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Molecular characterization and expression of equine testicular cytochrome P450 aromatase☆

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Abstract

We characterized testicular equine aromatase and its expression. A 2707 bp cDNA was isolated, it encoded a polypeptide of 503 residues with a deduced molecular mass of 57.8 kDa. The sequence features were those of a cytochrome P450 aromatase, with a 78% polypeptide identity with the human counterpart. The gene has a minimal length of 74 kb comprising at least 9 exons and expresses a 2.8 kb mRNA in the testis. Transient cDNA transfections in E293 cells and in vitro translations in a reticulocyte lysate system allowed aromatase protein and activity detections. The activity increased with androstenedione as substrate in a dose-dependent manner. The isolation of testicular aromatase by a new immunoaffinity method demonstrated that the protein could exist either glycosylated or not with a 2 kDa difference. All these results taken together allow new structural studies to progress in the understanding of this cytochrome P450.

Keywords: Aromatase; Cytochrome P450; Horse; Testis; cDNA cloning; Steroid

1. Introduction

Aromatase is the unique enzyme complex (E.C.1.6.2.4) known to be responsible for the irreversible estrogen biosynthesis from androgens [1]. To function in vertebrates, it has to be composed in fact of two protein moieties. The first one is an ubiquist reductase using flavine mononucleotide and flavine adenine dinucleotide and serving as electron donor in multiple metabolic reactions [2]. The second one is the cytochrome P450 aromatase, briefly aromatase, specific for the estrogen biosynthesis, containing the heme and the steroid binding pocket, and inserted by its N-terminal end in the smooth reticulum endoplasmic membrane [3].

Aromatase is encoded by the *CYP19* gene in humans, at least 75 kb long [4], and among the longest genes coding for steroidogenic enzymes. It is also the only member of the family 19 of cytochromes P450, which are encoded by more than 481 genes, classified in 74 families [5]. The knowledge of aromatase genes and cDNAs in different species [6–12] is thus interesting to an evolutionary point of view because estrogens are widely implicated in crucial physiological functions including development, cell differentiation and reproduction. Bone structuration [13], brain function and behavior [14,15] and breast cancer for which aromatase inhibitors are always developed [16] are also modulated by these hormones. Similarly, male reproductive function in mammals is under estrogen dependence [17,18] and in the stallion testicular estrogen synthesis is particularly elevated [19,20]. In this species, aromatase is mainly located in the Leydig cells [21] and the study of its structure–function relationships and comparative inhibition with human [22,23] has been pursued by our group. In this work, we have further characterized equine testicular aromatase by cloning and sequencing, by studying its gene structure and expression and, finally, by purification with a new immunoaffinity method.

2. Materials and methods

2.1. Chemicals

Coomassie brilliant blue G-250 dye and anti-rabbit IgG-peroxydase were from BioRad (Ivry-sur-Seine), and all companies were settled in France when not precised; SDS

☆ These sequence data have been submitted to the EMBL database under accession number AJO12610.

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was from Peypin; agarose from Gibco BRL (Cergy Pontoise); reverse transcriptase, specific buffer, dNTP- and TNT-coupled reticulocyte lysate systems from Promega (Charbonnières-les-Bains); synthetic oligonucleotides from Eurobio (Les Ulis); Rnasin- and CNBr-activated Sepharose 4B from Pharmacia Biotech (Orsay); methanol from Merck (Nogent-sur-Marne); [1β,2β-^{3}H]androst-4-ene-3,17-dione from Dupont de Nemours (Les Ulis); peptide *N*-glycosydase F (PNGase) from *Flavobacteriurn meningosepticum* of Boehringer-Mannheim (Meylan). The solvents were from Carlo Erba (Val de Reuil), and all other products were at the best purity available from Sigma (Saint Quentin Fallavier).

2.2. Library construction, screening and sequencing

Poly(A)-containing RNA from 2-year-old stallion testis was used with oligo(dT) linker primer to synthesize corresponding cDNAs, which were ligated into UniZAP-XR phage arms and packaged using Gigapack Gold phage extract (Stratagene, San Diego, CA). Approximately 2×10^7 recombinant clones were transferred on Hybond-N′ filters (Amersham.) and screened at high stringency with the fluorescein-labeled 2.8 kb Human P450 arom cDNA using the ECL Random Primer Labeling and Detection System (Amersham). After an overnight hybridization in buffer (5 × SSC, 0.1% SDS, 5% Dextran sulphate, 100 μg/ml salmon testes DNA), filters were washed with 0.5 × SSC, 0.1% SDS for 15 min at 60 °C. The following detection step was performed with anti-fluorescein-HRP conjugates according to Amersham ECL detection protocol. Fifteen positive clones were isolated, replated and rescreened. They presented the same restriction maps for the overlapping sequences, and the five longest positive phage clones were subcloned into pBluescript phagemid SK (Stratagene). The DNA inserts were analyzed at first by restriction endonuclease cleavages, then sequenced in both directions by the dideoxy-sequencing chain termination procedure [24]. For oligonucleotides designing the OLIGO v.3.4 software was used. Sequence entering and analysis were performed employing PC/GENE software (release 6.8, IntelliGenetics).

2.3. 5′-RACE procedure

To obtain a larger 5′-region of the cDNA encoding equine aromatase, we used the 5′-RACE procedure (GibcoBRL). First strand cDNA was synthesized from equine testicular poly(A)$^+$ RNA using aromatase-specific antisense primer I (5′-TGCCTCTTCAACTTTAGGGTGC-3′). The ssDNA was purified with GlassMAX spin cartridge, poly(dC)-tailed using terminal deoxytransferase and, subsequently, amplified by PCR with nested aromatase-specific primer II (5′-CAUCAUCAUCAUCACTGGTTCACATTCTCTTTGG-3′) and the anchor primer supplied. The PCR product of about 1 kb was gel-purified and subcloned into pAMP1 plasmid vector using uracil DNA glycosylase cloning procedure (CloneAmp pAMP1 system, Gibco BRL). Finally, the insert was analyzed by dideoxy DNA sequencing method.

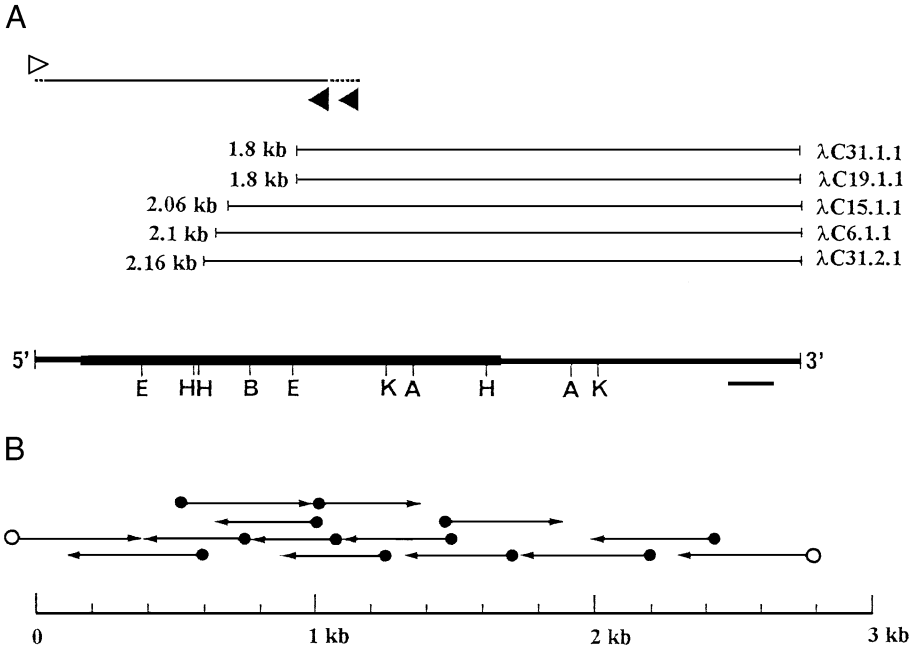

Fig. 1. (A) Common restriction endonuclease map of 5 equine P450 aromatase cDNA clones (λC6 to λC31). The coding region is thicker, the main endonucleases restriction sites used are *Eco*RI (E), *Hin*dIII (H), *Bam*HI (B), *Kpn*I (K), *Apa*I (A). Above is represented the fragment obtained with the 5′-RACE procedure, aromatase-specific primers are shown by black arrows, the anchor primer is at the 5′-end. (B) Sequencing strategy with the scale in kb below, indicating the directions and extent of readings using synthetic primers (●) or universal primers (○).

2.4. Recombinant aromatase expression and controls

The total aromatase cDNA was checked by RT-PCR and sequencing in three different adult horse testes with aromatase activity. The expression of protein corresponding to the cloned equine aromatase cDNA was performed according to TNT-coupled reticulocyte lysate systems, and [^{35}S]Met was visualized by autoradiography. The in vitro transcriptions and translations were performed with pBluescript SK alone, or with the vector containing linearized (3′-end) or circular sense cloned cDNA, and with the vector linearized at the 5′-end of the cDNA as control and with luciferase cDNA. Incubations were performed at 30 °C during 120 min. These reactions together with glycosylated and nondeglycosylated purified equine aromatase, for molecular weight controls, were electrophoresed on SDS polyacrylamide gel (gradient

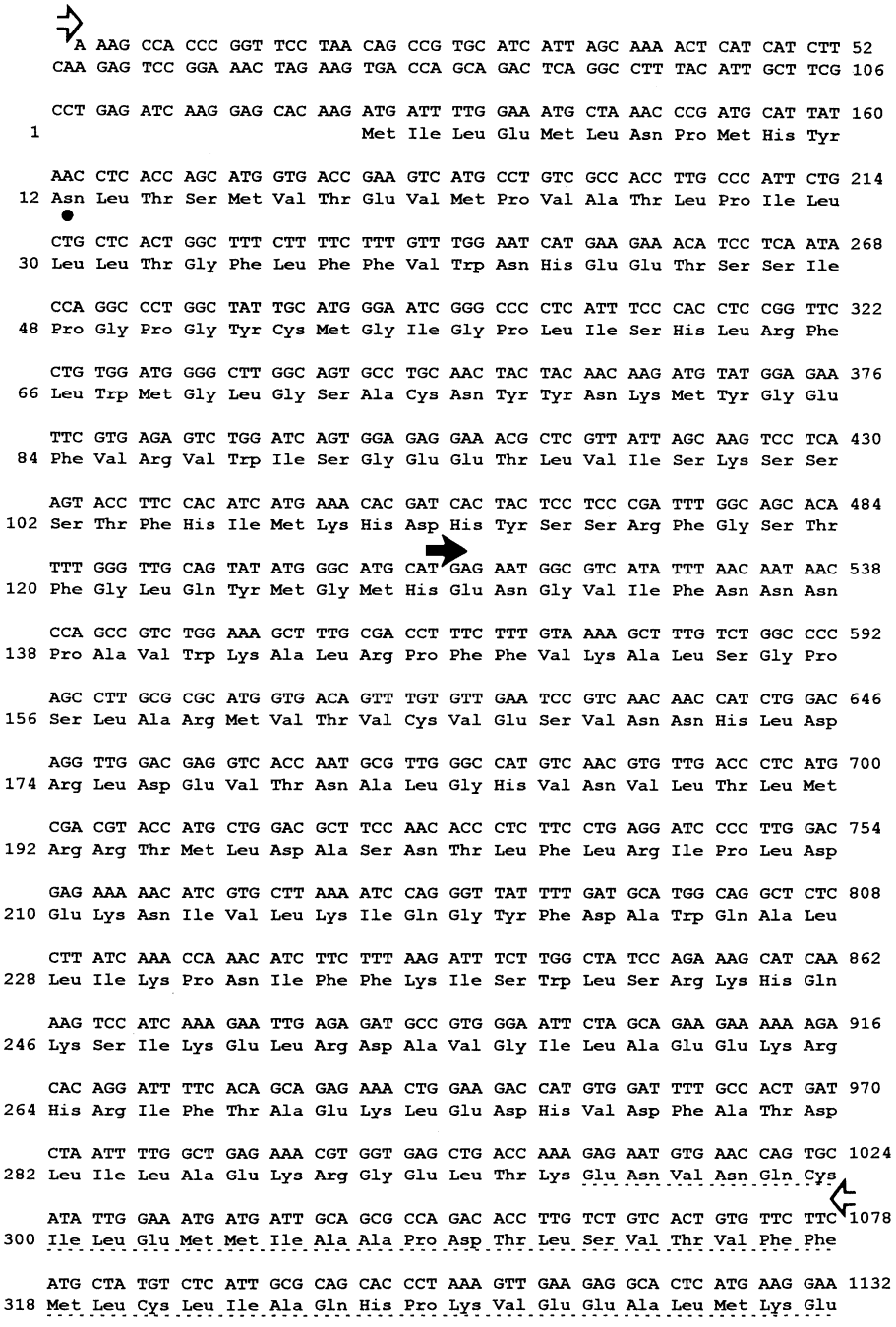

Fig. 2. Nucleotide and deduced amino acid sequences of equine P450 aromatase. The nucleotides are numbered on the right, residues on the left. The nucleotide sequence obtained by the 5′-RACE procedure is indicated by white arrows (1 to 1078), and it perfectly overlaps the longest clone (511 to 2707, black arrows). Two potential glycosylation sites are indicated by dots on Asn 12 and 361. From homologies with other species, sequence candidates for a part of the substrate-binding pocket, the aromatase-specific region and the heme-binding region are underlined by dots, one and two lines, respectively.

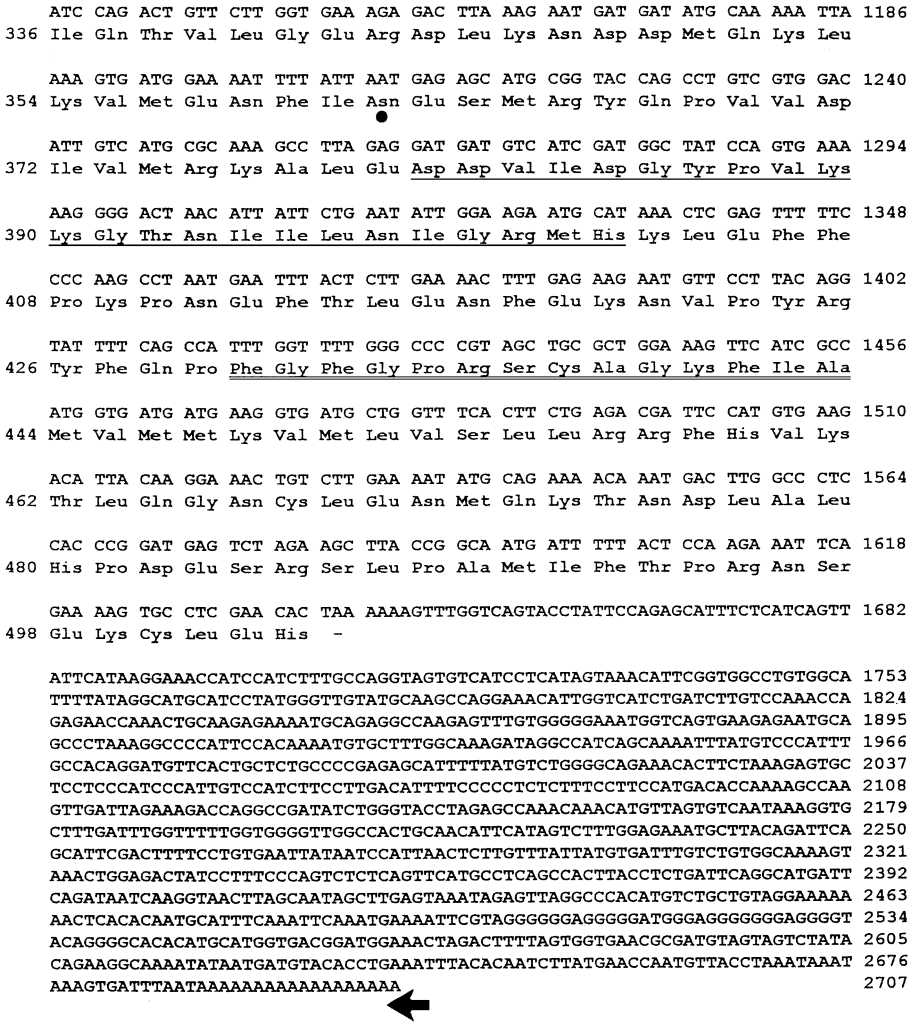

Fig. 2 (*continued*).

10–30% or 10% polyacrylamide). The gels were stained with Coomassie blue and dried before autoradiography. For deglycosylation, 10 μg of purified equine aromatase were incubated with 1U PNGase (or without, for negative control) at 37 °C overnight in 300 mM phosphate buffer containing 10 mM EDTA.

2.5. Southern and northern blot analyses

Equine genomic DNA was isolated from testis [25] digested with endonucleases, fragments were separated by electrophoresis in a 0.6% agarose gel (40 μg/lane), and then transferred on a Hybond N+ membrane by capillarity [26]. The membrane was washed and hybridized overnight with a random primed fluorescein-labeled 2.7 kb equine aromatase cDNA in 5 × SSC, 0.1% SDS, 5% dextran sulfate and 100 μg/ml salmon testes DNA at 60 °C. The membrane was then washed up to 0.5 × SSC, 0.1% SDS for 15 min at 62 °C. The detection was performed with anti-fluorescein-HRP as described for library screening. For northern blotting, total RNA was extracted [27], size-fractionated on a 1% agarose-formaldehyde gel electrophoresis (40–50 μg/lane), blotted and probed as described previously with the longest 2.16 kb equine aromatase cDNA clone.

2.6. cDNA expression in E293-transfected cells

The 2707 bp equine aromatase cDNA was subcloned into the pCMV *Eco*RI site. Orientation was then checked by sequencing. The plasmid-cDNA was purified from transformed JM109 bacterial strain by using the Qiagen Plasmid Mega kit. The length, the concentration and the purity of the plasmid-cDNA construction were verified by 1% agarose electrophoresis and ethidium bromide staining. A stable reductase-transfected human Embryonic kidney E293 cell line (gift of Dr. Van Luau-The, CHUL Québec) was transiently transfected with the construction as described previously [28].

Total cellular RNA were isolated from E293 cells transfected with pCMV–cDNA, pCMV–human aromatase (used as positive control) or pCMV alone (as control) using TRIzol reagent (Gibco BRL). Five hundred ng of total RNA was

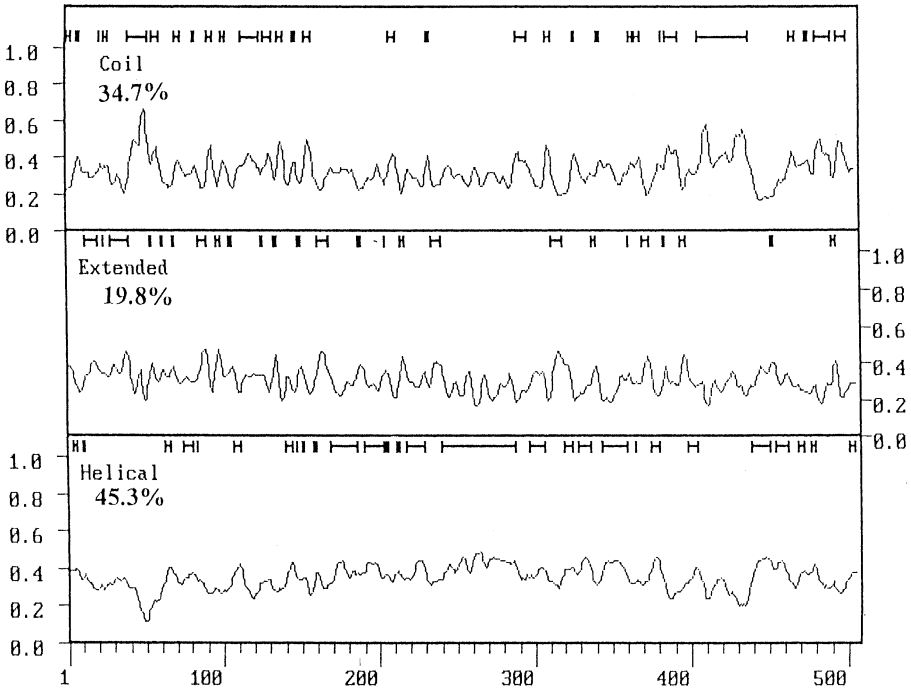

Fig. 3. Secondary structure prediction of equine aromatase. The probability for coiled, extended or helical conformations is indicated by the profiles according to Garnier et al. [38], along the 503 amino acid residues.

reverse transcribed with 4 U of moloney leukemia virus reverse transcriptase for 1 h at 37 °C in a total volume of 20 μl. PCR amplification of 2 μl of the reverse transcribed sample was carried out in 20 μl PCR Buffer (10 mM Tris–HCl, pH 8.3) with 1.7 mM $MgCl_2$, 200 μM dNTPs, 2 U of Taq polymerase (Sigma) and 10 pmol of each primer. Amplification was performed on a Stratagene Robocycler through 28 PCR cycles with the profile: 94 °C (30 s), 58 °C (30 s) and 72 °C (1 min). A last step of elongation was realized 5 min at 72 °C. PCR products were resolved on 1.5% agarose gel and visualized by ethidium bromide staining. Primers for equine aromatase were 5′-CATTCTGCTGCTCACTGGCTT-3′ and 5′-GGTTGTTGCGCATTCAACAC-3′; primers for human aromatase 5′-GTTTTGGAAATGCTGAACCCGATAC-3′- and 5′-TGTGTTGAGAGCATGCAGAAGATACAC-3′; and primers for the positive control, actine, 5′ GACTA-GACTACCTCATGAAGATCCT3′ and 5′-TTGCTGATCC-ACATCTGCTG-3′, which produced 420, 460 and 620 pb, respectively.

The aromatase activity "in cell" was measured by the conversion of androstenedione to estrone in E293 cells supernatant by radioimmunoassay according to Auvray et

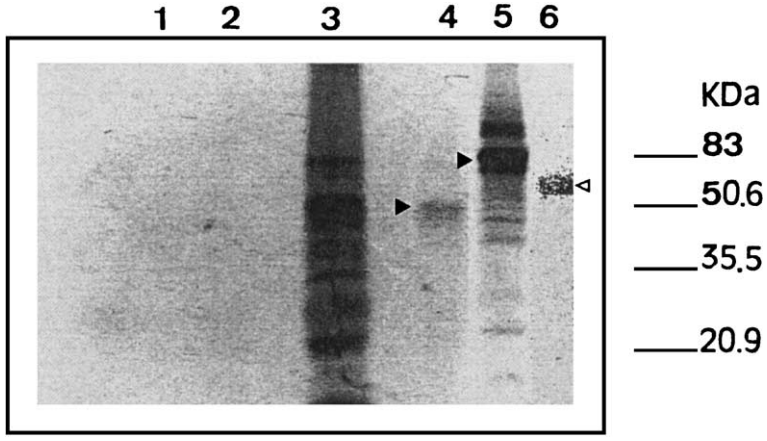

Fig. 4. Expression of equine aromatase cDNA in vitro. The autoradiography after incorporation of [^{35}S] Met and SDS-PAGE revealed neosynthesized proteins (lanes 1–4, 48-h exposure), by the TNT-coupled reticulocyte lysate system. Equine aromatase is expressed from pBluescript SK vector containing the cDNA linearized at the 3′-end (lane 4), or from circular plasmid showing less specific expression (3). Controls include incubation with vector alone (2), or linearized at the 5′-end of the cDNA (1). Expression with the same method of luciferase is on lane 5 (6-h exposure), and purified glycosylated equine aromatase from testicular tissue, and Coomassie-stained, is shown as a standard (6). Other standards are indicated on the right.

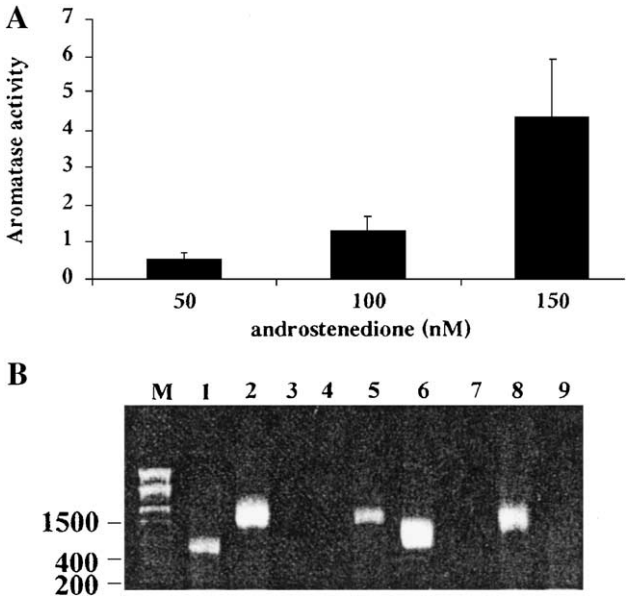

Fig. 5. (A) Equine aromatase activity in E293 cells. Transfections are performed with 2 μg of cDNA in pCMV and incubated for 45 min with 0–150 nM of androstenedione. Estrone is assayed in pg/ng aromatase min by RIA in the medium. Results are the mean of 3 assays ± S.D. (B) Amplification by RT-PCR of RNA isolated from E293 cells and cDNA fragments are visualized on 2% agarose gel stained by ethidium bromide. Transfections are performed with human aromatase cDNA in pCMV: (1) amplification with aromatase primers, (2) with actine primers, (3) with human aromatase primers but without reverse transcriptase. Transfections with equine aromatase cDNA in pCMV: (4) amplification with equine aromatase primers but without reverse transcriptase, (5) with actine primers, (6) with equine aromatase primers. Transfections with pCMV alone: (7) amplification with equine aromatase primers but without reverse transcriptase, (8) with actine primers, (9) with equine aromatase primers. Fragment sizes in bp are shown on the left. M: molecular markers.

al. [29]. The protein was also quantified by a direct sandwich enzyme-linked immunosorbent assay (ELISA) method in transfected E293 cells as described previously [30].

2.7. Polyclonal antibodies raising and aromatase immunoaffinity purification

Specific polyclonal antibodies against purified stallion testicular aromatase were raised in Hyla female rabbits, 10 weeks old, as previously described [21]. These antibodies (Ab I) were purified out of the antisera by ammonium sulfate precipitation according to Ternynck and Avrameas [31]. Briefly, antisera were diluted in PBS and precipitated in 33% ammonium sulfate, centrifuged at 1300 × *g* during 20 min at 4 °C and dialyzed overnight with PBS. The IgG were chromatographed on a DEAE cellulose column; the fractions were tested on immunoblots with equine testicular microsomes prepared as previously [32], the positive ones were able to inhibit aromatase activity in vitro [21]. For aromatase purification, equine testicular microsomes were diluted to 20 mg/ml and then solubilized by adding CHAPS 10% to a final concentration of 1% under agitation during 30 min at 4 °C. This solution was then centrifuged at 100,000 × *g*, and the supernatant was diluted twofold before being chromatographed on an aminohexyl Sepharose 4B column [33], previously equilibrated with 20 mM phosphate buffer containing 20% glycerol, 0.3% CHAPS, 1 mM DTT and 4 μM androstenedione (buffer A). The cytochrome P450 aromatase and the NADPH cytochrome P450 reductase were differently eluted by a 0–1 M NaCl gradient. The fractions containing high aromatase activity were adjusted to 0.15 M NaCl and chromatographed on a CNBr Sepharose 4B

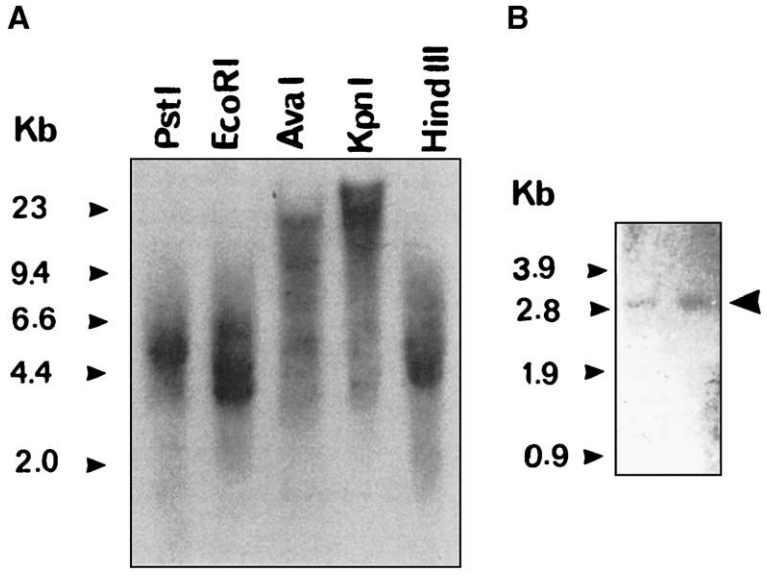

Fig. 6. (A) Southern blot of equine genomic DNA restricted with endonucleases indicated on the top and probed with the aromatase cDNA. (B) Northern blot of equine testicular RNA (two lanes) probed with the cloned aromatase cDNA, evidencing a 2.8 kb mRNA (arrow). In both instances, molecular markers are indicated on the left.

immunoaffinity column, containing the Ab I coupled according to Pharmacia. After washing, aromatase was eluted out of this column with buffer A and 2 mM NaCl. The detergent *n*-octyl glucopyrannoside was then added to a final concentration of 1%, giving the best aromatase activity. The purity was checked by SDS-PAGE and silver staining [34].

3. Results

3.1. cDNA and deduced protein structure

Following the last round of library screening, five positive clones were inserted into pBluescript SK and mapped. All inserts were then sequenced in both orientations according to the strategy presented (Fig. 1). Their lengths varied in size from 1.8 to 2.16 kb, and they appeared to derive from the same mRNA due to the common 1.8 kb region, identical at 75% to the human aromatase. The 5′-RACE procedure allowed to isolate a longer 5′-region; an anchor PCR amplified a 1.03 kb fragment overlapping at its 3′-end all the clones obtained (Fig. 1). The common 0.5 kb region with the longest clone 31.2.1 was perfectly identical; thus, we constructed a 2707 bp cDNA and deduced from the longest open reading frame the protein sequence (Fig. 2). It extended from nucleotides 128 to 1636 before the stop codon to encode a polypeptide of 503 residues with a total cDNA presents a 83% identity with the human in the

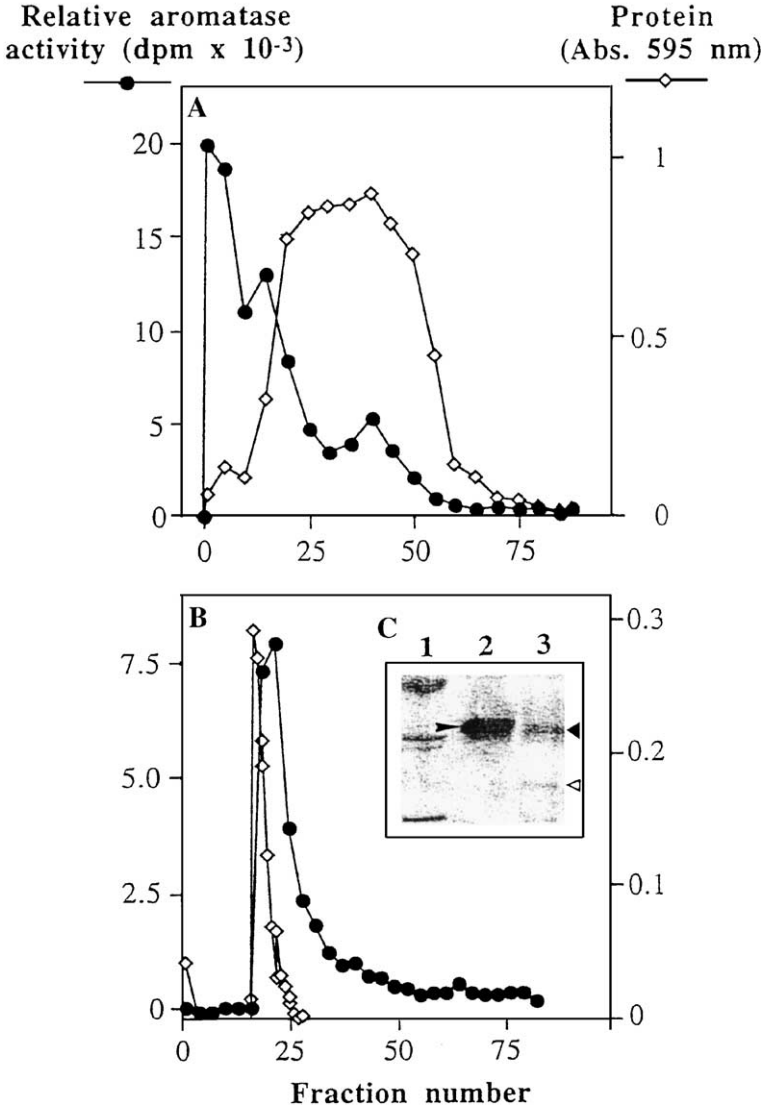

Fig. 7. Purification of equine testicular aromatase. (A) Relative aromatase activity after aminohexyl Sepharose 4B separation of solubilized microsomes (●) for the different fractions obtained of 6 ml each. Fraction number is indicated below as well as total protein absorbance at 595 nm (◇). (B) Relative aromatase activity in 2-ml samples after immunoaffinity chromatography of the most active fractions obtained in A. (C) SDS-PAGE of purified equine testicular aromatase. Molecular weight standards are from top to bottom 66.2, 45.0 and 31.0 kDa (lane 1). The equine aromatase was purified from adult testis by immunoaffinity chromatography, electrophoresed on a 10% acrylamide-bis gel, stained by Coomassie (lane 2, arrow at approximately 53 kDa). The deglycosylation with PNGase overnight evidenced a 2-kDa shift (lane 3, black arrow) and the PNGase is also visible (lane 3, open arrow at 35 kDa).

common part, while the equine polypeptide shows 78%, 78%, 75%, 75%, 74%, 72% and 70%, respectively, identity with human, bovine, rabbit, porcine, mouse, rat [35] and chicken [36] aromatases. It is also identical to the sequence deduced from an equine preovulatory follicle cDNA library [37], albeit there are distinct mRNAs and the full-length equine aromatase cDNA has not been published. The known aromatase-specific region (residues 380 to 402) was fully conserved as well as the heme-binding domain, and only the first potential glycosylation site (Asn 12) is common with the human sequence. Calculated according to Garnier et al. [38], the secondary structure (Fig. 3) predicts that most of the equine protein presents an helical (45.3%) or a coiled conformation (34.7%), the remaining is extended (19.8%). Such feature is compatible with a membrane-bound protein, overall with two out of four potential membrane-buried α helixes in the N-terminal domain, this domain being most probably inserted within the endoplasmic reticulum membrane. The third helix spans the potential substrate-binding pocket (Fig. 2).

3.2. In vitro translation

When expressed in vitro in the reticulocyte lysate system with labeled Met, a protein was visualized on SDS-PAGE out of the 2707 bp construct, comigrating with the deglycosylated form of purified equine testicular aromatase. This was important to check because the apparent molecular mass of this membrane-bound and globular protein was only 51 kDa (Fig. 4), below the 57.8 kDa deduced one from the sequence. The aromatase purified from testicular tissue is glycosylated and evidences an apparent molecular mass of 53 kDa (see below).

3.3. Expression in transfected cells

Forty-eight-hour post-transfection, E293 cells were incubated during 45 min with increasing concentrations of androstenedione (0, 50, 100 and 150 nM). As illustrated by Fig. 5A, the equine recombinant protein is active in this expression system, and the aromatase activity, measured by RIA and expressed in picograms estrone formed per nanograms aromatase per minute, increases in dose-dependent manner. Aromatase mRNA from the transfected cells was analyzed by RT-PCR (Fig. 5B). The protein was detected by ELISA only in cells transfected with the equine construction (1.75 ± 0.22 ng aromatase/well), or with the human one (0.99 ± 0.26 ng/well).

3.4. Gene structure and mRNA

Southern blotting evidenced a most probably unique gene for equine aromatase, with a minimal length of 74 kb comprising at least 9 exons (Fig. 6A), by comparison of the genomic restricted fragments with the cDNA endonuclease mapping. This gene appears to be expressed as a unique testicular mRNA of approximately 2.8 kb (Fig. 6B), which was detected with the longest clone isolated as probe.

3.5. Testicular aromatase purification

After rabbit immunizations, the IgG were precipitated out of the antisera and separated on a DEAE cellulose column. The fractions able to specifically detect aromatase in testicular microsomes on immunoblots were pooled and coupled to the immunoaffinity column. On the other hand, testicular equine microsomes containing high levels of aromatase activity (5.1 pmol/min mg) were solubilized with CHAPS 1% and chromatographed on a first aminohexyl Sepharose 4B column (Fig. 7A); this allows reductase separation. The first peak of active proteins (200 ml, 145 mg proteins, 19.9 pmol/min mg) was pooled and chromatographed on the immunoaffinity column (Fig. 7B). Out of this column, 1.5 mg of purified aromatase was collected in 20 ml with a specific activity of 68.2 pmol/min mg. This was a rapid method to purify testicular aromatase as evidenced by SDS-PAGE. A deglycosylation of this protein slightly reduced its molecular weight of about 2 kDa (Fig. 7C), without any significant loss of activity in comparison to control.

4. Discussion

We report here an analysis of the equine aromatase gene structure, the testicular cDNA cloning and in vitro expression, and testicular aromatase purification by a new immunoaffinity method. Clones with 5′-ends of various lengths were isolated, but on the basis of their identities in overlapping sequences confirmed by the 5′-RACE procedure, a unique coding sequence was found. Aromatase-specific region, heme-binding domain, and even N-terminal predicted membrane-spanning domains are comparable to human. Moreover, N-terminal sequencing of the purified enzyme [33] confirmed our present data for the first 25 residues and the use of this initiation codon. The global amino acid composition [33] and the secondary structure prediction mostly in helical and coiled conformation were also in agreement. The length of 503 residues was comparable to most cloned aromatases in other species [35], and the calculated exact molecular weight for equine aromatase is 57800. This is slightly above the apparent one on SDS-PAGE (53000) because globular membrane-bound proteins, purified with detergents, usually migrate a little faster than expected. In vitro expression of the recombinant aromatase confirmed that it had the same electrophoretical mobility as deglycosylated purified aromatase; the protein is specifically revealed on immunoblots by our antibodies.

The study of horse aromatase gene structure by Southern blot with our 2707 bp probe revealed at least 9 exons spanning 74 kb. This could correspond to the 9 coding exons characterized for the human gene, which is also at least 75 kb long [4]. Like the human gene, and in contrast to the pig [11],

the equine gene appears to be unique according to the Southern analysis. The mRNA length of 2.8 kb was comparable to the human placental coding mRNA of 2.9 kb [39], but the sequence of the 5′ -untranslated region was not homologous to the partial ovarian, placental and blastocyst equine sequences published [37,40,41]. This is in favor of the existence of an alternative splicing of the horse aromatase gene with tissue-specific promoters and corresponding untranslated exons [37], like it was characterized in detail for human [8,39,42], and in other species.

Transient transfections of equine aromatase cDNA were performed in E293 cells. The corresponding mRNA, protein and activity were assayed. Our results indicated a metabolization of androstenedione in estrone. This is the first demonstration that this sequence corresponds to an active protein. Estrone and its conjugated derivatives are also very important in equine semen [43], produced by the testis which is by far the major source of aromatase among equine tissues [20]. This justifies the use of this organ for this protein purification.

A purification of equine aromatase by five chromatographic steps involving aminohexyl Sepharose 4B, concanavalin A, hydroxyapatite and DEAE Sepharose CL6B columns was already in use in our laboratory [33]. However, we wanted to shorten the process and to purify also the nonglycosylated form of aromatase. This goal was reached after rabbits immunization with the highly purified enzyme, purification of the reactive IgG and coupling to a CNBr-activated Sepharose gel. The resulting immunoaffinity column allowed us to demonstrate that active equine testicular aromatase could exist either glycosylated or nonglycosylated in its native form. The deglycosylation revealed a 2 kDa carbohydrate moiety. We suggest that it is linked to Asn 12 on the protein because it is the only potential glycosylation site conserved at the same position between human and equine aromatases and because this Asn was not always detectable by direct protein sequencing like if it was charged with a sugar chain (data not shown).

In conclusion, the knowledge of equine testicular aromatase structure could be of importance because this enzyme presents very well-characterized biochemical properties, and moreover it allows the synthesis of very high estrogen levels by the stallion, hundred folds more elevated than in most other mammalian species. Obtaining equine aromatase in the milligram range out of one testis and the new possibility of recombinant testicular aromatase expression may allow the exploitation of this physiological particularity for further structural studies of this protein. This could be interesting because mammalian membrane-bound cytochromes P450 still need further spatial and functioning characterizations.

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