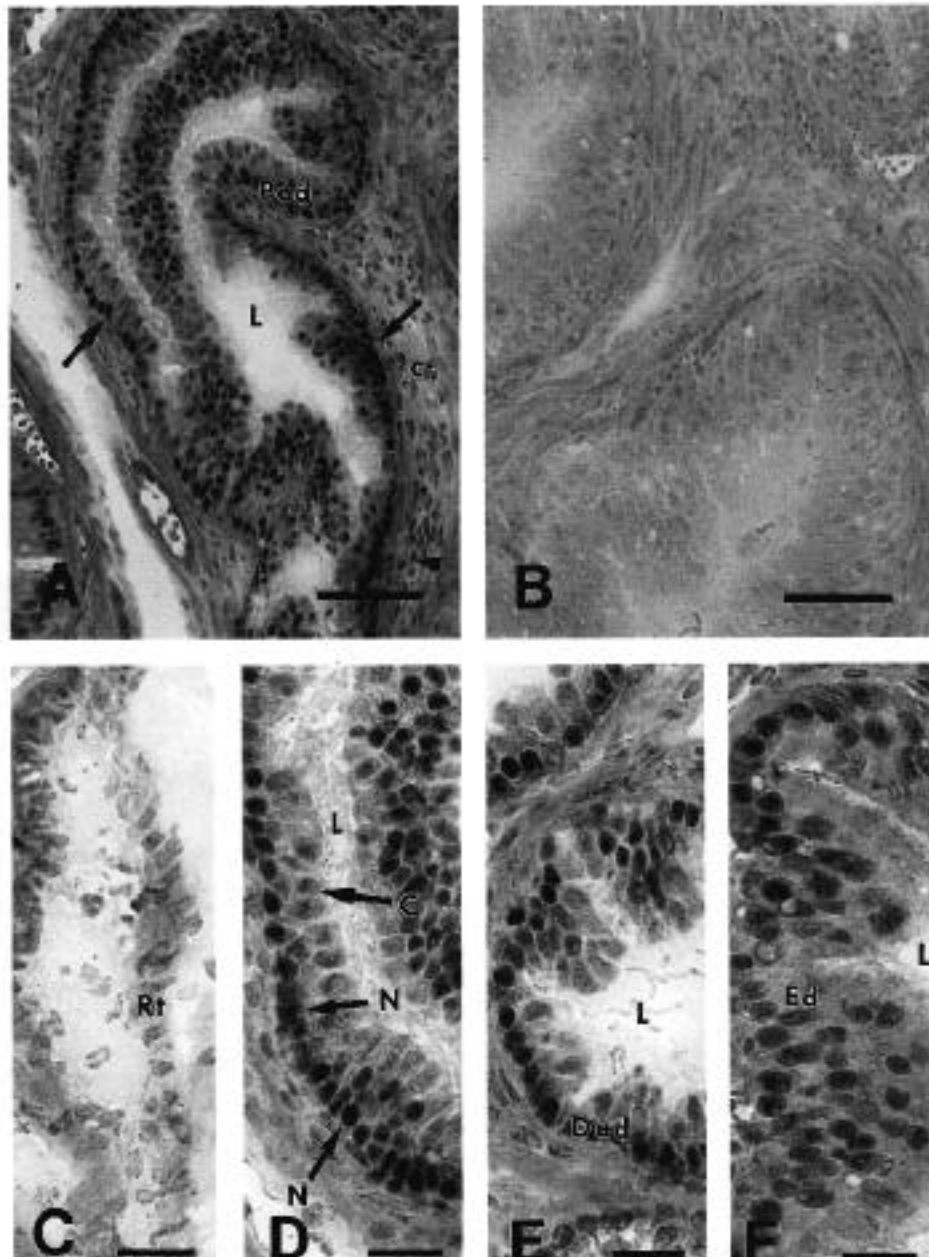


FIG. 2. (A-F), Immunocytochemical localization of estrogen receptors (ER) in the rete testes and the epididymis of the rooster. (A), This low magnification shows immunostaining for ER (a brown-colored reaction product) in the nuclei of epithelial cells (arrows) of the proximal efferent ductules (Ped). Connective tissues (ct) also exhibited weak staining (arrowhead). (B), In this control section of the epididymis, rat IgG was substituted for the primary antibody. No specific staining was found in this tissue. (C-F), are higher magnifications of different regions. (C), Very slight nuclear staining was found in the nuclei of the rete testis (Rt). (1), In the proximal efferent ductules (Ped), strong immunostaining was present in the nuclei of nonciliated cells (N). However, ciliated cells (C) appeared to be slightly positive. (E), Nuclear immunostaining was detected in the distal efferent ductules (Ded). (F), The epididymal duct (Ed) also exhibited immunostaining in the nuclei of epithelial cells, although less intense than seen in efferent ductules. (A, B), Bar = 100 μ m; (C-F), bar = 20 μ m. L-lumen.

3G,H, higher magnification). Connective tissue showed labeling for ER mRNA (Fig. 3A,B,G,L). In the control section, in which the plasmid DNA was used to make the cRNA probe, no specific signal for ER mRNA was observed (Fig. 3C,D). The rete testis did not exhibit

specific hybridization (Fig. 3E,F). In the distal efferent ductules (Fig. 3I,J), specific hybridization signals were abundant in epithelial cells. Similar abundance of silver grains was present in epithelial cells of the epididymal duct (Fig. 3K,L).

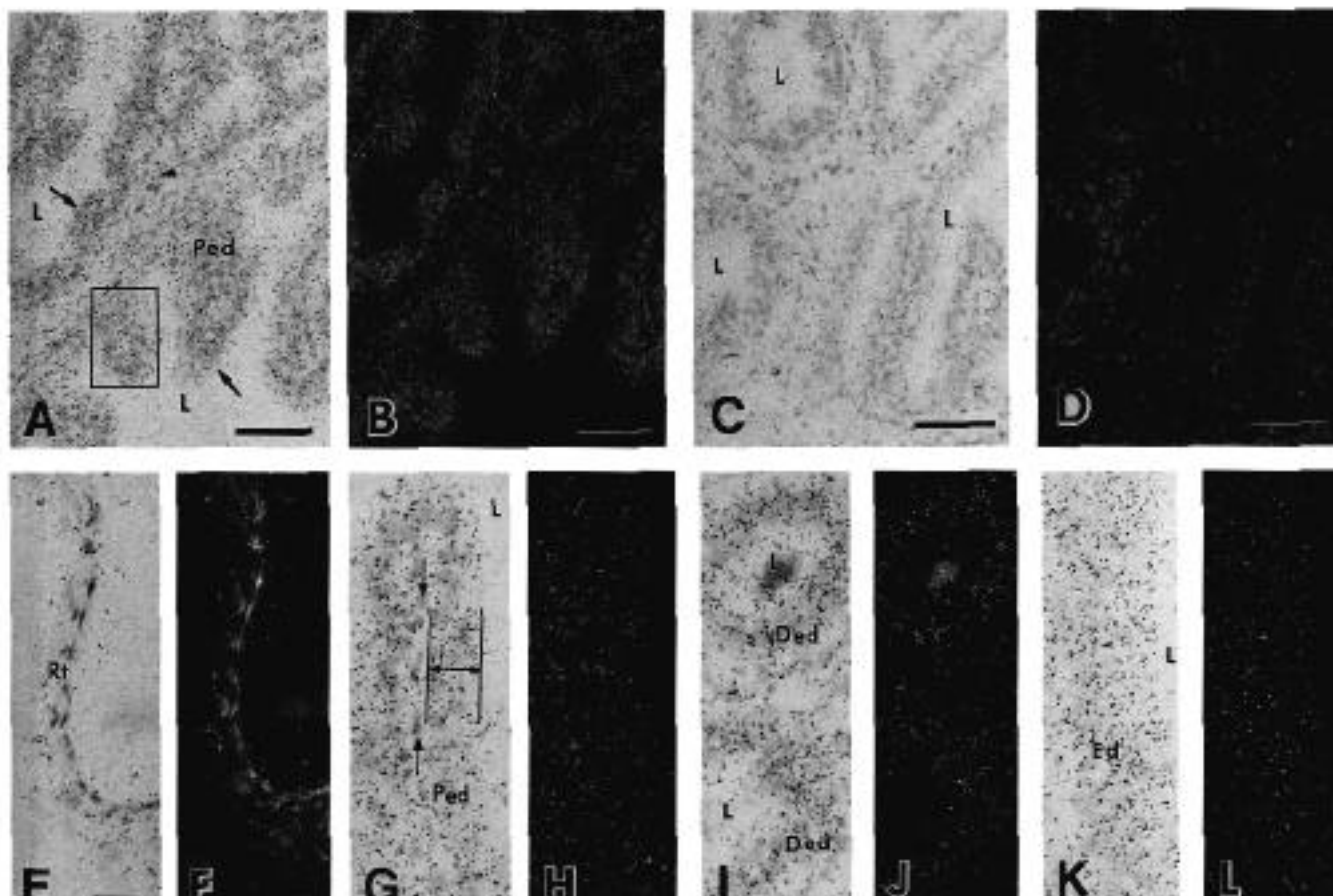


FIG. 3. (A-L), In situ hybridization of estrogen receptors (ER) mRNA in the rete testis and epididymis of the rooster. Comparative brightfield (A, C, E, G, I, K) and darkfield (B, D, F, H, J, L) microphotographs of each tissue are presented. (A, B), At low magnification, specific hybridization signals for ER mRNA were present primarily in epithelial cells of the proximal efferent ductules (Ped) (arrows). Connective tissues showed less labeling (arrowhead). (C, D), No specific signal for ER mRNA was observed in the negative control section. (E, F), The rete testis (Rt) did not show specific hybridization signal for ER mRNA. (G, M), In the proximal efferent ductules (Ped), specific hybridization signals were present in the epithelial cells. The box contains a mucosal fold that contains epithelium and connective tissue (see also Fig. 1), which is present in (G) and (H). The area between the two vertical lines connected by a horizontal arrow contains epithelial cells. The area directly to the left of the vertical line demarcated by two vertical arrows is a narrow band of connective tissue, similar to that shown in Fig. 1, (I, J). The distal efferent ductules (Ded) exhibited specific hybridization signal. (K, L), Specific hybridization signal was detected in the

Northern Blot Analysis

A single mRNA band corresponding to approximately 7.8 kb was detected in epididymal tissue and chicken oviduct (positive control) (Fig. 4). The relative abundance of ER mRNA in the epididymis was similar to that in chicken oviduct.

Discussion

This is the first report of the presence of ER and ER mRNA in the epididymis of the rooster. Strong immunostaining for ER was found in the nuclei of nonciliated epithelial cells of proximal and distal efferent ductules and the epididymal duct. Weak immunostaining for ER was also observed in the connective tissues. There was

very slight immunostaining for ER in the rete testis. Strong specific hybridization signals for ER mRNA corresponded to the same areas exhibiting immunocytochemical localization of ER. Northern blot analysis of the epididymis showed a single band corresponding to approximately 7.8 kb, which is in agreement with the published size of the chicken ER mRNA in the oviduct (Maxwell et al, 1987).

In contrast to the primate reproductive tract, the rooster epididymis exhibited ER in both epithelial cells and connective tissues, similar to the mouse. In the adult mouse, ER were found in epithelial cells of the efferent ductules and epididymis (Iguchi et al, 1991). In the primate, only the efferent ductule epithelium exhibited ER (West and Bremer 1990). Thus, there appear to be some species differences in the presence of ER within the male

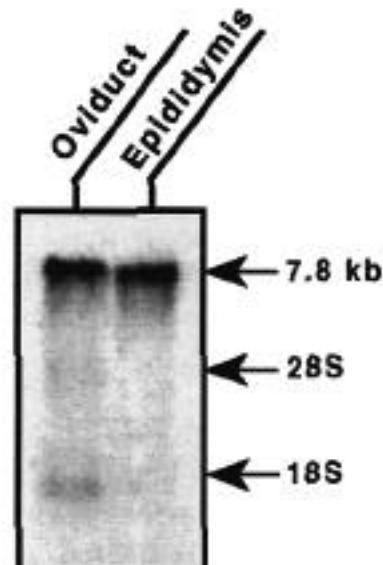


FIG. 4. Northern blot analysis of estrogen receptor (ER) mRNA in the epididymis of the rooster. Equal amounts of poly A+RNA (5 µg) from the epididymal tissues of the rooster and chicken oviduct were electrophoresed on a 1.5% agarose gel, transferred to a nylon membrane, and hybridized with a cDNA probe for chicken ER. The

The connective tissue of the avian epididymis also showed immunostaining for ER and a specific hybridization signal for ER mRNA. However, the intensity of immunostaining for ER and the density of specific hybridization signal for ER mRNA in the connective tissue were less intense than in the epididymal epithelium. The presence of ER in the connective tissue of the reproductive tract has been reported in the fetal and adult mouse reproductive tract (McLachlan et al, 1975; Stumpf et al, 1980; Schleicher et al, 1984; Cooke et al, 1991; Greco et al, 1992). Estrogen receptors were observed in mesenchymal cells of the Wolffian duct as early as 13 days in the fetal male mouse (Greco et al, 1992); however, there was a gradient of immunostaining in the connective tissue cells, which was less intense at later fetal and neonatal ages.

Autoradiographic methods have also been used to determine the cellular and tissue distribution of ER in the male reproductive tract. Specific nuclear labeling of ³H-diethylstilbestrol, which is indicative of a functional ER, has also been found in the mesenchyme surrounding the epididymis of 16-day-old male mouse fetus (Stumpf et al, 1980). Therefore, the presence of ER in early stages of mouse embryos suggests that there is a direct role for estrogen in the developing reproductive tract and that the effects of estrogen on the epididymal epithelium may be mediated through mesenchymal ER (Cunha et al, 1985; Pasqualini and Sumida, 1986).

The presence of ER in the epididymis of the rooster suggests that this tissue is a target for estrogen. In an

earlier publication, we reported that rooster germ cells and sperm contain an active aromatase (Kwon et al, 1995). Thus, the amount of estrogen being produced would be proportional to the number of germ cells and sperm. Whereas the concentration of estradiol in the rete testis fluid of the rooster has not been determined, data in the rat indicate that the estradiol concentration of the rete testis fluid is approximately 10-fold greater than the estrogen concentration in the serum of male rats (Free and Jaffe, 1979). The adult rooster has approximately 25 pg/ml of estradiol in its serum (unpublished).

However, the role of estrogen in the regulation of the male reproductive system remains unknown. Because ER immunostaining was more intense in the efferent ductules than in the epididymal duct of the rooster, and ER are also abundant in efferent ductules of mammals (Schleicher et al, 1984; West and Brenner, 1990; Iguchi et al, 1991), it is reasonable to hypothesize that estrogens may participate in the regulation of the major function of this region, i.e., fluid resorption (Clulow and Jones, 1988; Ilio and Hess, 1992). It is well established that epithelial cells of the epididymis are involved in the resorption of approximately 90% of the rete testis fluid and proteins in both mammalian and avian species (Morales and Hermo, 1983; Jones and Jurd, 1987; Robaire and Hermo, 1988; Ilio and Hess, 1994). In particular, the nonciliated cells of efferent ductules have been shown to contain a prominent endocytotic apparatus of microvilli, coated vesicles, and lysosomes, which are indices of active fluid uptake (Herme et al, 1988; Robaire and Hermo, 1988; Veeramachaneni and Amann, 1991; Rio and Hess, 1994).

We have chosen to examine the epididymis of the rooster for the presence of ER and its mRNA because the avian species had not previously been examined. Furthermore, the rooster is an excellent model to study the action of estrogen in the male reproductive tract of other avian species. There are several dysfunctions of the epididymis in the bird that significantly reduce fertility, suggesting the importance of the epididymis in the production of fertile sperm. Finally, there is a reasonable concern about the effect of estrogen-like compounds in our environment on the avian species. These compounds have the potential to have a negative impact on fertility of wild avian species.

In conclusion, we have used several techniques to demonstrate the presence of ER and its mRNA in the epididymis of the rooster. The strongest immunostaining and specific signals were found in the nonciliated cells of the efferent ductules. Future studies will be directed toward an understanding of the role of estrogen in regulating avian epididymal function.

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