BIOLOGY OF REPRODUCTION 56, 102–109 (1997)

Localization of Class I and Class IV Alcohol Dehydrogenases in Mouse Testis and Epididymis: Potential Retinol Dehydrogenases for Endogenous Retinoic Acid Synthesis[1]

Louise Deltour, Robert J. Haselbeck, Hwee Luan Ang, and Gregg Duester[2]

The Burnham Institute, La Jolla, California 92037

ABSTRACT

The vitamin A metabolite retinoic acid plays an essential signaling role in spermatogenesis by acting as a ligand for nuclear retinoic acid receptors. However, little is known about the regulation of retinoic acid synthesis from vitamin A (retinol). Here we have examined mouse testis and epididymis for the presence of endogenous retinoic acid and for the expression of genes encoding class I and class IV alcohol dehydrogenases (ADH), both of which catalyze retinol oxidation, the rate-limiting step in the conversion of retinol to retinoic acid. Using a bioassay we found that mouse testis and epididymis both have significant levels of retinoic acid ranging from 7 to 8 pmol/g, an amount known to be sufficient to optimally activate retinoic acid receptors. In situ hybridization analysis of mouse testis revealed that class I ADH mRNA was localized in Sertoli cells and Leydig cells, while class IV ADH mRNA was confined to late spermatids. In the epididymis, class I ADH mRNA was detected in both principal and basal cells, whereas class IV ADH mRNA was limited to basal cells. Immunohistochemical analyses of testis indicated that class I ADH protein was localized in Sertoli and Leydig cells, whereas class IV ADH protein was observed only in late spermatids. Class I ADH protein was localized in principal and basal cells of the cauda epididymidis but only in basal cells of the caput epididymidis. Class IV ADH protein was limited to basal cells along the entire length of the epididymis. These results support a role for ADHs during spermatogenesis, potentially as retinol dehydrogenases catalyzing local retinoic acid synthesis in the testis and epididymis.

INTRODUCTION

Vitamin A (retinol) is known to play an essential role in spermatogenesis and maintenance of epithelia including those of the reproductive tract [1]. Recent studies have shown that the active form of vitamin A is retinoic acid, an oxidative metabolite of retinol, which serves as a ligand for nuclear retinoic acid receptors [2, 3]. Administration of retinoic acid via the bloodstream to vitamin A-deficient animals restores epithelia to their normal state but does not support spermatogenesis, possibly owing to its inability to efficiently cross the blood-testis barrier [4]. It has been demonstrated that the bloodstream normally contains a low concentration of retinoic acid that can be taken up in significant amounts by most organs but that the testis is extremely poor in retinoic acid uptake [5]. However, it has recently been demonstrated that high repeated doses of retinoic acid administered intraperitoneally can restore spermatogenesis [6, 7]. Furthermore, male mice with a knockout mutation of the retinoic acid receptor α gene exhibit testis degeneration and sterility, indicating that retinoic acid is indeed the active retinoid required for spermatogenesis [8]. A mechanism has been proposed for the active transport of retinol from the bloodstream into the Sertoli cells within the seminiferous tubules, where it is stored as retinyl esters [9–12]. This suggests that retinoid signaling during spermatogenesis normally involves the local conversion of retinol to retinoic acid in the seminiferous tubule, although the cells performing this function have not been identified. Maturation of spermatozoa in the epididymis may also involve retinoic acid, since this molecule has been detected in rat epididymal tissue [13] and since mice carrying retinoic acid receptor mutations exhibit epididymal abnormalities [14].

Little is known about the regulation of retinoic acid synthesis in the testis or epididymis. On the basis of studies performed in other organs, it is assumed that synthesis occurs by two sequential oxidation steps in which retinol is first oxidized to retinal (the rate-limiting step) and retinal is then oxidized to retinoic acid [15–17]. The enzyme catalyzing retinol oxidation in mouse epidermis, another retinoid target tissue, has been identified as an isozyme of alcohol dehydrogenase (ADH) [18], now known as class IV ADH [19]. The epidermis was also found to have a retinal-oxidizing activity with the properties of an aldehyde dehydrogenase [18]. Several classes of ADH exist, and studies have shown that class I ADH and class IV ADH catalyze retinol oxidation in both humans and rodents [18, 20–22]. These ADHs are characterized by high affinities for retinol, as indicated by K_m values in the low micromolar range, but by low affinities for ethanol, as indicated by K_m values in the millimolar range. This suggests that some forms of ADH may normally play a role in endogenous retinol metabolism instead of (or in addition to) a role in metabolism of toxic alcohols such as ethanol. Further studies are needed to determine whether there are links between ADHs and retinoic acid production.

In the present study, we have examined the levels of endogenous retinoic acid in mouse testis and epididymis, as well as the expression of class I and IV ADH genes, in order to investigate the potential role of ADH in retinoic acid synthesis during spermatogenesis in the mouse.

MATERIALS AND METHODS

Bioassay Detection of Retinoic Acid

The retinoic acid reporter cell line F9-RARE-*lacZ* (Sil-15) was used as a bioassay to monitor the presence of retinoic acid. F9-RARE-*lacZ* was derived from stable transfection of mouse F9 teratocarcinoma cells with a transgene consisting of a promoter linked to a retinoic acid response element driving the expression of *lacZ* encoding β-galactosidase [23]. Through the monitoring of β-galactosidase activity produced in response to retinoic acid induction of the transgene promoter, this reporter cell line has been used

Accepted September 4, 1996.
Received June 21, 1996.
[1]Supported by NIH Grants AA07261 and AA09731.
[2]Correspondence: Gregg Duester, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037. FAX: (619) 646-3195; e-mail: greggd@ljcrf.edu

previously to quantitate retinoic acid in tissue homogenates added to the growth media [24, 25]. This assay is particularly useful for the analysis of tissues with a limited sample size.

F9-RARE-*lacZ* cell stocks were maintained in L15 CO_2 medium as described previously [23]. Retinoic acid was quantitated in testis and epididymis from adult male mice (strain FVB/N) by a modification of methods previously described [25]. Tissue was dissected, immediately weighed, and frozen. Tissue (10–20 mg) was diluted with 100 μl of L15 CO_2 medium and homogenized in a 200-μl ground-glass micro tissue grinder. The homogenate was transferred to a 1.5-ml microcentrifuge tube, subjected to three cycles of freeze/thaw (dry ice/ethanol bath for 5 min followed by 37°C bath for 5 min), incubated for 1 h at 4°C, and then centrifuged at 12 000 × *g* for 5 min at 4°C. The supernatant (100 μl) was removed and serial dilutions were made using L15 CO_2 medium. Dilutions of the homogenates (100-μl total volume) were added to F9-RARE-*lacZ* cells growing in 96-well microtiter plates; after 18-h incubation the cells were fixed in 1% glutaraldehyde, and β-galactosidase activity was visualized with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as described previously [26]. The intensity of the blue reaction product was measured with an ELISA microtiter plate reader at an absorbance of 650 nm.

To estimate the concentration of retinoic acid in the tissue homogenates, a standard curve was created by treating F9-RARE-*lacZ* cells with known concentrations of all-trans-retinoic acid and plotting the concentration vs. the A_{650} as previously described [25]. The linear range for accurate estimation of retinoic acid was 0.1–10 nM. The retinoic acid concentration in the diluted homogenates was determined from the standard curve, and this value was converted to the retinoic acid concentration in the tissue (pmol/g) by using the weight of the tissue prior to homogenization and the dilution factors caused by homogenization and addition of homogenates to media for the bioassay. Tissues having a retinoic acid concentration of at least 1 pmol/g prior to dilution can be effectively measured with this bioassay.

As indicated previously, this assay is optimal for detection of all-trans-retinoic acid but will also detect the 9-cis and 13-cis isomers of retinoic acid with lower efficiency [25]. Also, it has recently been discovered that 4-oxo retinoids can activate the retinoic acid receptor [27, 28] and may activate the reporter gene in F9-RARE-*lacZ* cells. Thus, a limitation of the assay is that the results reflect the combined levels of all biologically active ligands in the tissues examined. Also, since this assay measures changes in absorbance at 650 nm as an index of β-galactosidase activity, there may be other factors present in tissues that effect 650-nm absorbance.

Tissue Sectioning and In Situ Hybridization

Testis and epididymis from adult (> 8 wk old) male mice (strain FVB/N) were fixed, embedded in paraffin, sectioned at 6–10 μm, and processed for in situ hybridization under high-stringency conditions as described previously [29]. The hybridization probes consisted of ^{35}S-labeled antisense RNAs derived from the full-length cDNAs for mouse class I ADH [30, 31] and class IV ADH [19] subcloned in pBluescript II KS (Stratagene Cloning Systems, Inc., La Jolla, CA). Antisense RNA was synthesized using [α-^{35}S]UTP and T3 or T7 RNA polymerase as described earlier [29]. As a control to monitor background detection we used a sense RNA probe transcribed from the plasmid containing the mouse class I ADH cDNA. We have previously shown that no cross-hybridization is observed between the ADH classes under high-stringency conditions in either Northern or Southern blots, probably because of the low ADH interclass sequence identity, which is in the 60% range [19].

Slides were exposed to emulsion for 2–3 wk prior to development. Staining of hybridized sections was performed with neutral red, and adjacent nonhybridized sections were stained with hematoxylin and eosin as described previously to observe tissue morphology [32]. Hybridization results were observed by darkfield microscopy, and stained adjacent sections were observed by brightfield microscopy.

Immunohistochemistry

Rabbit polyclonal antisera against mouse class I ADH and class IV ADH were generated from four monthly injections of 200 μg of each protein expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase (GST) using the pGEX-4T-2 plasmid vector system (Pharmacia Biotech, Uppsala, Sweden). To remove residual cross-reactivity to the other class of ADH, each crude antiserum was preabsorbed by incubation either with an acetone powder of a crude lysate of *E. coli* expressing an unspecific GST-ADH fusion protein, or with 100 μg of an unspecific GST-ADH fusion protein resolved by SDS-PAGE and electroblotted onto filter strips. Antisera so treated were observed by Western blot analysis to react with the GST fusion protein of the specific class of ADH used to generate the antisera, but they lacked cross-reactivity either to purified GST or to the GST fusion protein of the other class of ADH (data not shown).

Affinity purification of either class I or class IV ADH-specific antibodies was performed using 100-μg aliquots of GST-ADH fusion protein electroblotted to polyvinylidiene fluoride membrane. These small filter strips were first blocked for 1 h with 5% BSA in PBS and then added to a 1-ml volume of preabsorbed antiserum diluted 1:10 in the aforementioned solution. After incubation for 24–72 h with gentle agitation at 4°C, the filters were washed three times, 10 min each, with excess PBS and then placed in 1 ml ice-cold 100 mM glycine (pH 2.2). The filter was incubated for 3 min on ice with occasional gentle up and down pipetting to facilitate elution of bound antibodies. The 1-ml volume was removed from the filter and neutralized by addition of a titrated amount of 2 M Tris base. BSA was added to a level of 5% as a stabilizing agent, and the preparations were routinely stored for a month at 4°C with no appreciable loss of immunoreactivity. Concentrations of purified antibodies were estimated by comparison of Western blot signals of serially diluted preparations to known concentrations of commercially available rabbit IgG detected by horseradish peroxidase-conjugated goat anti-rabbit antiserum (Sigma Chemical Co., St. Louis, MO).

Sections of adult mouse testis and epididymis embedded in paraffin were subjected to immunohistochemical analysis using the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). For epididymis, sections of the caput, corpus, and cauda were examined. Incubation was performed for 30 min with purified antibody serially diluted in normal blocking solution provided in the Vectastain kit. Incubation with diaminobenzidine for color detection was performed for 3 min; this was followed by dilution in tap

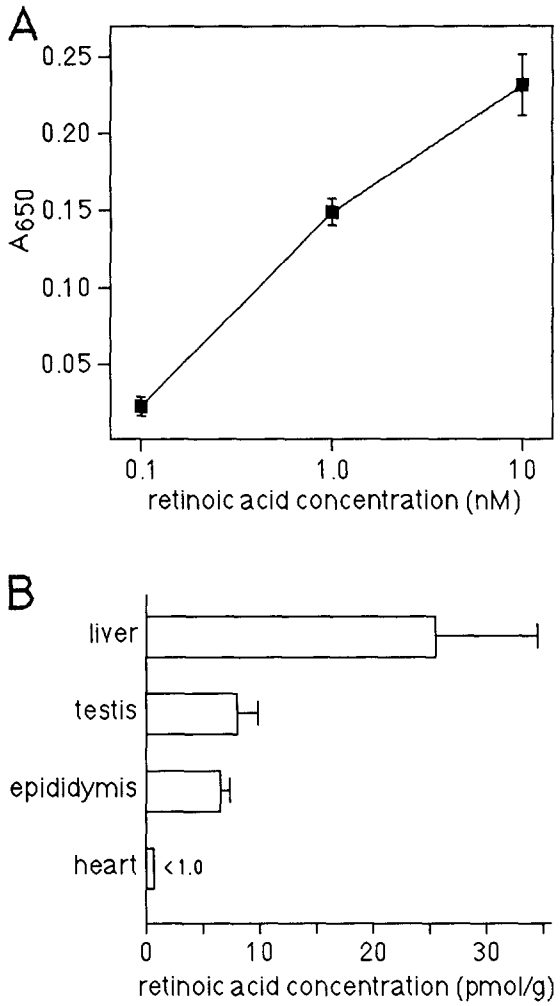


FIG. 1. Measurement of retinoic acid in testis and epididymis using a bioassay. **A**) Shown is a standard curve for retinoic acid detection generated by treating F9-RARE-*lacZ* reporter cells with known concentrations of all-trans-retinoic acid, then measuring the induction of β-galactosidase activity spectrophotometrically at A_{650}. **B**) Retinoic acid concentrations are shown for adult mouse liver, testis, epididymis, and heart. These values were determined by treatment of reporter cells with tissue homogenates, conversion of the determined A_{650} value to retinoic acid concentration in the homogenate using the standard curve, and conversion of this value to the concentration in the tissue by knowing the dilution factor (at least 10-fold for all tissue examined here). Heart homogenates gave values below the linear range of the assay (below 0.1 nM), indicating that the retinoic acid concentration in heart tissue was below 1.0 pmol/g. Values are averages of four independent homogenates, ± SD.

water to stop the reaction. Slides were not counterstained prior to mounting and observation by brightfield microscopy. Optimal class I ADH staining in testis or epididymis was obtained using class I ADH affinity-purified antibody at 0.05 μg/ml. Optimal class IV ADH staining in the testis was obtained using class IV ADH purified antibody at 0.1 μg/ml, while a concentration of 0.01 μg/ml gave optimal staining in the epididymis. Control incubations with serially diluted preimmune serum failed to detect signals specific to either class I ADH or class IV ADH in testis or epididymis.

RESULTS

Bioassay Measurement of Retinoic Acid Levels in Testis and Epididymis

A sensitive bioassay using a retinoic acid reporter cell line (F9-RARE-*lacZ*) was used to quantitate retinoic acid levels in homogenates of adult mouse testis and epididymis. Figure 1A shows the results of β-galactosidase activity in reporter cells that was induced by treatment with known concentrations of all-trans-retinoic acid to create a standard curve. The retinoic acid concentrations in adult mouse liver, testis, epididymis, and heart were determined by treating the reporter cells with homogenates of these tissues (Fig. 1B). The retinoic acid concentrations determined by this bioassay were as follows: liver (24.8 ± 9.2 pmol/g), testis (7.8 ± 1.7 pmol/g), epididymis (6.6 ± 0.4 pmol/g), and heart (< 1.0 pmol/g).

Expression of Class I ADH and Class IV ADH Genes in Testis

ADH mRNA was detected in sections of adult mouse testis by in situ hybridization. A hematoxylin/eosin-stained section through the mouse testis, observed under brightfield, shows the morphology of this structure; the seminiferous tubule shown has numerous late spermatids along the lumen (Fig. 2A). An adjacent section hybridized to an antisense RNA probe specific for the mouse class I ADH gene indicated that mRNA was localized in some of the interstitial cells (Leydig cells), with also a diffuse signal throughout the seminiferous tubule (Fig. 2B). Conversely, an antisense RNA probe specific for the class IV ADH gene indicated that this mRNA was localized only in the seminiferous tubule, primarily in the luminal half of the epithelium containing spermatids, with no detection in seminiferous epithelial cells near the basal lamina (Fig. 2C). Analysis of seminiferous tubules at various stages of spermatogenesis indicated that class IV ADH mRNA was detected only in sections containing numerous late spermatids, further indicating an absence of expression in Sertoli cells, spermatogonia, and spermatocytes, which are present at all stages (data not shown). A sense RNA probe used as a control shows the level of background detection during in situ hybridization (Fig. 2D). Spermatozoa present in the lumen of the seminiferous tubule contained neither class I ADH mRNA nor class IV ADH mRNA (Fig. 2, B and C).

Epididymal Expression of Genes for Class I and Class IV ADHs

Sections of adult mouse epididymis were examined for ADH gene expression by in situ hybridization. A mouse cauda epididymal section stained with hematoxylin/eosin and observed under brightfield shows the morphology of this structure (Fig. 3A). An adjacent section hybridized to an antisense RNA probe specific for the mouse class I ADH gene indicated that mRNA was present throughout the cauda epididymal epithelium including the principal cells lining the lumen and the basal cells along the basal lamina (Fig. 3B). Whereas class I ADH mRNA was detected in both principal and basal cells of the cauda epididymidis (Fig. 3B), it was detected in only basal cells of the caput epididymidis (data not shown). In contrast, an antisense RNA probe specific for the class IV ADH gene indicated that in the cauda epididymidis this mRNA was localized exclusively in basal cells and was absent from the principal cells (Fig. 3C). Class IV ADH mRNA was also absent from the principal cells of the caput and corpus epididymidis but remained in the basal cells (data not shown). A control sense RNA probe did not detect a specific signal in the cauda epididymidis during in situ hybridization (Fig. 3D). Neither class I ADH nor class IV ADH mRNAs were de-

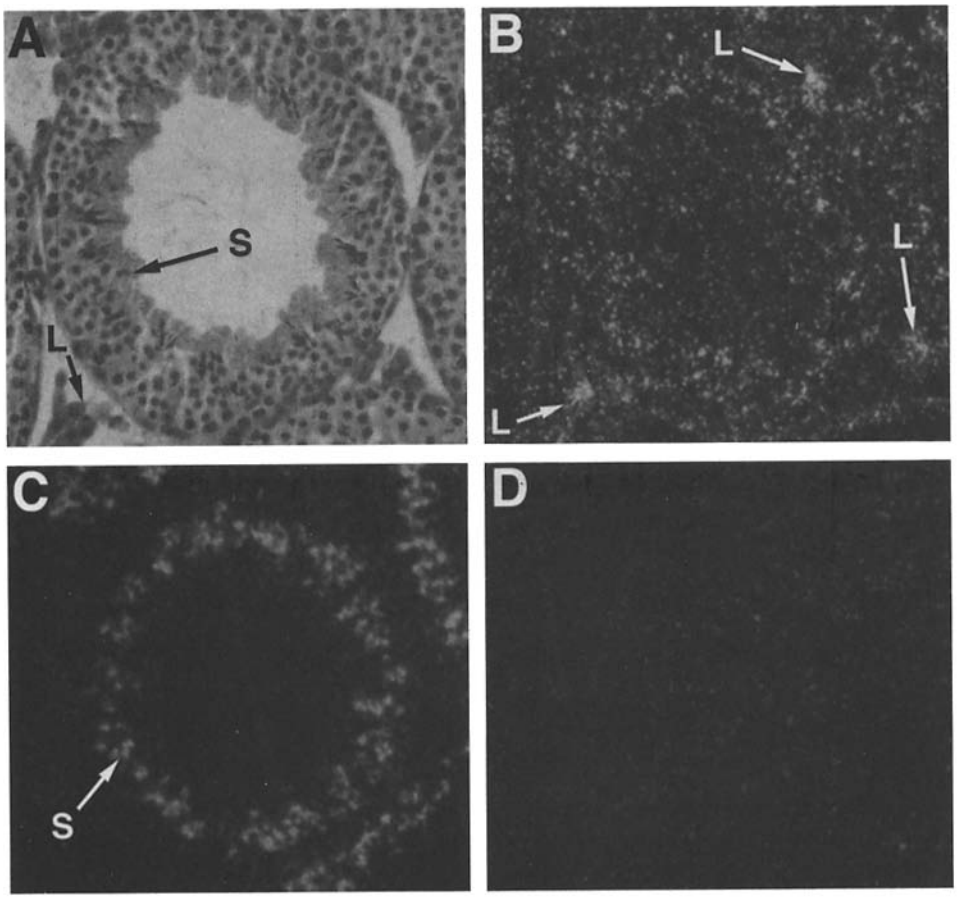


FIG. 2. Localization of class I ADH and class IV ADH mRNAs in the testis. **A**) Brightfield view of testicular section stained with hematoxylin/eosin showing an accumulation of elongating spermatids along the lumen of the seminiferous tubule, and adjacent interstitial Leydig cells. **B**) Darkfield view of in situ hybridization results for class I ADH gene expression showing mRNA detection within some interstitial Leydig cells as well as a diffuse signal throughout the seminiferous tubule. **C**) In situ hybridization results for class IV ADH gene showing mRNA localization in spermatogenic cells within the luminal half of the seminiferous tubule. **D**) Results of in situ hybridization with a sense RNA control probe. L, Leydig cells within interstitial region; *S*, spermatids. ×144.

tected in spermatozoa present within the lumen of the cauda epididymidis (Fig. 3, B and C).

Localization of ADHs in Male Reproductive Tract by Immunohistochemistry

Sections of adult mouse testis and epididymis were examined for ADH protein localization using affinity-purified antibodies specific to class I ADH or class IV ADH. The morphology of the testis is shown by a section stained with hematoxylin/eosin (Fig. 4A). Immunodetection of an adjacent section with class I ADH-specific antibodies revealed a specific signal in both Sertoli cells and Leydig cells but no detection in spermatogenic cells (Fig. 4B). Not all Leydig cells appeared to stain for class I ADH, indicating some heterogeneity in the interstitial cells. In contrast, immunodetection of an adjacent testis section with class IV ADH-specific antibodies revealed a specific signal in late spermatids along the luminal portion of the seminiferous tubule, with no detection above background in other germ cells or Sertoli cells or Leydig cells (Fig. 4, C and D). A preimmune serum control indicated no specific immunostaining in the testis (Fig. 4E). A class IV ADH signal was observed only in seminiferous tubules containing numerous late spermatids and appears to be in the residual bodies of spermatids that, upon release of spermatids, are phagocytosed by Sertoli cells (Fig. 4D).

The morphology of the caput epididymidis is shown by a section stained with hematoxylin/eosin (Fig. 4F). Class I ADH immunodetection of an adjacent section through the caput epididymidis revealed the presence of protein only in basal cells (Fig. 4G), and the same was true for class IV ADH immunodetection in the caput (Fig. 4H). However, class I ADH immunodetection in the cauda epididymidis indicated a specific signal in both principal and basal cells (Fig. 4I), whereas class IV ADH protein was still confined to only basal cells (data not shown). As a control, incubation with preimmune serum led to no specific immunodetection in the cauda epididymidis (Fig. 4J). Basal cell immunodetection for class I ADH appeared to be less prominent in the cauda than in the caput epididymidis (Fig. 4, G and I); the corpus appeared to represent a transition zone where basal cell detection became less apparent than in the caput whereas principal cell detection became more apparent than in the caput (data not shown).

DISCUSSION

The data reported here demonstrate the existence of retinoic acid in testis and epididymis of the mouse. We found that retinoic acid was present at a concentration of 8 pmol/g in the testis and 7 pmol/g in the epididymis. Mouse liver was found to have a retinoic acid concentration of 25 pmol/g, but mouse heart retinoic acid was not detectable above 1 pmol/g, the limit of detection with the bioassay. The concentrations of retinoic acid we detected in mouse testis and epididymis (i.e., 7–8 pmol/g or approximately 7–8 nM) are sufficient to optimally activate nuclear retinoic acid receptors that have ED_{50} values of approximately 1–10 nM in biological assays [33–35]. The measurements of biologically active retinoic acid in tissues using this bioassay can be compared to the results obtained by others using chromatographic methods to quantitate retinoic acid. Previous measurements from adult rat tissues indicated that all-trans-retinoic acid is present at a concentration of 11 pmol/g in the testis, 14 pmol/g in the epididymis, and 11

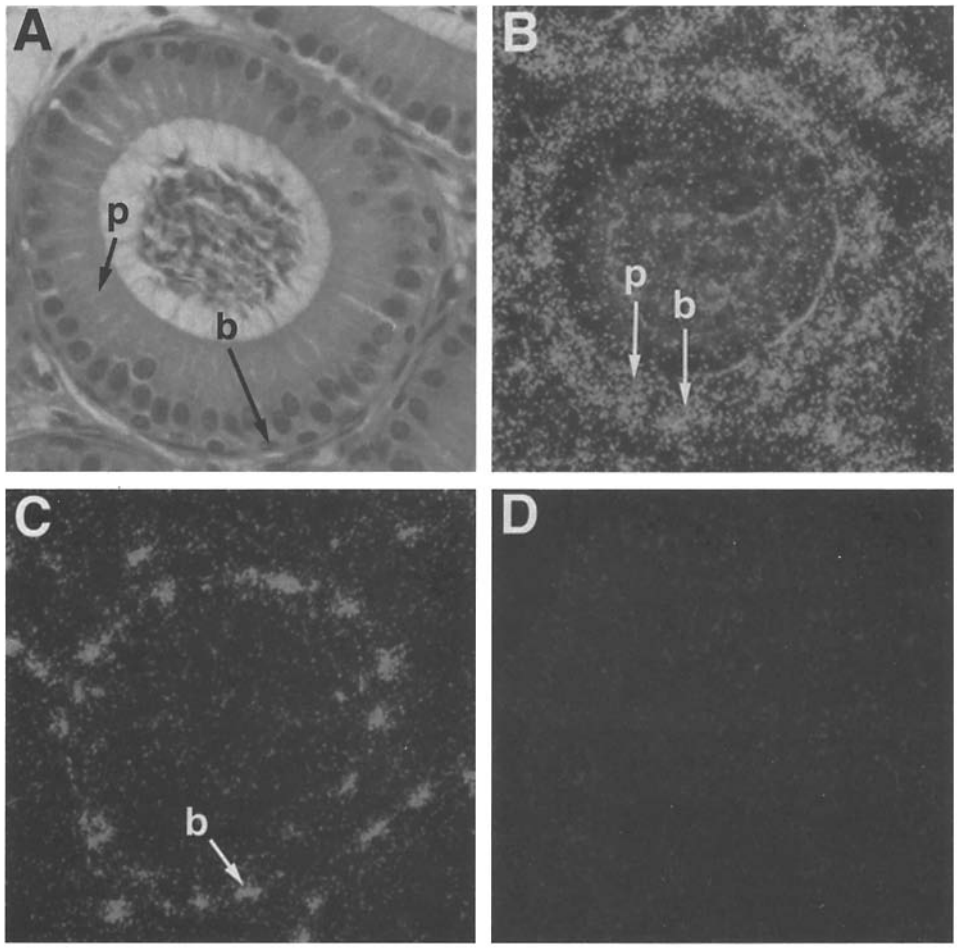
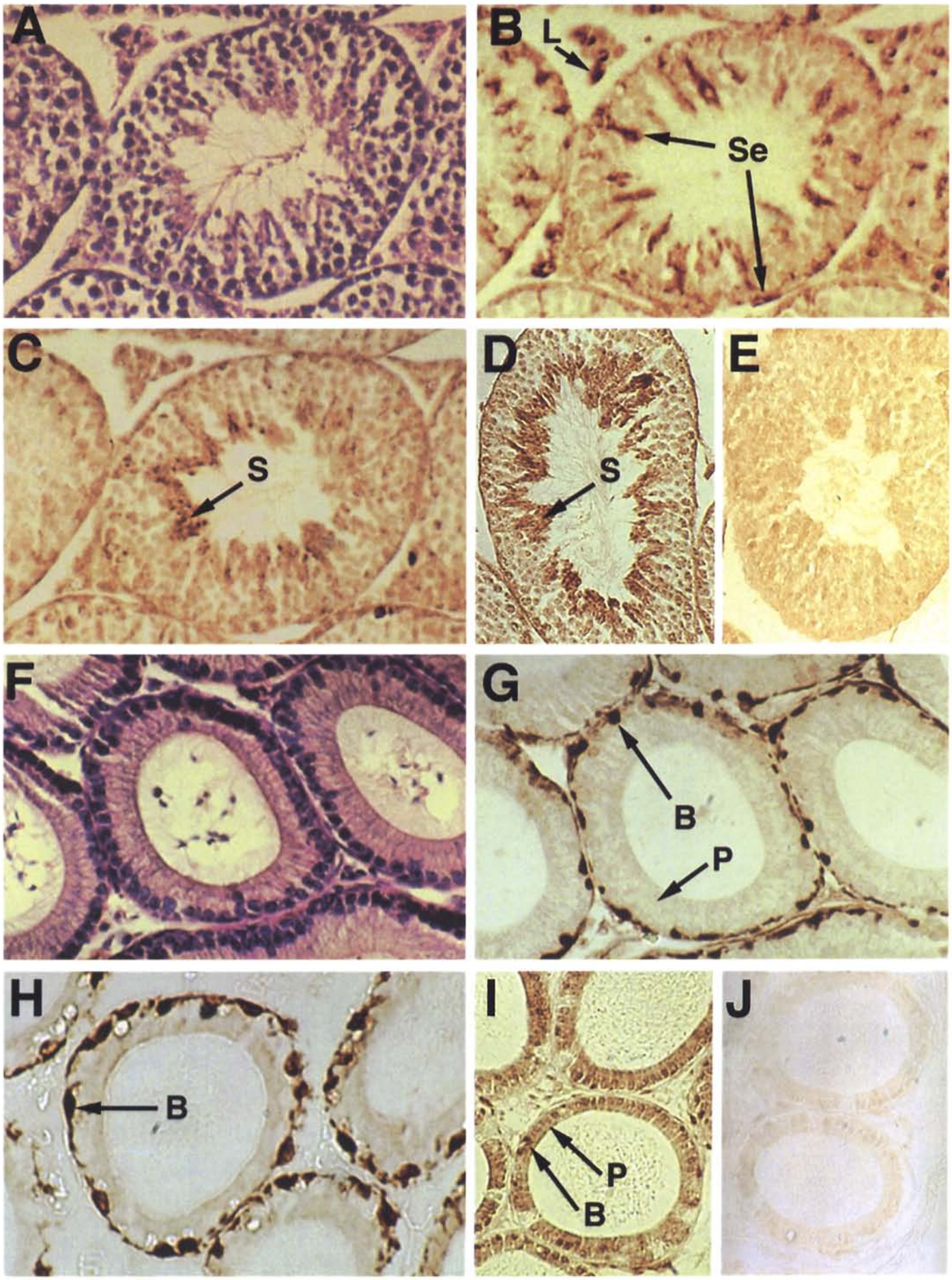


FIG. 3. ADH mRNA localization in mouse cauda epididymidis. **A**) Brightfield view of section stained with hematoxylin/eosin showing the principal cells and basal cells, as well as spermatozoa in the lumen. **B**) Darkfield view of in situ hybridization results for class I ADH gene expression showing mRNA detection in both principal and basal cells. **C**) In situ hybridization results for class IV ADH gene showing mRNA localization limited to the basal cells of the epithelium. **D**) Results of in situ hybridization with a sense RNA control probe. b, basal cells; p, principal cells. ×300.

pmol/g in the liver [5]. In another study, retinoic acid was quantitated at 13–35 pmol/g in the rat epididymis [13]. Our bioassay measurements for testis, epididymis, and liver in the mouse were thus similar to those determined by chromatographic methods for the rat, differing by approximately twofold. Some of this variation may result from rat/mouse species differences in retinoic acid levels.

In order to determine whether ADHs are present in the correct location to participate in retinoic acid synthesis in the male reproductive tract, we examined the expression patterns for class I and class IV ADHs, both of which are known to function as retinol dehydrogenases in vitro [18, 20, 21]. In the seminiferous tubules, class I ADH mRNA was scattered throughout the epithelium, and immunohistochemical analysis indicated that class I ADH protein was limited to Sertoli cells. In contrast, class IV ADH mRNA and protein were localized in spermatids along the luminal half of the epithelium, being absent in other germ cells and Sertoli cells. In particular, immunodetection of class IV ADH protein indicated that localization was primarily in late spermatids, suggesting that this is the site of action for this form of ADH. Since the testis has been shown to be very poor in retinoic acid uptake from the circulation [5], it is very likely that this organ must synthesize its own retinoic acid from retinol. Testicular peritubular cells have been shown to import retinol from serum retinol-binding protein and may facilitate retinol transport into the seminiferous tubule [12]. Retinol can be transported into cultured Sertoli cells where it accumulates to a concentration of 75 nmol/g [10], an amount quite sufficient to serve as a substrate for retinoic acid synthesis. However, previous studies have not determined whether Sertoli cells or spermatogenic cells can oxidize retinol to retinoic acid. Detection of class I ADH protein in Sertoli cells as well as class IV ADH protein in late spermatids provides evidence that both of these cells may be sites of retinoic acid synthesis needed to support spermatogenesis. Many epithelia require retinoic acid to maintain their differentiated state, including those of the testis [1, 8], and class I ADH in Sertoli cells may function to produce retinoic acid for the seminiferous tubule epithelium. The presence of class IV ADH in late spermatids, as opposed to spermatogonia or spermatocytes or Sertoli cells, suggests that this enzyme may function to

FIG. 4. Immunohistochemical localization of ADH in male reproductive tract. **A**) Hematoxylin/eosin-stained section of adult mouse testis. **B**) Class I ADH immunodetection of adjacent testis section showing specific protein localization in the Sertoli cells and some Leydig cells. **C**) Class IV ADH immunodetection of adjacent testis section indicating specific protein detection within the seminiferous tubule in late spermatids but not in Leydig cells, Sertoli cells, or spermatogonia, which exhibited background. **D**) Another view of class IV ADH immunodetection in a testis section limited to late spermatids. **E**) Control testis section treated with preimmune serum. **F**) Hematoxylin/eosin-stained section of caput epididymidis. **G**) Class I ADH immunodetection in adjacent section of the caput epididymidis showing localization in only the basal cells; principal cells lining the lumen show no protein detection. **H**) Class IV ADH immunodetection in the caput epididymidis showing localization only in basal cells. **I**) Class I ADH immunodetection in the cauda epididymidis localized in principal cells and to a lesser extent in basal cells. **J**) Preimmune serum control of cauda epididymidis. B, basal cells; L, Leydig cells; P, principal cells; S, late spermatids; Se, Sertoli cells. **A–E**), ×200; **F–H**), ×400; **I–J**), ×200.


A
B
L
Se
C
S
D
S
E
F
G
B
P
H
B
I
P
B
J

provide retinoic acid specifically for spermatid cell maturation during spermiogenesis.

Detection of class I ADH mRNA and protein in the testicular interstitial regions was heterogeneous, but their presence in some of the Leydig cells was clear. In addition to its ability to catalyze retinol oxidation, class I ADH has been found to function in vitro as a 3β-hydroxy-5β-steroid dehydrogenase in the testosterone metabolic pathway [36, 37]. Thus, it is possible that class I ADH may play a role in testosterone production and secretion within Leydig cells. This proposed function of class I ADH could be limited to steroid modification, since we know of no evidence implicating Leydig cells in testicular retinoic acid production. Class IV ADH does not metabolize 3β-hydroxysteroids in vitro [22], and its mRNA was clearly not expressed in Leydig cells.

Spermatozoal maturation occurs in the epididymis, and there is evidence that retinoic acid is involved in this process as well as in the maintenance of the epididymal epithelium [1, 13, 14]. Retinol, the precursor of retinoic acid, has been demonstrated to exist in the epididymis at a concentration of 2.7 nmol/g [13]. We observed both class I ADH and class IV ADH gene expression in the mouse epididymal epithelium, suggesting that either of these enzymes may play a role in local retinoic acid synthesis from retinol in this tissue. Also, our bioassay detected a significant amount of retinoic acid in mouse epididymal homogenates. Class IV ADH mRNA and protein were not detected in principal cells but were observed in basal cells along the entire length of the epididymis. Class I ADH mRNA and protein were observed in principal cells of the cauda region only and in basal cells of both the caput and cauda regions; however, the immunointensity was much stronger in the caput than in the cauda. Our results suggest that the caput and cauda epididymal epithelia may have different properties related to the function of ADH. One such property may be efficiency of retinoic acid synthesis. This is supported by studies of rat epididymis in which it was demonstrated that cauda epididymidis has approximately 2.7-fold more retinoic acid than caput epididymidis [13]. Class I ADH is much more abundant in the cauda than in the caput, primarily because of its presence in the principal cells of the cauda. Thus, it is possible that class I ADH in principal cells may function as a retinol dehydrogenase to provide an increased level of retinoic acid synthesis in the cauda relative to the caput. Class IV ADH is not differentially expressed along the length of the epididymis, but its expression in the basal cells may help provide a baseline level of retinoic acid needed along the entire length of the epididymis. Interestingly, retinoic acid receptor α is also differentially expressed in the epididymis, being high in the caput and cauda but low in the corpus [38]. Further investigation is needed to determine whether differential expression of enzymes and receptors is significant for retinoid signaling in the epididymis.

We did not detect expression of class I ADH or class IV ADH (either mRNA or protein) in the spermatozoa present in the lumen of either the testis or epididymis. However, sperm isolated from rat epididymis have been found to contain retinoic acid at 7–10 pmol/g [13]. Thus, it appears unlikely that either ADH examined here could be responsible for local synthesis of retinoic acid in spermatozoa themselves, although class IV ADH in late spermatids may contribute to the production of retinoic acid that remains stored within spermatozoa. Another possibility is that spermatozoa acquire retinoic acid by uptake from the epididymal lumen, since there is an epididymal retinoic acid-binding protein secreted into the lumen that may shuttle retinoic acid from the epithelium to spermatozoa [39]. Perhaps class I or class IV ADHs present in the epididymal epithelium participate in the synthesis of retinoic acid that is transported to this luminal binding protein.

In summary, we have used a bioassay to measure retinoic acid levels in mouse testis and epididymis. We have found that these tissues contain levels of endogenous retinoic acid sufficient to optimally activate retinoic acid receptors, suggesting that they are competent to locally synthesize retinoic acid from retinol. Class I and class IV ADHs, both known to function as retinol dehydrogenases, were detected in specific cells of the testis and epididymis, suggesting that they may function in the generation of retinoic acid for these tissues. Genetic studies using the mouse can be used to further examine the potential roles of class I and class IV ADHs in retinoic acid synthesis during spermatogenesis.

ACKNOWLEDGMENTS

We thank M. Felder for the mouse class I ADH cDNA and M. Wagner for the F9-RARE-*lacZ* reporter cells.

REFERENCES

1. Wolbach SB, Howe PR. Tissue changes following deprivation of fat-soluble A vitamin. J Exp Med 1925; 42:753–777.
2. Kastner P, Chambon P, Leid M. Role of nuclear retinoic acid receptors in the regulation of gene expression. In: Blomhoff R (ed.), Vitamin A in Health and Disease. New York: Marcel Dekker, Inc.; 1994: 189–238.
3. Mangelsdorf DJ, Umesono K, Evans RM. The retinoid receptors. In: Sporn MB, Roberts AB, Goodman DS (eds.), The Retinoids: Biology, Chemistry, and Medicine, 2nd Edition. New York: Raven Press, Ltd.; 1994: 319–349.
4. Thompson JN, Howell JM, Pitt GAJ. Vitamin A and reproduction in rats. Proc R Soc London Ser B 1964; 159:510–535.
5. Kurlandsky SB, Gamble MV, Ramakrishnan R, Blaner WS. Plasma delivery of retinoic acid to tissues in the rat. J Biol Chem 1995; 270: 17850–17857.
6. Van Pelt AMM, De Rooij DG. Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology 1991; 128:697–704.
7. Gaemers IC, Van Pelt AMM, Van der Saag PT, De Rooij DG. All-trans-4-oxo-retinoic acid: a potent inducer of in vivo proliferation of growth-arrested A spermatogonia in the vitamin A-deficient mouse testis. Endocrinology 1996; 137:479–485.
8. Lufkin T, Lohnes D, Mark M, Dierich A, Gorry P, Gaub M-P, LeMeur M, Chambon P. High postnatal lethality and testis degeneration in retinoic acid receptor α mutant mice. Proc Natl Acad Sci USA 1993; 90:7225–7229.
9. Porter SB, Ong DE, Chytil F, Orgebin-Crist M-C. Localization of cellular retinol-binding protein and cellular retinoic acid-binding protein in the rat testis and epididymis. J Androl 1985; 6:197–212.
10. Bishop PD, Griswold MD. Uptake and metabolism of retinol in cultured Sertoli cells: evidence for a kinetic model. Biochemistry 1987; 26:7511–7518.
11. Schmitt MC, Ong DE. Expression of cellular retinol-binding protein and lecithin-retinol acyltransferase in developing rat testis. Biol Reprod 1993; 49:972–979.
12. Davis JT, Ong DE. Retinol processing by the peritubular cell from rat testis. Biol Reprod 1995; 52:356–364.
13. Pappas RS, Newcomer ME, Ong DE. Endogenous retinoids in rat epididymal tissue and rat and human spermatozoa. Biol Reprod 1993; 48:235–247.
14. Mendelsohn C, Lohnes D, Décimo D, Lufkin T, LeMeur M, Chambon P, Mark M. Function of the retinoic acid receptors (RARs) during development. (II) Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development 1994; 120:2749–2771.
15. Leo MA, Kim C, Lowe N, Lieber CS. Increased hepatic retinal de-

hydrogenase activity after phenobarbital and ethanol administration. Biochem Pharmacol 1989; 38:97–103.
16. Napoli JL. Retinol metabolism in LLC-PK1 cells: characterization of retinoic acid synthesis by an established mammalian cell line. J Biol Chem 1986; 261:13592–13597.
17. Kim C-I, Leo MA, Lieber CS. Retinol forms retinoic acid via retinal. Arch Biochem Biophys 1992; 294:388–393.
18. Connor MJ, Smit MH. Terminal-group oxidation of retinol by mouse epidermis: inhibition in vitro and in vivo. Biochem J 1987; 244:489–492.
19. Zgombic-Knight M, Ang HL, Foglio MH, Duester G. Cloning of the mouse class IV alcohol dehydrogenase (retinol dehydrogenase) cDNA and tissue-specific expression patterns of the murine ADH gene family. J Biol Chem 1995; 270:10868–10877.
20. Boleda MD, Saubi N, Farrés J, Parés X. Physiological substrates for rat alcohol dehydrogenase classes: aldehydes of lipid peroxidation, omega-hydroxy fatty acids, and retinoids. Arch Biochem Biophys 1993; 307:85–90.
21. Yang Z-N, Davis GJ, Hurley TD, Stone CL, Li T-K, Bosron WF. Catalytic efficiency of human alcohol dehydrogenases for retinol oxidation and retinal reduction. Alcohol Clin Exp Res 1994; 18:587–591.
22. Kedishvili NY, Bosron WF, Stone CL, Hurley TD, Peggs CF, Thomasson HR, Popov KM, Carr LG, Edenberg HJ, Li T-K. Expression and kinetic characterization of recombinant human stomach alcohol dehydrogenase. Active-site amino acid sequence explains substrate specificity compared with liver isozymes. J Biol Chem 1995; 270: 3625–3630.
23. Wagner M, Han B, Jessell TM. Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. Development 1992; 116:55–66.
24. McCaffery P, Lee M-O, Wagner MA, Sladek NE, Dräger UC. Asymmetrical retinoic acid synthesis in the dorsoventral axis of the retina. Development 1992; 115:371–382.
25. McCaffery P, Dräger UC. Hot spots of retinoic acid synthesis in the developing spinal cord. Proc Natl Acad Sci USA 1994; 91:7194–7197.
26. Lim K, Chae CB. A simple assay for DNA transfection by incubation of the cells in culture dishes with substrates for β-galactosidase. Biotechniques 1989; 7:576–579.
27. Blumberg B, Bolado J Jr, Derguini F, Craig AG, Moreno TA, Chakravarti D, Heyman RA, Buck J, Evans RM. Novel retinoic acid receptor ligands in *Xenopus* embryos. Proc Natl Acad Sci USA 1996; 93:4873–4878.
28. Achkar CC, Derguini F, Blumberg B, Langston A, Levin AA, Speck J, Evans RM, Bolado J Jr, Nakanishi K, Buck J, Gudas LJ. 4-Oxoretinol, a new natural ligand and transactivator of the retinoic acid receptors. Proc Natl Acad Sci USA 1996; 93:4879–4884.
29. Wilkinson DG, Nieto MA. Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. Methods Enzymol 1993; 225:361–373.
30. Edenberg HJ, Zhang K, Fong K, Bosron WF, Li T-K. Cloning and sequencing of cDNA encoding the complete mouse liver alcohol dehydrogenase. Proc Natl Acad Sci USA 1985; 82:2262–2266.
31. Ceci JD, Lawther R, Duester G, Hatfield GW, Smith M, O'Malley MP, Felder MR. Androgen induction of alcohol dehydrogenase in mouse kidney: Studies with a cDNA probe confirmed by nucleotide sequence analysis. Gene 1986; 41:217–224.
32. Kaufman MH. The Atlas of Mouse Development. San Diego: Academic Press, Inc.; 1992.
33. Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 1987; 330:444–450.
34. Brand N, Petkovich M, Krust A, Chambon P, De Thé H, Marchio A, Tiollais P, Dejean A. Identification of a second human retinoic acid receptor. Nature 1988; 332:850–853.
35. Zelent A, Krust A, Petkovich M, Kastner P, Chambon P. Cloning of murine α and β retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. Nature 1989; 339:714–721.
36. McEvily AJ, Holmquist B, Auld DS, Vallee BL. 3β-Hydroxy-5β-steroid dehydrogenase activity of human liver alcohol dehydrogenase is specific to gamma-subunits. Biochemistry 1988; 27:4284–4288.
37. Murono EP, Fisher-Simpson V. Ethanol directly increases dihydrotestosterone conversion to 5α-androstan-3β,17β-diol and 5α-androstan-3α,17β-diol in rat Leydig cells. Biochem Biophys Res Commun 1984; 121:558–565.
38. Akmal KM, Dufour JM, Kim KH. Region-specific localization of retinoic acid receptor-α expression in the rat epididymis. Biol Reprod 1996; 54:1111–1119.
39. Newcomer ME. Structure of the epididymal retinoic acid binding protein at 2.1 Å resolution. Structure 1993; 1:7–18.