Fish 3β-Hydroxysteroid Dehydrogenase/Δ5-Δ4Isomerase: Antibody Production and Their Use for the Immunohistochemical Detection of Fish Steroidogenic Tissues

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Fish 3β-Hydroxysteroid Dehydrogenase/Δ^5-Δ^4 Isomerase: Antibody Production and Their Use for the Immunohistochemical Detection of Fish Steroidogenic Tissues

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ABSTRACT—We have produced polyclonal antibodies against two oligopeptides corresponding to middle and c-terminal regions of amino acid sequences predicted from rainbow trout (*Oncorhynchus mykiss*) 3β-hydroxysteroid dehydrogenase (3β-HSD) cDNA (Sakai *et al.*, 1994, FEBS Letters 350, 309-313). Both antibodies (α-tr3β-M and α-tr3β-C) recognized recombinant rainbow trout 3β-HSD protein derived from rabbit reticulocyte lysate system and non-steroidogenic mammalian COS-1 cell lysate. Immunoblot analysis of rainbow trout ovarian follicle homogenates revealed specific recognition of 3β-HSD protein. In rainbow trout testis, furthermore, immunoreactive 3β-HSD localized in Leydig cells in the interstitium of immature testes and interrenal cells in the head kidney. These results indicate that both α-3β-HSD antibodies recognized rainbow trout 3β-HSD protein at the level of immunoblot and immunohistochemical analyses. Furthermore, both antibodies also recognized immunohistochemically 3β-HSD in various steroidogenic organs (ovary, testis, and interrenal glands) of several teleost fishes.

INTRODUCTION

The enzyme 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) is essential for the biosynthesis of most steroid hormones. 3β-HSD catalyzes pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone and androstendiol to progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone, respectively. Recently, recombinant 3β-HSD proteins from several mammalian species have been characterized (human, Lorence *et al.*, 1990a, b; mouse, Bain *et al.*, 1990; rat, Lorence *et al.*, 1991). Dynamics of 3β-HSD protein expression were examined using 3β-HSD antibody against purified human placental 3β-HSD protein (Clarke *et al.*, 1993a, b).

Although numerous studies of steroid metabolism have been reported (see Nagahama, 1987), analysis of 3β-HSD protein in lower vertebrates has little attention due to the lack of the specific 3β-HSD antibodies. Recently, Sakai *et al.* (1994) cloned rainbow trout 3β-HSD cDNA and consequently the derived amino acid sequence allows production of specific antibodies to help clarify the role and function of 3β-HSD in fish. This study determines the specificity of 3β-HSD antibodies produced and their use for the immunohistochemical detection of fish steroidogenic tissues.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Aichi Prefectural Fisheries Station, Toyokawa, Japan. These animals were maintained in the laboratory until use.

Production of polyclonal antibodies

The middle portion sequence (CTCALRPMYIYGEC: M) with an additional cysteine in the N- and C-terminus and C-terminal sequence (CTMDWVASQLPKERERIKV: C) in amino acid sequence with an additional cysteine in the N-terminal sequence predicted from rainbow trout 3β-HSD cDNA (Sakai *et al.*, 1994) were synthesized by the F-moc protocol on an Applied Biosystem model 431A peptide synthesizer, and purified by reversed-phase HPLC using a ODS-5 column (Develosil). To increase antigenicity, these peptides were coupled to bovine serum albumin (BSA: Fraction V, Sigma) or Keyhole limpet hemocyanin (KLM: Calbiochem), using EMCS (N-(ε-Maleimidocaproyloxy)succinimide) (Dojindo), following the cleavage of disulfide bonding within the molecules of BSA and KLM with dithiothreitol (DTT).

Female rabbits were immunized at 2-week intervals by four

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subcutaneous injections of peptide-linked protein (1 mg/rabbit/injection). These antigens were emulsified in Freund's complete adjuvant at the first injection and in Freund's incomplete adjuvant after the first injection. One week after the last injection, whole blood from rabbit was collected. From collected blood, serum was separated and then purified by affinity chromatography using synthetic peptides used as antigens.

Preparation of recombinant 3β-HSD protein

To obtain 3β-HSD protein, we tried to produce recombinant 3β-HSD protein using rabbit reticulocyte lysate system (Promega) and COS-1 cells transfected with rainbow trout 3β-HSD cDNA.

For rabbit reticulocyte lysate system, we used rainbow trout 3β-HSD cDNA ligated to pBluescriptII SK(–) as template. According to the instruction manual, lysate containing recombinant trout 3β-HSD protein was obtained and then this lysate was treated with Laemmli's SDS sample buffer (Laemmli, 1970) for immunoblot analysis. For expression of rainbow trout 3β-HSD in COS-1 cells, the rainbow trout 3β-HSD expression vector was constructed by ligating the blunt-ended cDNA fragment into the *Sma*I site of pSVL (Pharmacia LKB). Transfection of rainbow trout 3β-HSD cDNA construct to COS-1 cells was carried out as described previously (Sakai *et al.*, 1994). After this, transfected COS-1 cells were recovered and then homogenized in 0.25 M sucrose, 20 mM Hepes (pH 7.5). To demonstrate whether 3β-HSD activity was in these homogenates, a part of these homogenates was applied to steroid metabolism experiments as described previously (Kobayashi *et al.*, 1993), and the other was treated with SDS-sample buffer for immunoblot analysis.

Protein extraction and electrophoresis

To obtain native 3β-HSD proteins, proteins from testis and interrenal glands were extracted as a mitochondria and microsomal fraction after ultracentifugation (100,000 g, 1 hr, 4°C), then frozen in liquid nitrogen and stored at -80°C until use. Also ovarian follicles were frozen in liquid nitrogen and stored at -80°C. For electrophoresis, proteins from testis and interrenal glands and ovarian follicles were treated with Laemmli's SDS-sample buffer containing 10% β-mercaptoethanol, for 3 min at 100°C, and analyzed by SDS-PAGE with 12.5% gel (Laemmli, 1970).

Immunoblotting

Proteins separated by SDS-PAGE were transferred to Immobilon membrane (Millipore) by electroblotting (Towbin *et al.*, 1979). The membrane was rinsed in Tris-buffered saline (TBS: 20 mM Tris-HCl, 150 mM NaCl, pH 7.5), blocked with non-fat dry milk in TBS containing 0.1% Tween 20 (TTBS). After washed three times (5 min each) with TTBS, the membrane was incubated with a 1:1000 dilution of serum for 2 hr. After washing three times (5 min each) with TTBS, the membrane was incubated with a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Tago). Following further three washes with TTBS, phosphatase activity was visualized by treating the membrane with 0.2 mM 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitroblue tetrazolium (Sigma) in 100 mM diethanolamine buffer (pH 9.5) containing 5 mM $MgCl_2$. All incubations were preformed at room temperature.

Immunohistochemistry

Ovary, testis and interrenal glands were dissected from rainbow trout, tilapia (*Oreochromis niloticus*), goldfish (*Carassius auratus*) and Japanese eel (*Anguilla japonica*), then fixed in Bouin's fixative solution and embedded in paraffin. Serial cross sections were cut at 6 μm. The antibodies were used at 1:1600 dilution. The procedure of this immunohistochemistry in details was described in a previous report (Kobayashi and Iwasawa, 1992).

RESULTS

Immunoblotting with anti-tr3β-M and tr3β-C antibodies

To characterize anti-tr3β-M and tr3β-C antibodies, first, proteins extracted from rainbow trout ovarian follicles were immunoblotted. As shown in Fig. 1, both antibodies recognized specifically 45 kDa band. This immunoreactive 45 kDa band was disappeared after adsorption of each peptide coupled with carrier protein (data not shown). Next, we examined whether these antibodies could detect recombinant 3β-HSD protein. Rabbit reticulocyte lysate system using rainbow trout 3β-HSD cDNA as complement produced only 45 kDa protein (Fig. 2a). Immunoblot analysis demonstrated that recombinant 3β-HSD protein was recognized by both anti-tr3β-M and anti-tr3β-C antibodies (Fig. 2b). COS-1 cell lysates transfected with rainbow trout 3β-HSD cDNA produced a bioactive 3β-HSD protein which was also detected by these antibodies (Fig. 3a, b). These results indicated that the anti-tr3β-M and tr3β-C antibodies specifically recognized 3β-HSD protein from rainbow trout.

To demonstrate whether the multiple forms of 3β-HSD are present, immunoblot analysis of several steroidogenic organs (i.e., testis, ovary and interrenal glands) from rainbow trout has completed. As shown in Fig. 4, a single and immunoreactive protein of equivalent size was detected in all organs tested.

Immunohistochemistry with anti-tr3β-M and tr3β-C antibodies

To examine the availability of anti-tr3β-M and tr3β-C antibodies for immunohistochemistry, we applied these antibodies to immature and mature testes, immature ovaries, and interrenal glands of several teleost fishes including rainbow trout (Fig. 5a-f). In rainbow trout, immunoreactive 3β-HSD

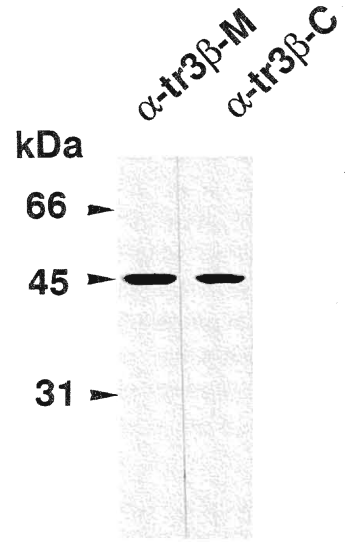

Fig. 1. Immunoblot analysis of ovarian follicle homogenates from rainbow trout with α-tr3β-M and tr3β-C antibodies. For SDS-PAGE, 5 μg of protein from each sample was applied. Both antibodies recognized specifically 45 kDa band.

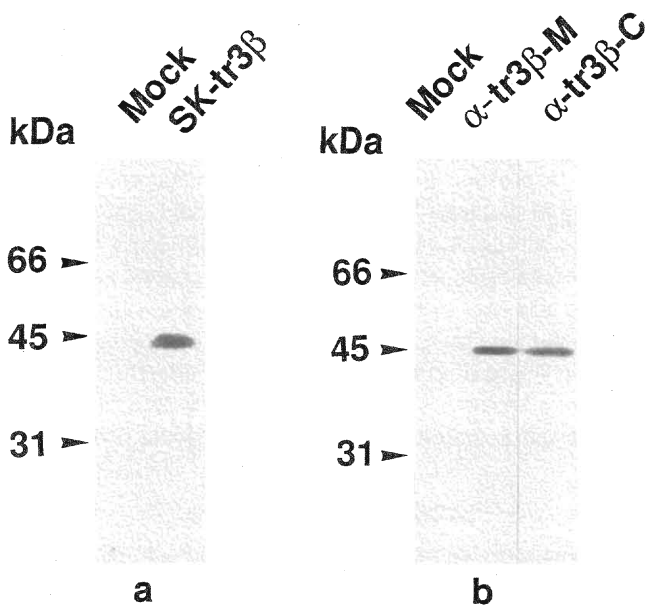

Fig. 2. Immunoblot analysis of recombinant rainbow trout 3β-HSD protein with α-tr3β-M and tr3β-C antibodies. Rabbit reticulocyte lysate system was used for the production of 3β-HSD protein. a, Incorporation of ^{35}S-methionine at production of rainbow trout 3β-HSD protein. SK-tr3β, rainbow trout 3β-HSD cDNA ligated to pBluescriptII SK(−). b, Immunoblot analysis of recombinant rainbow trout 3β-HSD protein with α-tr3β-M and tr3β-C antibodies. For SDS-PAGE, 5 μg of protein from each sample was applied.

localized in Leydig cells in the interstitium of immature testes (Fig. 5a) and interrenal cells in the head kidney (Fig. 5d). These immunoreactive cells have typical steroidogenic features such as smooth endoplasmic reticulum, mitochondria with tubular cristae and lipid droplets (data not shown). Immunoreactive 3β-HSD was also found in Leydig cells of the mature testis (Fig. 5b) and interstitial cells of the immature ovary of tilapia (Fig. 5c); a weak immunoreaction was found in the cytoplasm of immature oocytes. Positive staining for 3β-HSD was also detected in interrenal cells of goldfish (Fig. 5e) and Japanese eel (Fig. 5f). There was no immunostaining associated with non-steroidogenic cell types such as chromaffin cells, endothelial cells and blood cells.

DISCUSSION

We described the characteristics of anti-tr3β-M and tr3β-C antibodies that recognized 3β-HSD proteins and the localization of 3β-HSD proteins in ovary, testis and interrenal glands. To our knowledge, this report is the first examination on the localization of 3β-HSD using homologous 3β-HSD antibodies in nonmammalian vertebrates.

Previous reports indicated that multiple forms of 3β-HSD proteins are present in mammals. Although immunoblot analyses were performed by two distinct antibodies, the present study indicated a single immunoreactive 3β-HSD protein. However, other forms of 3β-HSD protein in rainbow trout may not be recognized by these antibodies. In contrast to the multiple related 3β-HSD isoenzymes in human (Lorence

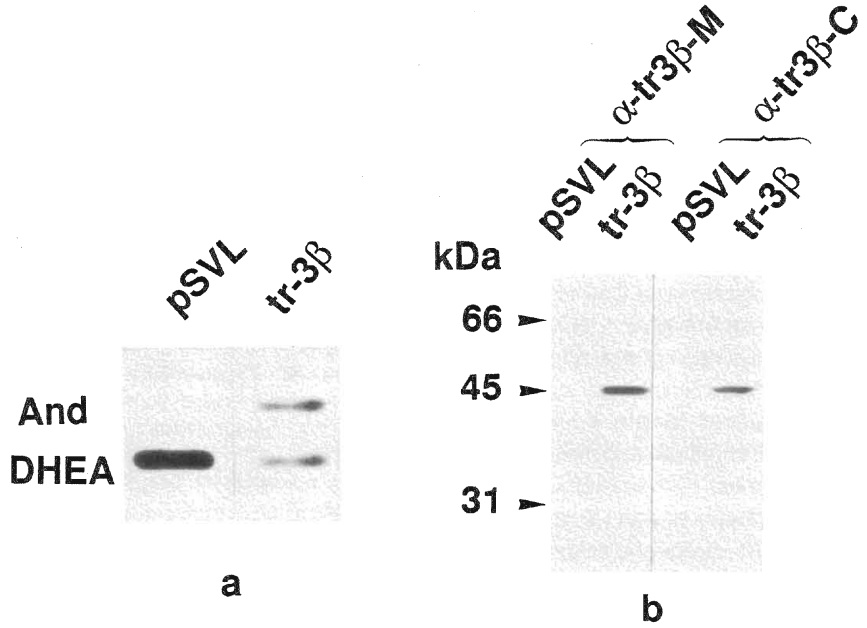

Fig. 3. Immunoblot analysis of recombinant rainbow trout 3β-HSD protein with α-tr3β-M and tr3β-C antibodies. COS-1 cell lysates transfected with rainbow trout 3β-HSD cDNA. a, Bioactivity of COS-1 cell lysates transfected with rainbow trout 3β-HSD cDNA. To determine 3β-HSD activity of transfected COS-1 cell lysates, dehydroepiandrosterone (DHEA) was used as substrate. The extracted steroid metabolites were applied on thin layer chromatography with benzene : acetone (4 : 1). And, Androstenedione. b, Immunoblot analysis of COS-1 cell lysates transfected with rainbow trout 3β-HSD cDNA. For SDS-PAGE, 5 μg of protein from each sample was applied.

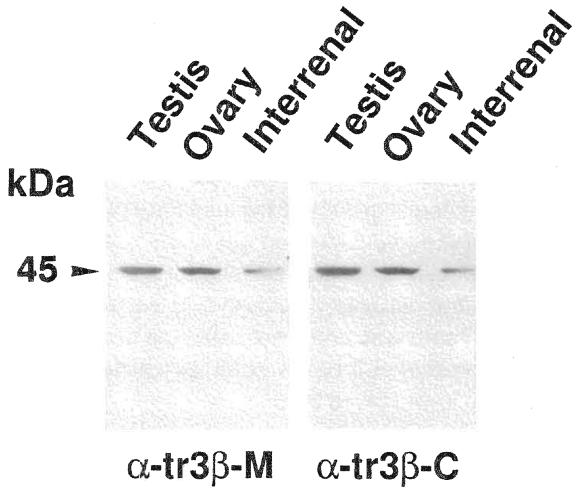

Fig. 4. Immunoblot analysis of several steroidogenic tissues from rainbow trout with α-tr3β-M and tr3β-C antibodies. Testis, lysates of mitochondria and microsome-rich fractions from testis; Ovary, homogenates of ovarian follicles from ovary; Interrenal, lysates of mitochondria and microsome-rich fractions from head kidney. For SDS-PAGE, 5 μg of protein from each sample was applied.

et al., 1999a, b), rat (Naville *et al.*, 1991; Zhao *et al.*, 1990, 1991) and mouse (Bain *et al.*, 1990), Sakai *et al.* (1994) indicated that a single gene encoded 3β-HSD in rainbow trout from Southern hybridization analysis of genomic DNA in rainbow trout. Thus, these results suggest that rainbow trout 3β-HSD protein is not multiform.

Although numerous studies on the histochemical detection of 3β-HSD and 3β-HSD activity at the level of steroid metabolism were reported (Lofts and Bern, 1972; Guraya, 1976; Nagahama, 1987), there is a little information in lower vertebrates on the localization of 3β-HSD protein in steroidogenic tissues at the level of immunohitochemistry and immunoblotting. In the present study, both 3β-HSD antibodies recognized immunohistochemically 3β-HSD in immature ovaries, immature and mature testes, and interrenal glands of several teleost fishes. In the immature ovary of tilapia, strong immunostaining for 3β-HSD was observed in the interstitial cells which had previously been shown to be steroidogenic by ultrastructural observations (Nakamura *et al.*, 1993). A weak 3β-HSD immunoreaction was also seen in the cytoplasm of immature oocytes of tilapia. Further biochemical studies are necessary to determine whether tilapia oocytes are steroidogenic. Leydig cells in both immature and mature testes were positive to 3β-HSD antibodies. These findings are in agreement with previous histochemical and ultrastructural observations on the Leydig cells of several teleost fishes (Nagahama *et al.*, 1978; Nagahama, 1987). Immunoreactive 3β-HSD in Sertoli cells was not detected in the present study, though some previous reports suggested the presence of 3β-HSD activity in Sertoli cells (*Cymatogaster aggregata*, Wiebe, 1969; *Fundurus heteroclitus*, Bara, 1969; *Salmo gairdneri*, van den Hurk *et al.*, 1978a, b). Further studies are necessary whether Sertoli cells are steroidogenic in fish. In the present study the immunohistochemical localization of 3β-HSD in the head kidney was confined only to the interrenal cells; immunoreaction was not observed in either chromaffin cells or blood cells. These findings are consistent with previous results for the restricted distribution of 3β-HSD in interrenal cells in the teleost head kidney (Hanke and Chester Jones, 1966; Lofts and Bern, 1972; Kagawa and Nagahama, 1989).

In the present study, we demonstrated that rainbow trout anti-tr3β-M and tr3β-C antibodies which could detect the 3β-HSD protein specifically were available for immunoblot analysis and immunohistochemistry. Recently we obtained the results that the tr3β-M-antibody was able to recognize the 3β-HSD protein in many animals including mammals, birds, reptiles and amphibians (Kobayashi *et al.*, unpublished). Thus it seems that these antibodies are also available for detection of 3β-HSD protein in steroidogenic tissues throughout vertebrates.

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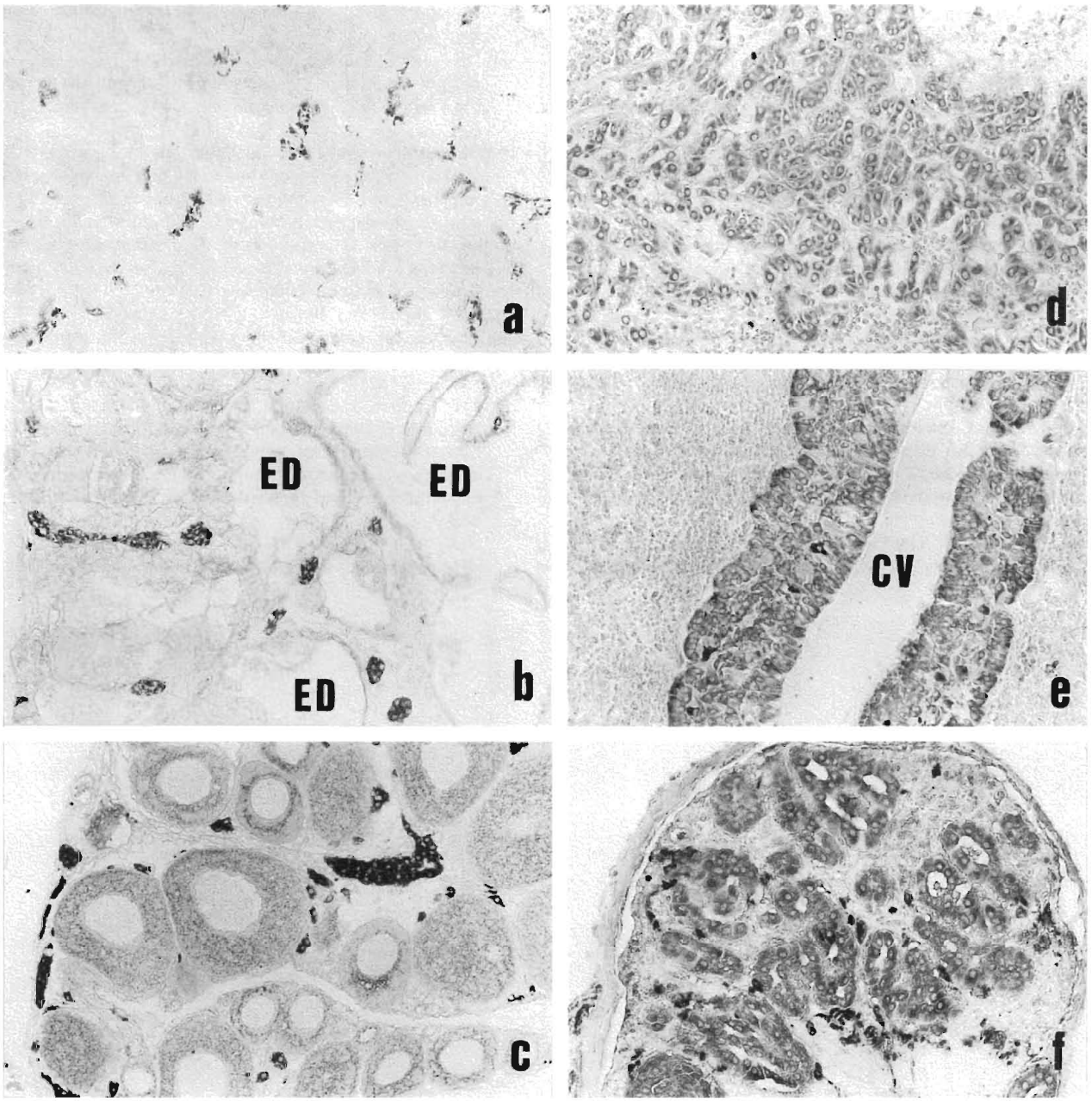

Fig. 5. Immunohistochemistry of several steroidogenic tissues with 3β-HSD antibodies. a, Immature testis from young rainbow trout was stained with α-tr3β-M antibody. Seminiferous tubules were filled with spermatogonia and poorly-developed Leydig cells. b, Mature testis from tilapia was stained with α-tr3β-M antibody. Note strong immunoreaction for 3β-HSD in only Leydig cells (a and b). ED, intratesticular efferent duct. c, Immature ovary from young tilapia was stained with tr3β-C antibody. 3β-HSD positive cells are found in the interstitium. d, Interrenal gland in the head kidney from female rainbow trout was stained with tr3β-C antibody. e, Interrenal gland in the head kidney from female goldfish was stained with tr3β-C antibody. CV, cardinal vein. f, Interrenal gland in the head kidney from male Japanese eel was stained with α-tr3β-M antibody. Note strong immunoreactive 3β-HSD in interrenal cells (d, e and f). × 270.

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