BIOLOGY OF REPRODUCTION 58, 919–926 (1998)

Expression and Immunolocalization of Functional Cytochrome P450 Aromatase in Mature Rat Testicular Cells[1]

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ABSTRACT

Aromatase activity has been measured in Leydig cells and Sertoli cells from both immature and mature rats. Cytochrome P450 aromatase ($P450_{arom}$) has been immunolocalized in germ cells of the rodent, bear, and rooster. Our purpose was to investigate expression of and to immunolocalize $P450_{arom}$ in adult rat testicular cells. After Western blotting with a specific anti-cytochrome $P450_{arom}$ antibody, we demonstrated the presence of a 55-kDa protein in mature rat seminiferous tubules and crude germ cell preparations. Immunoreactive aromatase was detected both in cultured rat Leydig cells and in testis sections (interstitial tissue and elongated spermatids showed positive immunoreactivity for $P450_{arom}$). We next used reverse transcription-polymerase chain reaction to localize and quantify the $P450_{arom}$ mRNA in the various testicular cells. In rat Leydig cells, the amount of $P450_{arom}$ mRNA was 15 times higher than in Sertoli cells (34.1 ± 3.2 to 2.3 ± 0.2 × 10^{-3} amol/10^6 cells, respectively). In pachytene spermatocytes, round spermatids, and testicular spermatozoa the $P450_{arom}$ mRNA levels were 38.7 ± 8.1, 20.4 ± 3.8, and < 1.3 × 10^{-3} amol/10^6 cells, respectively. The aromatase activity was 2.5–4 times higher in testicular spermatozoa (8.48 ± 1.98 fmol/10^6 cells per hour) than in other germ cells. These results indicate that in mature rats, not only Leydig cells and Sertoli cells but also germ cells have the capacity to express functional $P450_{arom}$. According to the germ cell maturation state, there was an inverse relationship between $P450_{arom}$ mRNA content and the biological activity of the protein. The expression of the functional $P450_{arom}$ in mature rat germ cells confirms the existence of an additional source of estrogens within the genital tract of the male.

INTRODUCTION

Testosterone is the main sex steroid produced by the mammalian testis. Together with gonadotropins, it is responsible for induction and maintenance of spermatogenesis. In addition, the ability of the male gonad to convert androgens into estrogens is well known; the microsomal enzymatic complex involved in this transformation is named aromatase and is composed of two proteins. One of them is a specific microsomal heme glycoprotein, the cytochrome P450 aromatase ($P450_{arom}$), the product of a unique gene that is a monooxygenase enzyme, a member of the cytochrome P450 superfamily. It is responsible for binding and catalyzing the modification of steroid substrates. By contrast, the second moiety is a ubiquitous, nonspecific microsomal flavoprotein, NADPH-cytochrome P450 reductase, required to transfer electrons from NADPH to any cytochrome P450 [1, 2]. The aromatase complex is located in the endoplasmic reticulum in the cells in which it is expressed, and there appears to be an increasing list of tissues now known to express aromatase [3]. The precise location of $P450_{arom}$ inside the testis has been the subject of controversy during recent decades [4–8]: in the rat there is an age-related change in the cellular location of the aromatization site, mainly in Sertoli cells in immature animals and in Leydig cells in adults [9]. At the testicular level, aromatase has been immunohistolocalized in Leydig cells in humans [10], in young and adult rats [11], in rams [12], and in stallions [13]. Striking species differences exist: in the mouse [14], brown bear [15], and rooster [16], aromatase is present not only in Leydig cells but also in the seminiferous tubules, predominantly in spermatids. In contrast, aromatase activity can be measured in cultured human Sertoli cells [17] and in immature rat Sertoli cells [9] but also in rat germ cells [18] and mouse sperm [19]. The differences noticed between the immunohistolocalization of the $P450_{arom}$ on the one hand, and the measurement of $P450_{arom}$ activity in the cell cultures on the other, could be related to the absence of endocrine or paracrine regulation, due, for example, to germ cell depletion or a lack of cell-cell contacts in culture dishes [20–22]. These observations led us to carefully evaluate the source of estrogens in the various testicular cells of the mature rat. For that purpose we 1) immunolocalized $P450_{arom}$ in testicular cells, 2) determined the biological activity of the $P450_{arom}$, and 3) investigated the testicular cell distribution and then quantified $P450_{arom}$ mRNA using a highly sensitive reverse transcription-polymerase chain reaction method.

MATERIALS AND METHODS

Animals and Enriched Testicular Cell Preparations

The testes of mature (80–100 days old) Sprague-Dawley rats were coarsely minced and then subjected to an enzymatic treatment with collagenase-dispase (0.05%), soybean trypsin inhibitor (0.005%), and deoxyribonuclease (0.001%) in Ham's F-12/Dulbecco's Modified Eagle's (DME) (1:1, v:v) medium for 10 min at 32°C. The Leydig cells were purified on discontinuous Percoll gradients [23]; only the most enriched fraction was collected and characterized for presence of Leydig cells by histochemical staining for 3β-hydroxysteroid dehydrogenase (3β-HSD). We observed that 98% of the cells present stained positive for 3β-HSD. The pelleted seminiferous tubules were assayed for Western blotting studies or used for tubule cell preparations. After an additional enzymatic treatment with the cocktail mentioned above, the supernatant obtained was labeled mixed tubular cells (MTC), and the pellet was sub-

Accepted November 12, 1997.
Received April 28, 1997.
[1]B.B. was a recipient of a fellowship from the French Ministry of Education and Research. Part of this work was presented as an abstract at the XIII Congrès de la Société Française d'Endocrinologie, Nancy (September 1995) and at the IV International Aromatase Conference, Tahoe City, California (June 1996).
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mitted to a third enzymatic digestion in the presence of hyaluronidase (0.01%), soybean trypsin inhibitor (0.005%), and DNase type I (0.001%) [24, 25]. The cells were filtered, washed, and plated for 48 h under an air/CO_2 (5%) atmosphere at 37°C in Ham's F12/DME medium supplemented with rat serum (5%) in order to attach the Sertoli cells. On Day 2, the culture medium containing germ cells was removed (mixed germ cells) and replaced by fresh culture medium without serum. On Day 4 the cells were submitted to a hypotonic treatment in order to remove the remaining germ cells, and the culture dish was washed with fresh medium and then incubated for 24 h.

Immunohistochemical Staining

Paraffin-embedded testicular tissue sections were mounted on slides precoated with polylysine and then deparaffinized and rehydrated. Rat Leydig cells were cultured for 48 h on round glass coverslips in Ham's F12/DME (1:1, v:v) medium supplemented with rat serum (0.2%) under an air: CO_2 (5%) atmosphere at 32°C. Tissue sections and Leydig cells were both fixed for 15 min in freshly prepared paraformaldehyde (4% and 2%, respectively); the cultured cells were then permeabilized with 0.1% Triton X-100. To inhibit endogenous peroxidase activity, hydrogen peroxide (1.2% in absolute methanol for sections and 3% in distilled water for cultured cells) was used for 30 min. Then the cells were incubated for a further 30 min with 20% normal goat serum to block the nonspecific binding sites. After that, sections and cells were both processed for visualization of the aromatase using an immunocytochemical technique described elsewhere [12]. They were incubated with a primary antibody, rabbit polyclonal antiserum against human placental P450 aromatase (1:500), generously provided by Dr. Yoshio Osawa (Hauptman-Woodward Medical Research Institute, Buffalo, NY). (This antibody was developed with the support of U.S. Public Health Service Research Grant #HD P4945 from NICHHD). Sections as well as cultured cells were then incubated with secondary antibody, biotinylated goat-anti-rabbit (1:600) IgG (Vector Laboratories, Burlingame, CA), for 1 h followed by avidin-biotin-horseradish peroxidase complex (Strept ABC complex/HRP) without treatment (Dako/AS, Denmark). The peroxidase reaction was developed by means of 3–3′-diaminobenzidine tetrachloride dihydrate (Stable DAB; Research Genetics Inc., Huntsville, AL). A portion of the experiments dealing with aromatase immunolocalization were done with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG instead of Strept ABC complex/HRP.

As the control, the primary antibody was replaced by normal rabbit serum at the same concentration. After each step of this procedure, sections and cultured cells were both carefully rinsed with Tris-buffered saline (0.05 M Tris-HCl plus 0.15 M NaCl; TBS, pH 7.6); the antibodies were also diluted in TBS buffer.

Purification of Germ Cells

Germ cells were prepared from adult rat testes by the trypsin-DNase method [26]. Briefly, the testes were decapsulated, and after mechanical disruption in PBS (pH 7.2), the interstitial cells were removed by four successive decantations. Tubules were submitted to enzymatic digestion (trypsin 0.025%, DNase 0.1% in PBS) during 45 min at 32°C. The testicular cells were washed in PBS supplemented with glucose (1.5 mM) and pyruvate (1.2 mM). The spermatozoa were purified by centrifugation on a Percoll/PBS gradient (70:30, v:v). After the removal of some Sertoli cell aggregates by filtration through fine nylon mesh, the pachytene spermatocytes and round spermatids containing fractions were purified by unit gravity sedimentation on a continuous BSA gradient (2.75–0.2%) [27]. The pachytene spermatocyte- and round spermatid-containing fractions were identified under a phase-contrast microscope, and the highly homogeneous fractions were pooled and washed. The cells were counted and used immediately for RNA preparation or frozen until used for microsomal extraction.

Western Blot Analysis

Cells were homogenized in 50 mM Tris-maleate buffer (pH 7.4), and the protein concentration was estimated using Bradford's method [28]. The proteins were separated on SDS-polyacrylamide (9%) gel and then electroblotted onto nitrocellulose membrane. The blots were probed overnight at 4°C with a rabbit polyclonal antiserum against the human placental aromatase (a generous gift from Dr. Fernand Labrie and Dr. Van Luu-Thé, MRC, CHUL Research Center, Laval, Quebec, Canada) diluted to 1:700; the antigen-antibody complexes were revealed at room temperature with goat anti-rabbit IgG coupled to peroxidase (Bio-Rad, Ivry sur Seine, France).

Measurement of Aromatase Activity

Microsomes were prepared from either testicular tissue or purified testicular cells as previously described [29]. The aromatase activity was assessed by measurement of ^3H_2O released [30] from [1β-^{3}H]androst-4-ene-3,17-dione (2000 pmol; New England Nuclear, Les Ulis, France). Incubations were performed at 37°C for 1 h in Tris-maleate buffer. The reaction was started by the addition of NADPH (0.3 mM) to a final volume of 0.5 ml and stopped by the addition of 1 ml chloroform. After centrifugation (2700 × *g*, 5 min, 4°C), 500 μl aqueous phase was removed, and 500 μl activated-charcoal (7%) suspension containing dextran (1.5%) was added. After 10 min the charcoal was separated from the aqueous phase by centrifugation (2700 × *g*, 15 min, 4°C), and the radioactivity of the supernatant was measured.

Microsomal fractions from human placental and rat leg muscles were used as positive and negative controls, respectively. The activity was expressed either in pmol ^3H_2O produced per milligram of protein per hour or in fmol ^3H_2O released per 10^6 cells per hour.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay

The mRNA present in the total RNA, isolated by the Chomczynski and Sacchi [31] guanidium thiocyanate-derived method, was reverse transcribed to cDNA as follows: 30 min at 37°C with 200 IU Moloney murine leukemia virus reverse transcriptase (Life Technology, Eragny, France), 500 μM dNTP, 0.2 μg oligo-dT (12–18 mer), and 24 IU RNasin. The cDNAs obtained were further amplified by PCR using selected oligonucleotides. PCR was performed during 35 cycles (30 sec at 94°C, 30 sec at 60°C, and 1 min 72°C with 2 sec delay at 72°C for each cycle) in the presence of 200 μM dNTPs, 1 μM of primer set, and 1.5 IU *Taq* polymerase in a final volume of 50 μl (Eurobio, Les Ulis, France). $P450_{arom}$ primers (5′ARO: 5′-GCTTCTCATCGCAGAGTATCCGG-3′ at position 1555–1577 and 3′ARO: 5′-CAAGGGTAA-ATTCATTGGGCTTGG-3′ at position 1821–1844 [GenBank

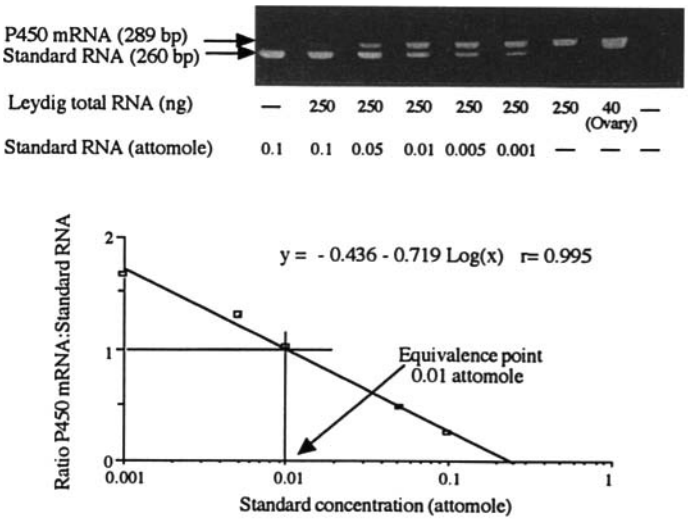

FIG. 1. Quantitative competitive RT-PCR of total RNA from adult rat Leydig cells. $P450_{arom}$ mRNA was co-amplified with serial dilutions of RNA standard. The amplified products were subjected to electrophoresis on 4% agarose gel, and the gel was photographed and subjected to scanning densitometry. The $P450_{arom}$ mRNA content was estimated by the determination of the equivalence point according to the equation established by Menzo et al. [37].

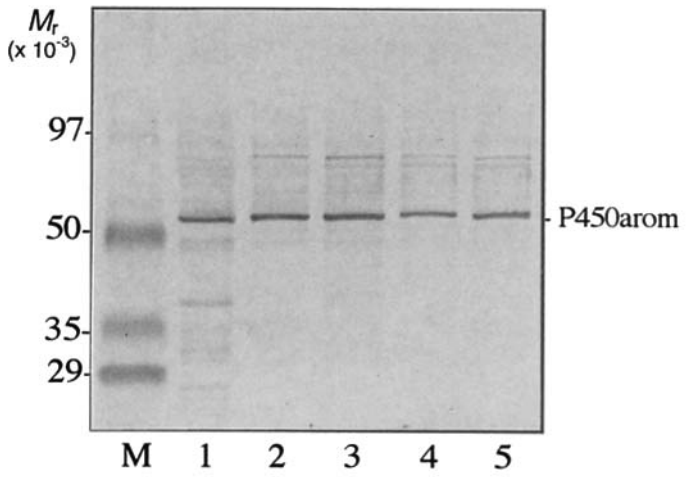

FIG. 2. Western immunoblotting of $P450_{arom}$ in adult rat testis and cell suspensions. Total proteins were extracted from whole testis (lane 2), seminiferous tubules (lane 3), and from two cell fractions obtained during Sertoli cell purification. The MTC containing Sertoli cells, peritubular cells, and germ cells (lane 4) and the mixed germ cells (spermatocytes and spermatids) (lane 5). Human placental extract (5 μg) was used as positive control (lane 1). On left are low-range prestained SDS-PAGE standards.

Accession no. M33986]) were chosen to amplify a highly conserved sequence (289-base pair [bp] length) including helical and aromatic regions [32]. To estimate the Sertoli cell contamination of the germ cell preparations, two androgen-binding protein (ABP; [33]) gene-specific primers were synthesized (5′-ABP: 5′-ACAATCTCTGGGCTCGGCTT-3′ at position 365–384 and 3′ABP: 5′-CCCAAAGGTCACTCAGGCAA-3′ at position 1119–1139).

To sequence the amplified products, the 3′ARO primer was previously phosphorylated (30 min, 37°C; 10 min, 65°C) in the presence of 10 IU T4-kinase and 10 mM ATP. After PCR, the phosphorylated strand was digested by 10 IU Lambda exonuclease (Pharmacia Biotech, Saclay, France; 20 min, 37°C), and the single remaining strand was then purified on a Microcon 100 column (Amicon, Grace, France) and sequenced according to Sanger's method [34] using the Sequenase kit version 2 (USB, Cleveland, OH).

RNA Standard Preparation and Quantitative Competitive RT-PCR

The RNA standard was prepared by internal nucleotide deletion of the 289-bp amplified product [35, 36]. Briefly, total RNA from rat ovary was subjected to RT and PCR with the appropriate primers. The 5′STD primer contained three specific sequences including 1) the complementary sequence for the DNA-binding region of T7 RNA polymerase, 2) the primer 5′ARO (underlined), and 3) a 27-base sequence (position 1607–1634) located 29 bp downstream from the 5′ARO primer (bold). The 3 STD primer resulted from the association of the oligo(dT) and the specific 3 ARO sequence (underlined): (5 STD: 5′-TAATACGACTCACTATAGGGAGAGCTTCTCATCGCAGAGTATCCGG**CACACTGTTGTTGTTGACAGAGACATA**-3′ and 3 STD: 5′-TTTTTTTTTTTTTTTTTTCAAGGGTAAATTCATTGGGCTTGG-3′). The cDNA of interest now contained sequences for the T7 polymerase; it has a 29-bp deletion compared to the native mRNA strand. Using 20 IU RNasin, 2.5 mM of each ribonucleotide, and 20 IU T7 RNA polymerase (Riboprobe Systems, Promega, France) this cDNA strand was then transcribed to an internal competitive RNA standard of 260 bp [36]. The RNA standard was treated with 1 IU RQ1 RNase-free DNase (Promega, Charbonnières, France) to digest the cDNA templates. The RNA was extracted [31] and resuspended in diethyl pyrocarbonate-treated water supplemented with 0.1 mg/ml tRNA. In order to verify that the PCR-amplified product was in the linear part of the amplification curve, a 1:10 serial dilution was reverse transcribed and amplified with the 5′ARO-3′ARO set of primers. The amplified products were subjected to electrophoresis on 2% agarose gel; the gel was then photographed and submitted to densitometry scanning analysis (Bio-Profil System, Vibert-Lourmat, France). The concentration of RNA was plotted against the density of the bands. With 35 cycles of PCR, the amplification was linear from 10^{-4} amol (lower limit of detection) to 100 amol ($r = 0.98$, $p < 0.001$) [36]. The absence of contaminating cDNA template in the RNA standard preparation was checked by PCR without RT using the same primers (at the same serial dilution); an amplified product was first detected in 100 amol of RNA standard.

For quantitative RT-PCR experiments, increasing dilutions of the RNA standard were added to a known amount of total RNA. After RT and PCR with the $P450_{arom}$-specific primers, the amplified products were run on a 4% agarose gel stained with ethidium bromide, then photographed and analyzed by densitometry (Fig. 1).

RESULTS

Western Blot Analysis

Using an antibody against human placental $P450_{arom}$, a positive signal (Fig. 2) was detected in protein extracted from whole testis (lane 2), seminiferous tubules (lane 3), and mixed germ cells (pachytene spermatocyte and round spermatid; lane 5). With that $P450_{arom}$ antibody, the major bands detected in rat testis and germ cells comigrated with a 55-kDa protein revealed in the human placenta extract (5 μg) used as a positive control (lane 1). Only one nonspecific band was detected at 48 kDa in the presence of a 10-

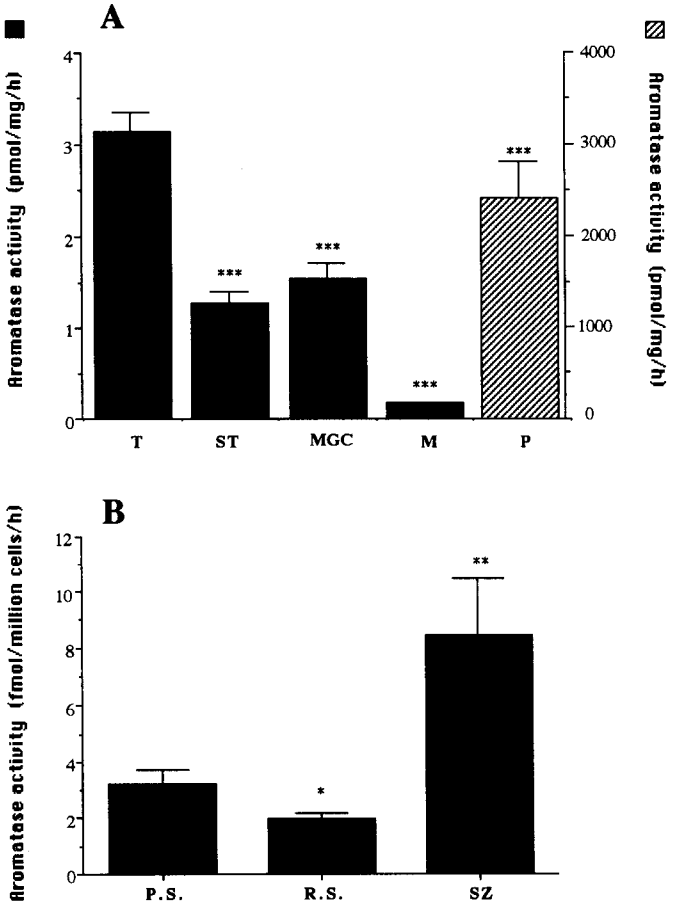

FIG. 3. P450 aromatase activity in adult rat testis. **A**) Whole testis (T), seminiferous tubules (ST), and mixed germ cells (MGC). Human placenta (P) and muscle (M) were used as positive and negative controls, respectively. **B**) Pachytene spermatocytes (P.S.), round spermatids (R.S.), and spermatozoa (SZ). Increasing concentrations of microsomal proteins were assayed. Values are means ± SEM (n = 3); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, comparing to whole testis in **A** and to pachytene spermatocytes in **B**.

fold-higher protein concentration of rat leg muscle used as a negative control (data not shown).

Aromatase Activity in Rat Testicular Compartments and in Purified Germ Cells

To confirm the presence of the $P450_{arom}$ shown by Western blot analysis, aromatase activity was first measured in microsomal preparations from whole testis, seminiferous tubules, and mixed germ cells (Fig. 3A). The ^3H_2O assay for $P450_{arom}$ activity was validated using increasing concentrations of each microsomal preparation; a linear relationship ($r > 0.96$) was observed between the amount of ^3H_2O released and the quantity of protein incubated (not shown). In the presence of increasing amounts of proteins from rat leg muscle, no change in ^3H_2O output was noticed compared to the blank value (microsomal proteins incubated without NADPH). The aromatase activity in germ cells was 1.52 ± 0.21 pmol/mg protein per hour and was not significantly different from that of whole seminiferous tubules (1.24 ± 0.17 pmol/mg protein per hour) but was about half that in whole testis (2.86 ± 0.34 pmol/mg protein per hour). In human placenta this enzyme activity was about 1000 times higher (2755.37 ± 574 pmol/mg protein per hour) than in the rat testicular extract. In a second step we determined the $P450_{arom}$ activity in purified germ cells from mature rat testes. For that purpose, three preparations of purified germ cells were studied, and for each of these, three concentrations of microsomal proteins were used. As shown in Figure 3B, there was a significant difference in aromatase activity as determined in either pachytene spermatocytes or round spermatids (3.16 ± 0.55 and 1.95 ± 0.20 fmol/10^6cells per hour, respectively) and that in spermatozoa (8.48 ± 1.98 fmol/10^6 cells per hour). These data were confirmed when the aromatase activity was expressed in micrograms of microsomal proteins.

Leydig cell aromatase activity measured in a pool of purified Leydig cells was around 20 fmol/10^6 cells per hour (data not shown).

Localization of Immunoreactive P450 Aromatase

Immunoreactive $P450_{arom}$ was detected both in cultured rat Leydig cells and in paraffin-embedded sections of mature testes. The intensity of cell immunoreactivity was not the same in all cells examined; however, it was always strong or very strong (Fig. 4C). The immunostaining pattern was not homogeneous, being either granular or diffusive; no nuclear staining was observed either in cultured Leydig cells (Fig. 4C) or in the cells of the interstitial area (Fig. 4E). In testicular sections, a high degree of immunoreactivity was observed in the interstitial cells as well as in the adluminal compartment of the seminiferous tubules, mainly in the elongated spermatids (arrowheads) shortly before their release into the tubular lumen (Fig. 4E). With a higher magnification we were able to localize the immunostaining in the elongated spermatids and in more mature germ cells within the lumen, likely spermatozoa, which exhibited the immunoreactivity both in the heads and along flagella. It is of note that the cytoplasm of less mature spermatids was even slightly positive (data not shown). In order to check the staining specificity, the immunoreactivity of testicular cells was studied using immunofluorescence technique. With the antibody coupled with FITC, strong immunoreactivity for P450 aromatase was observed not only in the cytoplasm of Leydig cells (Fig. 4D, arrowhead) but also in elongated spermatids that were obviously present around the lumen of the seminiferous tubules (Fig. 4F, arrowhead). Blood vessels were negative, and no immunohistochemical staining was observed when cells or sections were incubated without primary antibody (Fig. 4, G and H).

Presence of Cytochrome $P450_{arom}$ mRNA

The total RNA isolated from each highly purified cellular fraction was subjected to reverse transcription and amplification with the specific $P450_{arom}$ set of primers. As illustrated in Figure 5A, after RT-PCR, a single band was found in purified Leydig cells and Sertoli cells (lanes 1 and 2) but also in various germ cell populations such as pachytene spermatocytes, round spermatids, and spermatozoa (lanes 3–5), as well as in rat ovary used as a positive control (lane 7). When total RNA from rat leg muscle (lane 6) and water added instead of RNA (lane 8) were treated in the same conditions, no amplified signal was observed, so they were used as negative controls.

Whatever the source of RNA, the amplified product evidenced the correct size of 289 bp according to that expected with the chosen primers. To further confirm the nature of the signal, the cDNA fragments obtained in Leydig and Sertoli cells, pachytene spermatocytes, and ovary were sequenced and then compared to the corresponding frag-

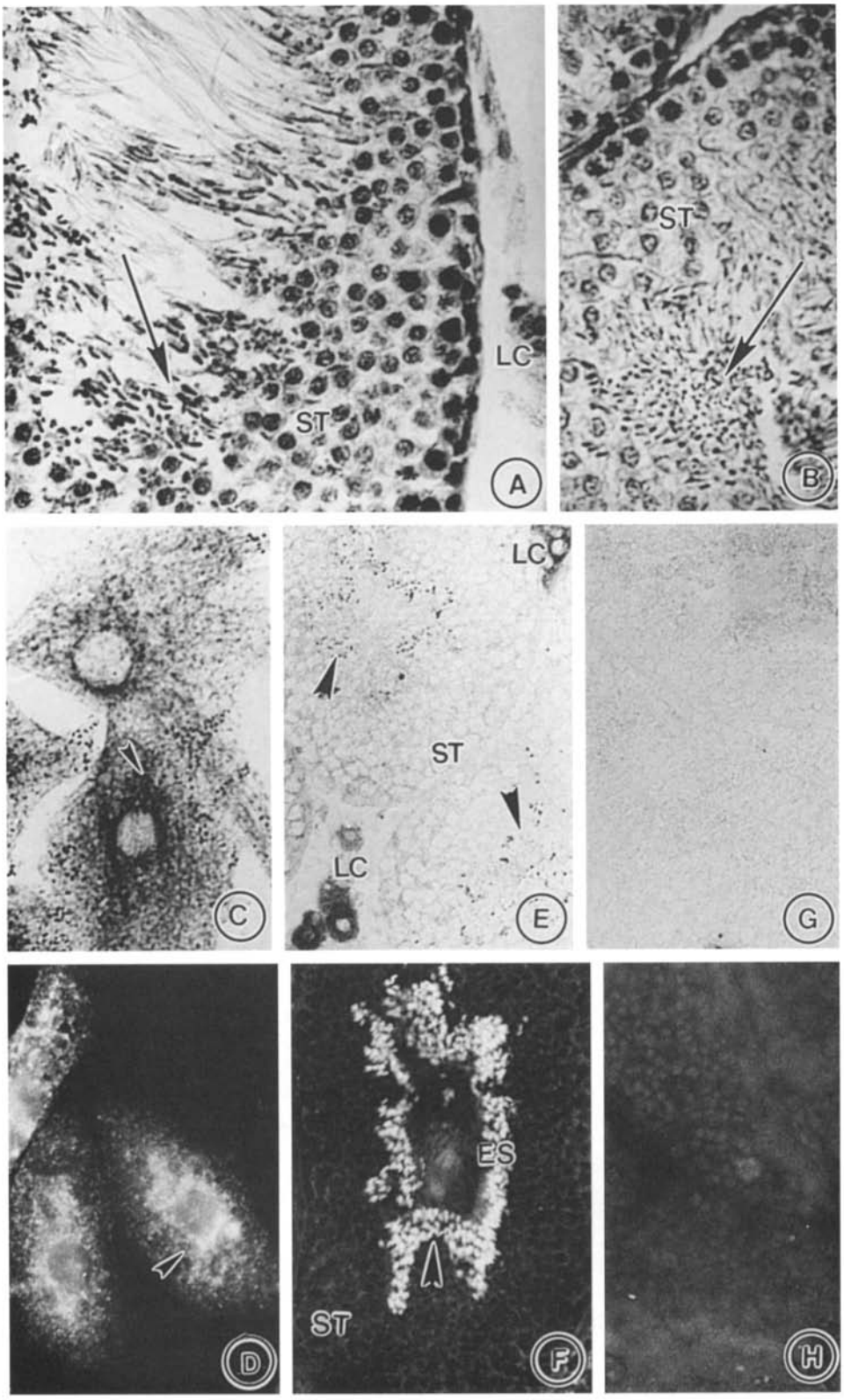

FIG. 4. Morphology and immunoreactivity of the aromatase in Leydig cells and testis sections from adult rats. Immunostaining was performed using a polyclonal antibody specific for the human placental cytochrome P450 aromatase and Strept ABC complex/HRB visualized by DAB (**C, E**) or indicated by FITC-conjugated anti-rabbit IgG (**D, F**). **A, B**) Light micrographs of seminiferous tubules. Note the elongated spermatids shortly before their release into the tubular lumen (arrows, hematoxylin-eosin staining). LC, Leydig cells; ST, seminiferous tubules. **C, D**) Localization of immunoreactive aromatase in cultured rat Leydig cells. Strong aromatase immunoreactivity in whole cytoplasm (arrowheads). No nuclear staining was observed. **E, F**) Sections of rat testis. Immunoreactive aromatase in elongated spermatids (ES) around the lumen of seminiferous tubules (ST) (arrowheads). **G, H**) Sections of rat testis. No immunopositive staining was detected when the primary antibody was replaced by normal rabbit serum. **A, B**: ×620; **C, D**: ×360; **E-H**: ×320. Reproduced at 95%.

ments of rat ovarian granulosa cell cDNA published by Hickey et al. [32]; they showed 100% homology.

After Sta-Put, the purity of each germ cell fraction was first estimated under a microscope, and then the cell preparations were submitted to 3β-HSD histochemical staining; no positive cells were observed. In both pachytene spermatocytes and round spermatids the purity appeared to be higher than 95%, and it was around 98% in the spermatozoa fraction; the major contamination of these preparations was by other germ cells. However, it is well known that rat Sertoli cells express aromatase activity [9]. Therefore, to check for possible contamination of the various germ cell fractions (especially in spermatozoa for which a weak signal was detected) we co-amplified the $P450_{arom}$ with ABP used as a specific Sertoli cell marker (Fig. 5B). The specific 774-bp ABP band was highly detectable in the Sertoli cell

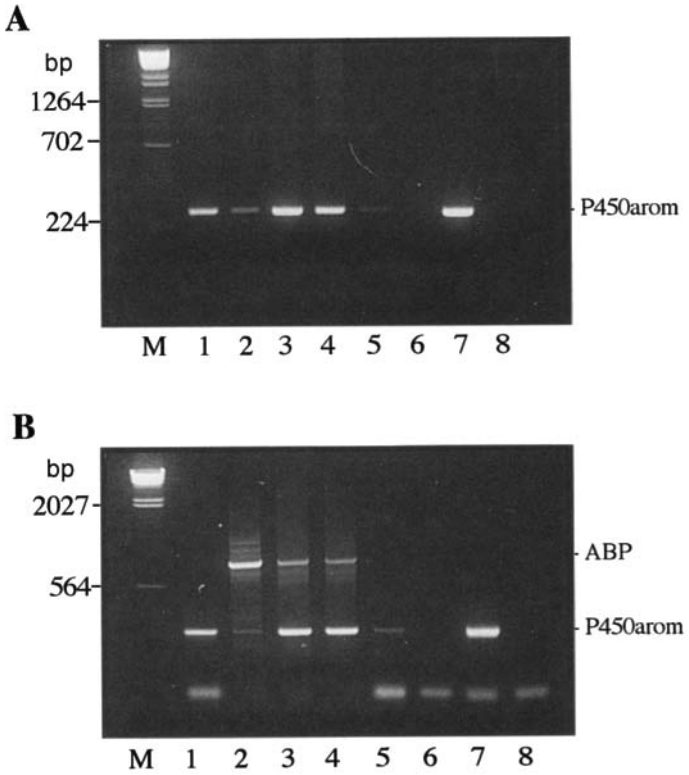

FIG. 5. Amplification by RT-PCR of RNA isolated from purified testicular cells of adult rats including Leydig cells (lane 1), Sertoli cells (lane 2), pachytene spermatocytes (lane 3), round spermatids (lane 4), and spermatozoa (lane 5). The muscle (lane 6) and ovary (lane 7) were used as negative and positive controls, respectively. Lane 8 represented an internal control (water added instead of RNA). For each sample, 250 ng of total RNA was used for RT except in the case of the ovary (40 ng). **A**) The primers used were designed to amplify $P450_{arom}$ mRNA. **B**) Co-amplification of $P450_{arom}$ mRNA and ABP mRNA. The cDNA fragments were separated on 2% agarose gel stained by ethidium bromide. In **A**, lane M contained the λ/*Bst* EII marker and in **B**, λ/*Hin*dIII.

fraction (lane 2), and to a lesser extent in the pachytene spermatocytes and round spermatids (lanes 3 and 4, respectively), but no signal was observed in the other preparations studied, in particular in spermatozoa and Leydig cells.

Quantification of the $P450_{arom}$ mRNA

As shown in Figure 5A, the intensity of the $P450_{arom}$ mRNA signal in the various testicular cell fractions appeared to be greater in pachytene spermatocytes and round spermatids than in Sertoli cells or spermatozoa. To estimate the P450 mRNA level, a quantitative method using a truncated wild-type RNA as an internal standard was developed. In the testicular somatic cells, the $P450_{arom}$ level was $34.1 \pm 3.2 \times 10^{-3}$ amol/10^6 Leydig cells compared to $2.3 \pm 0.2 \times 10^{-3}$ amol/10^6 Sertoli cells (Fig. 6). The $P450_{arom}$ mRNA level decreased in germ cells from $38.7 \pm 8.1 \times 10^{-3}$ amol of $P450_{arom}$ mRNA/10^6 pachytene spermatocytes to $20.4 \pm 3.8 \times 10^{-3}$ amol/10^6 round spermatids and less than 10^{-3} amol/μg total RNA in spermatozoa, corresponding to less than $1.36 \pm 3.2 \times 10^{-3}$ amol/10^6 spermatozoa.

DISCUSSION

In this work, four complementary approaches were used to demonstrate that not only somatic cells but also germ cells should be considered a new source of estrogens in the testicular tissue of the adult male rat; thus our data confirm those reported elsewhere in germ cells of rodents [14, 18] and other species [15, 16]. Both in purified Leydig cells and in testicular slices, $P450_{arom}$ was immunolocalized using two different techniques of enzyme visualization: the Strept ABC complex/HRP developed by means of stable DAB, or FITC-conjugated IgG. Within the seminiferous tubules, only cells closely located to the lumen (elongated spermatids) were positively stained, thus providing strong evidence for the presence of $P450_{arom}$ in the adluminal compartment of the rat testis, as reported in the mouse [14], brown bear [15], and rooster [16], and supporting findings of $P450_{arom}$ activity in mouse and rat sperm cells as demonstrated by Janulis et al. [19, 38] and Janssen et al. [39]. Moreover, after Western immunoblotting we showed the presence of a 55-kDa specific protein in mixed germ cell preparations, and these data correlate with functional $P450_{arom}$ as confirmed by the measurement of ^3H_2O released after incubation of purified germ cells with labeled androstenedione.

In addition, using the RT-PCR method we revealed $P450_{arom}$ mRNA in Sertoli cells, Leydig cells, and germ cells, in agreement with recent reports [36, 38]. The specificity of the amplification was validated by the absence of

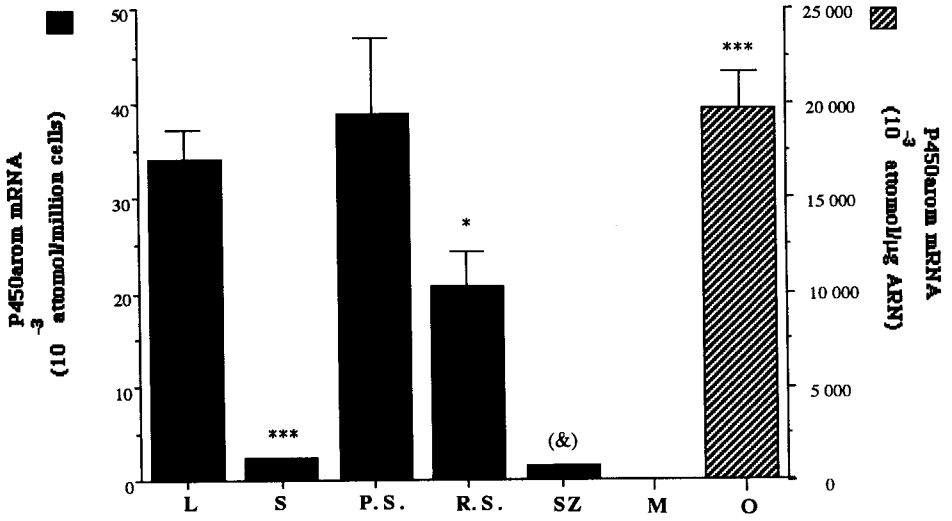

FIG. 6. Levels of $P450_{arom}$ mRNA in adult rat purified germ cells and Leydig and Sertoli cells as determined by quantitative competitive RT-PCR. Total RNA of Leydig cells (L), Sertoli cells (S), pachytene spermatocytes (P.S.), round spermatids (R.S.), spermatozoa (SZ), muscle (M), and ovary (O) were reverse transcribed and amplified by the 5'ARO and 3'ARO primers for 35 cycles with serial dilution of RNA standard. The values except for those of the ovary are expressed as 10^{-3} amol/million cells. Values are means ± SEM of triplicate determinations in three separate experiments. $*p < 0.05$, $*** p < 0.001$ compared to the Leydig cell mRNA value; (&): value at the lower limit of mRNA determination (1×10^{-3} amol/μg RNA [d'ARN on figure]).

mRNA signal in rat leg muscle and peritubular cells (not shown), which are both unable to produce estrogens, and by a strong positive signal in rat ovary. The sequence of the amplified product in Leydig or Sertoli cells and in pachytene spermatocytes shows 100% homology with that of rat granulosa cell $P450_{arom}$ cDNA [32]. This 289-bp fragment contains the I-helix and the aromatic region that confer high specificity for that $P450_{arom}$ transcript. Therefore, in various rat germ cells, $P450_{arom}$ mRNA is present and translated in a full-length protein with aromatase activity. In order to evaluate testicular $P450_{arom}$ mRNA content of rat germ cells and somatic cells, we used a quantitative competitive RT-PCR method. Because of the high sensitivity of that assay, the cellular fractions should be highly purified; accordingly, ABP mRNA was measured as a specific marker for Sertoli cell [33] contamination of the germ cell preparations, since we have demonstrated the presence of $P450_{arom}$ transcripts in adult rat Sertoli cells [36]. The co-amplification method, based on the competition between ABP and $P450_{arom}$ mRNA syntheses, the ratios between the amplified ABP and $P450_{arom}$ products in Sertoli cells and in germ cells (Fig. 5B), not only confirms the presence of aromatase in Sertoli cells but also supports the germ cell origin of the P450 aromatase (the absence of ABP mRNA in the spermatozoa fraction confirms the germ cell specificity of $P450_{arom}$ mRNA synthesis). In addition, we could not detect any 3β-HSD-positive cells in the various purified germ cell preparations; moreover, pachytene spermatocytes and round spermatids fractions were contaminated (< 5%) mainly by other germ cells and in part by Sertoli cells. In fact, it has been reported by Janulis et al. [38] that $P450_{arom}$ mRNA expression is detectable in rat germ cells before it is detected in Leydig cells.

We have observed that the amount of $P450_{arom}$ mRNA transcript decreases according to the maturation of the rat germ cells. In mouse, the aromatase transcript is present from the late spermatocytes until the round spermatids [40]. By contrast, if one considers $P450_{arom}$ immunolocalization and aromatase activity, $P450_{arom}$ is expressed at higher levels in elongated spermatids and spermatozoa (not shown) than in younger germ cells; this is in agreement with a lower enzyme activity in either in pachytene spermatocytes or round spermatids. This observation is in agreement with previous reports in the mouse, rooster, and brown bear [14–16]. This discrepancy between $P450_{arom}$ mRNA synthesis and aromatase activity was reported in the mouse; in round spermatids, it has been calculated that the message precedes the protein appearance by approximately 6 days and that the protein is still present in the absence of detectable $P450_{arom}$ mRNA for 12 days [39–40]. In rat germ cells, the Diazepan binding inhibitor (DBI) mRNA is intensively transcribed in round spermatids, whereas the DBI protein is detected in elongated spermatids and mature spermatozoa, suggesting again that the transcripts were untranslated [41].

Aromatase activity in rat and mouse spermatozoa decreases along the epididymal tract from the caput to the cauda [18, 39]. The high concentration of estradiol in rat rete testis fluid [42] and the presence of estrogen receptors in the epididymis [43] are likely related to an important role for estrogens in the male genital tract. After castration, androgens alone are unable to restore the head of the epididymis [44], and one or more nonandrogen sperm-associated factors are required [45]. So it is tempting to invoke the estrogens produced by the spermatozoa, which may well play this role in that proximal part of the rodent epididymis.

We have demonstrated that rat germ cells are able to produce estrogens; in addition to the synthesis of growth factors [46], germ cells should be considered part of the endocrine unit in the mammalian testis. Increased aromatase expression within the testis could be required at specific stages of germ cell development. For example, it is known that estradiol is able to regulate the expression of the protooncogene c-*fos*, largely present in both Sertoli and germ cells [47, 48]. Germ cell estrogens may well be involved also in paracrine regulation of Sertoli cell function, since it has been reported in rodents [49, 50] and in human [25, 51] that germ cells exert a negative influence on aromatase activity itself. The exact role of estrogens in the reproductive tract of the male and in the regulation of aromatase gene expression during rat testicular development remains to be clarified. There is evidence to suggest that estradiol improves rat gonocyte development [52] and that estrogens are absolutely required for complete maturation of spermatozoa and for fertility of male mice [53, 54].

ACKNOWLEDGMENTS

The French Ministry of Education and Research is gratefully acknowledged for financial help given to Dr. Bilinska during her 3-month stay in the CNRS Unit. We are greatly indebted to C. Deschildre and C. Edine for their valuable help with rat testicular cell preparations. The authors wish to thank Professors P. Leymarie and G.E. Séralini for their helpful discussions, and Mr. M. Jacobs for English revision.

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