0013-7227/98/$03.00/0
Endocrinology

Vol. 139, No. 4
Printed in U.S.A.

Identification of Multiple CYP19 Genes Encoding Different Cytochrome P450 Aromatase Isozymes in Brain and Ovary*

ANNA TCHOUDAKOVA† AND GLORIA V. CALLARD

Department of Biology, Boston University, Boston, Massachusetts 02215

ABSTRACT

Evidence to date indicates that the gene encoding cytochrome P450 aromatase (P450arom) in humans is a single member of the CYPl9 family, but multiple CYPl9 loci and isoforms have been identified in pigs. Here we report the cloning and characterization of a second member of the CYP19 family in goldfish. A search for P450arom variants was prompted by studies showing that a full-length P450arom complementary DNA (cDNA) isolated from a goldfish brain cDNA library hybridizes with a high abundance 3 kb transcript in brain RNA but fails to detect a message in ovarian RNA. A stepwise PCR cloning strategy led to isolation of a 1.9-kb cDNA, which encodes a protein of 518 amino acids and has a predicted mol wt of 58.7K. The ovary-derived P450arom (-A) shares 68–72% sequence identity with ovarian aromatases of other fish species, but only 62% identity with the homologous brain-derived P450arom (-B). Amino acid differences are distributed throughout the two goldfish P450arom forms, but presumptive functional domains are highly conserved. Both P450aromA and -B are able to aromatize [^{3}H]androgen to [^{3}H]estrogen when expressed in nonsteroidogenic COS cells. Southern analysis and PCR-restriction analysis of genomic DNA using discriminating probes and primers indicates that a single locus encodes the brain-derived P450aromB (CYPl9B), whereas one or two different loci encode the ovarian form (CYPl9A). Northern blot analysis revealed two P450aromA messenger RNAs (1.9 $\gg$ 3.0 kb) in ovary. Simultaneous PCR amplification with A- and B-specific primer pairs confirms that P450aromA is the only form expressed in ovaries, but shows overlapping expression of the two genes in neural tissues. Whereas P450aromB messenger RNA predominates in brain (B/A, $\approx$14:1), the ratios are reversed in retina (B/A, $\approx$1:25). Further studies are required to resolve the evolutionary and functional implications of multiple CYPl9 genes and P450arom isozymes in goldfish, their differential expression in brain and ovary, and whether observations can be generalized to other vertebrates. (*Endocrinology* **139:** 2179–2189, 1998)

IN BOTH males and females, estrogen programs and coordinates developmental, physiological, and behavioral responses essential for reproduction. Conversion of C_{19} androgens to C_{18} estrogens is the rate-limiting step in estrogen biosynthesis and is catalyzed by an aromatase enzyme complex comprising a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytochrome P450 reductase and a cytochrome P450 aromatase (P450arom), a product of the CYPl9 gene (for review, see Ref. 1). Although early studies focused on estrogen biosynthesis in placenta and ovaries, which are P450arom-rich and a major source of circulating estrogen, it is now understood that smaller amounts of estrogen are formed in close proximity to estrogen receptors in brain, fat, bone, gonads and other tissues, where it functions as a paracrine or autocrine factor (1). Based primarily on molecular cloning and characterization of P450arom complementary DNAs (cDNAs) from human tissues, the gene encoding aromatase is thought to be a single member of the CYP19 family, with regulatory complexity accomplished by tissue-specific usage of multiple promoters and untranslated first exons (1, 2).

The ability of the gonads and brain to aromatize androgen is an evolutionarily conserved characteristic of vertebrates (3, 4). However, teleost fish, including the goldfish *Carassius auratus*, are unique among vertebrates in having exceptionally high levels of brain aromatase activity: *e.g.* 100- to 1000-fold higher than mammalian brain but similar to mammalian ovary (5, 6). Enzyme activity is correspondingly high in teleost pituitary and retina, but ovarian aromatase is less than one-tenth that of brain (6, 7). To address the molecular mechanism of high constitutive neural aromatase expression, a full-length 3-kb P450arom cDNA was isolated from a goldfish brain library and used to show that high accumulated levels of P450arom messenger RNA (mRNA) in brain correspond to high enzyme levels (8). Paradoxically, the brain-derived cDNA failed to hybridize with ovarian mRNA under any condition. This observation led us to postulate the existence of different brain and ovarian mRNA variants. Although evidence from chickens (9) and medaka fish (10) supports studies in humans indicating that the CYPl9 gene exists as a single copy in the haploid genome (11, 12), multiple CYPl9 loci encoding different P450arom isoforms in placenta, ovary, and blastocysts were identified in pigs (13–15).

Here we report the isolation of a second P450arom cDNA from goldfish ovaries, and present evidence for multiple CYPl9 gene loci encoding the brain- and ovarian-derived P450arom forms. We show that both proteins are able to catalyze estrogen production from radiolabeled androgen, and are differentially expressed in neural and gonadal tis-

Received October 7, 1997.

Address all correspondence and requests for reprints to: Gloria V. Callard, Department of Biology, Boston University, 5 Cummington Street, Boston, Massachusetts 02215. E-mail: gvc@bio.bu.edu.

* This work was supported by grants from the National Science Foundation (IBN89–16809 and IBN96–01297). The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number AF020704.

† Recipient of an Endocrine Society Summer Fellowship.

sues. Preliminary findings have been reported elsewhere (16).

Materials and Methods

Oligonucleotides

Oligonucleotides (Ransom Hill Bioscience, Ramona, CA) used as PCR primers and probes for Southern analysis are listed below and shown in Fig. 1. Primers 1–11 were used with the ovarian P450arom cDNA, whereas 12–16 are complementary to brain P45Oarom cDNA:

Primer 1: nucleotides 1173–1197: 5′ AGG TWC CAK CCN GTB GTS GAC TTC 3′ (IUB group codes were used: D = A+G+T; S = G+C; N = A+G+C+T; Y = C+T; R = A+G; K = G+T; B = G+T+C; V = G+A+C; W = A+T).

Primer 2: nucleotides 1395–1416: 5′ CACCATNGCDATRWRYTT-NCC 3′.

Primer 3: nucleotides 1316–1337: 5′ TGGAAGTTGTCTAGACT-GAAC 3′.

Primer 4: nucleotides 1303–1324: 5′ GACTGAACTCATTCGGCTTGG 3′.

Primer 5: nucleotides 338–359: 5′ RGTBTGGATCWVYGGAGARGA 3′.

Primer 6: nucleotides 398–423: 5′ TGAGGTGTACAGAGATTTCCT-CAAC 3′.

Primer 7: nucleotides 1301–1329: 5′ CCCCAAGCCGAATGAGT-TCAGTCTAGAC 3′.

Primer 8: nucleotides 17–43: 5′ TCTTATGGCACGTGAACTTCTC-CAGC 3′.

Primer 9: nucleotides 1848–1874: 5′ CAGTAGAAGACACCAGTA-ATTCATAG 3′.

Primer 10: nucleotides 1220–1241: 5′ TGATGTCATCGAAGGCTA-CAA 3′.

Primer 11: nucleotides 1274–1299: 5′ GAATTCCGATCTGTGCATC-CGACCC 3′.

Primer 12: nucleotides 1241–1262: 5′ ACGACGAGATCGATGGC-TACC 3′.

Primer 13: nucleotides 1362–1383: 5′ AAGTAACGACTGGGAACG-GTG 3′.

Primer 14: nucleotides 1338–1359: 5′ TCGAAGTTCTCCAAGTT-GAAT 3′.

Primer 15: nucleotides 1206–1227: 5′ GGTTGTGGACTTCATCAT-GAG 3′.

Primer 16: nucleotides 1338–1359: 5′ TCGAAGTTCTCCAAGTT-GAAT 3′.

RT-PCR cloning of goldfish aromatase cDNA from ovaries

cDNA was synthesized from 5 μg total RNA with oligo(dT) primers using SuperScript II reverse transcriptase (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions. An aliquot (15%) of the first-strand reaction was amplified with degenerate primers 1 and 2. PCR was performed in 50-μl final vol containing 5 μl 10 $\times$ reaction buffer, 2 mM $MgCl_2$, 200 μM deoxynucleotide triphosphate, 2 μM of each primer, and 2.5 U *Taq* DNA Polymerase (Promega, Madison, WI). The following PCR conditions were used for the first 5 cycles: 94 C for 1 min, 37 C for 2 min, and 72 C for 3 min; during the remaining 30 cycles, the annealing temperature was increased from 37 C to 50 C. A 240-bp fragment was purified from 2% agarose with GLASSMILK (GENECLEAN III kit; BIO 101, Vista, CA), T-A cloned into pCR II vector (Invitrogen, Carlsbad, CA) and sequenced. Subsequently, the 1-kb PCR fragment was amplified using the RT-reaction primed with sequence-specific primer 3. The PCR was conducted as indicated above but with 0.2 μM of primer 4, 2 μM of degenerate primer 5, and *Taq*-mix consisting of 2.5 U *Taq* DNA polymerase and 0.25 U Pfu DNA Polymerase (Stratagene, La Jolla, CA). The annealing temperature during the first 5 cycles was 43 C; otherwise PCR conditions were as described above. After purification, the 1-kb PCR product was T-A cloned into pCR II vector and sequenced. The remaining cDNA was obtained with the Marathon Kit (Clonetech, Palo Alto, CA) according to the manufacturer's protocol. Primers 6 and 7 were used for 5′-and 3′-rapid amplification of cDNA ends (RACE), respectively. The amplification of double-stranded cDNA was done with Advantage KlenTaq polymerase mix containing a proof-reading polymerase (Clonetech). 5′-RACE was carried out for 30 cycles under the following conditions: 94 C for 30 sec, 60 C for 45 sec, and 72 C for 2 min. For 3′-RACE, a touch-down program was used (5 cycles: 94 C for 30 sec and 72 C for 2 min; 5 cycles: 94 C for 30 sec and 70 C for 2 min; and 25 cycles: 94 C for 20 sec and 68 C for 2 min). The full-length cDNA was generated by PCR using Advantage KlenTaq polymerase mix and primers 8 and 9, derived from the 5′ and 3′ ends of the cDNA. A PCR product of approximately 2 kb was silica purified (Geno-Bind; Clonetech) from a 1% agarose gel and T-A cloned into pGEM-T vector (Promega). The nucleotide sequences of three independent cDNAs derived from four separate amplification reactions, pooled together, were determined.

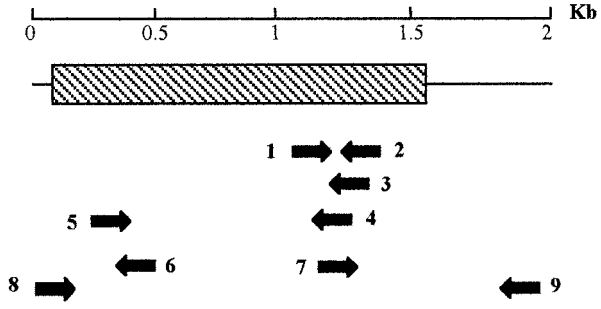

FIG. 1. RT-PCR cloning strategy of P450arom cDNA from goldfish ovaries. Location of degenerate and sequence-specific sense (→) and antisense (←) primers are shown and correspond to oligonucleotides 1–9 listed in *Materials and Methods*. *Hatched bar* indicates coding region.

Sequencing and computer analysis

Sequencing of ovarian aromatase cDNA was done on double-stranded DNA with Sequenase (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's recommendations. Complete sequence was obtained using Sp6 and T7 primers (Promega) complementary to the vector and sequential internal primers. The nucleotide and amino acid sequences were analyzed using the WI Package Version 9.0, Genetics Computer Group (GCG), Madison, WI. Phylogenetic analysis was done by multiple alignments of deduced amino acid sequences using the Neighbor-Joining method within the Clustal W program (17).

DNA isolation and Southern analysis

Genomic DNA was isolated from brains of two individual goldfish as previously described (8). Goldfish were purchased from Grassyfork Fisheries (Martinsville, IN). Approximately 10 μg DNA of each fish was digested with *Eco*RI, *Hin*dIII, *Pst*I, or *Xba*I (New England Biolabs, Boston, MA) restriction enzymes, electophoresed on a 0.8% agarose gel, and transferred to a Magnacharge nylon membrane (MSI, Westborough, MA). The filter was hybridized at 42 C with an ovary-derived P450arom cDNA probe (nucleotides: 1173–1322). The probe was generated by cutting the 240-bp PCR product (see above) with *Xba*I enzyme, and labeled by random priming to a specific activity 1×10^9 cpm/μg (18). The most stringent wash was performed at 60 C in 0.1 $\times$ SSC/0.1% SDS. After autoradiography, the membrane was reprobed with a brain-derived cDNA fragment, generated by *Eco*RI restriction enzyme digestion of the RT-PCR product amplified with primers 1 and 2. The amplified product was identical to the corresponding sequence of the reported goldfish P450arom cDNA (nucleotides: 1195–1336) (8).

RNA isolation and Northern analysis

Different tissues of 50 fish (mixed males and females) were pooled by type and total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH). Poly(A) RNA was purified from total forebrain and ovarian RNA with PolyATract mRNA isolation system (Promega), then 5 μg/lane poly(A) RNA was fractionated on a 1% formaldehyde agarose gel and transferred to Magnacharge nylon membrane (MSI, Westborough, MA). The RNA blot was prehybridized in 50% formamide, 5 $\times$ SSPE, 2 $\times$ Denhardt's solution, 100 μg yeast transfer RNA,

and 0.1% SDS at 42 C. The ovarian aromatase complementary RNA probe was derived from the 240-bp PCR product cloned into pCR II vector (Invitrogen) (see above). The plasmid was linearized with *Hin*dIII and labeled with [^{32}P]uridine triphosphate using the MAXIscript *In Vitro* Transcription kit (Ambion, Austin, TX) to a specific activity of approximately 1×10^9 cpm/μg. Hybridization was performed in the prehybridization buffer at 42 C with 4×10^6 cpm/ml riboprobe. The final stringent wash was at 68 C in 0.1 × SSPE/0.01% SDS. A 1-kb goldfish actin cDNA (derived by RT-PCR with degenerate primers, our unpublished data) was used as probe to standardize for RNA loading.

RT-PCR

Total RNA isolated from forebrain, mid/hindbrain, pituitary, ovary, and retina was DNase I-treated (Promega) according to the standard procedure (18). Each RNA (5 μg) was reverse transcribed as indicated above. PCR was performed with 10% of the RT reaction, diluted as follows: forebrain and ovary, 1:1, 1:3, 1:9, 1:27, 1:81, and 1:243, and retina, 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32. The PCR mix consisted of the same components as for amplification with primers 1 and 2, except two sets of primer pairs (10 and 3 plus 12 and 13) were added at a concentration of 0.2 μM. The reaction mixture was denatured for 3 min at 94 C. Thirty cycles of amplification were conducted with 30 sec denaturation at 94 C, 30 sec annealing at 60 C, and 1 min extension at 72 C. The reaction was incubated for an additional 10 min at 72 C to allow completion of extension. One half of each PCR reaction was analyzed on an 8% polyacrylamide gel. After staining with ethidium bromide (EtBr), gel image acquisition was performed with a Videk CCD camera (Kodak, Canandaigua, NY) and Bio Image's Low Light Scanning software (Bio Image, Ann Arbor, MI). A 600 ± 70-nm band-pass filter was used to reduce background fluorescence. Authenticity of PCR products was confirmed by Southern analysis of aliquots of forebrain and ovarian RT-PCR reactions, after separation on a 3% Nu-sieve GTG/1% agarose gel (FMC Bioproducts, Rockland, ME). The DNA blot was probed at 55 C with an oligonucleotide 11, labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega) (18). The membrane was washed for 5 min in 6 × SSC/0.1% SDS, autoradiographed, and then reprobed with an oligonucleotide 14 under the same conditions.

PCR of genomic DNA

Genomic DNA (200 ng) from two goldfish (different from individuals used for Southern analysis) was subjected to PCR with either brain (15 and 16) or ovarian (10 and 3) primer pairs (see legend of Fig. 5C). The PCR conditions were as described for RT-PCR, except that the final volume of PCR reaction was 100 μl. An aliquot (20%) of the reaction mixture was digested with the restriction enzymes *Eco*RI or *Cla*I to generate distinct size fragments for each amplified product. Undigested and digested products were resolved on an 8% polyacrylamide gel, EtBr-stained and photographed with FOTO/Phoresis UV documentation system (Fotodyne, Hartland, WI).

Plasmid constructs

For functional expression studies, an open reading frame (ORF) for brain-derived P450arom was generated by PCR with primers: TTCAGACGACGTGGTGATGG (nt: 93–113), and GACTGGTATGCGTCACTGAG (nt: 1751–1771) (8). Two substitutions were introduced in the sense primer (*underlined*) to remove ATGs located immediately upstream to the start of translation. The PCR product was T-A cloned into pCR II vector (Invitrogen), subsequently excised with *Eco*R V (vector site) and *Dra*I (nt: 1647), and ligated into the *Eco*R V site of pSGFLAG5 expression vector (19). Identity of the amplified neural P450arom cDNA insert was verified by sequencing. The resultant product was named pSGFLAG5-BRAROM. The ovary-derived P450arom cDNA was obtained from the pGEM vector (Promega) by restriction digestion with *Sal*I (filled-in with DNA polymerase I, Klenow fragment (Promega) and *Hpa*I (nt: 1605), and then inserted into the *Eco*R V site of the pSGFLAG5 and pcDNA3.1 (Invitrogen) expression vectors to give rise to pSGFLAG5-OVAROM and pcDNA3.1-OVAROM, respectively. The pcDNA3.1-BRAROM construct was produced by *Eco*R V/*Xho*I digestion of the pSGFLAG5-BRAROM and insertion into corresponding sites of the pcDNA3.1 expression vector.

Transient transfection

COS 7 African green monkey kidney cells were grown in Temin's modified Eagle's medium (TMEM) supplemented with 10% FBS (Sigma Chemical Co., St. Louis, MO). Transfections were done in 60-mm dishes (two dishes per construct, 65% confluent cells) with 10 μg/ml plasmid DNA construct (see above) and 30 μg/ml polybrene (Sigma) by the dimethylsulfoxide-
polybrene method as described by Kawai and Nishizawa (20). Mock-transfected cells were treated similarly only without addition of DNA constructs.

Aromatase assays

Aromatase activity was measured by [^{3}H]androgen to [^{3}H]estrogen conversion. Substrates were [7-^{3}H]androstenedione (-A; specific activity, 22.5 Ci/mmol) and [1α,2α-^{3}H]testosterone (-T; specific activity, 53.5 Ci/mmol; New England Nuclear, Boston, MA), repurified just before use by thin-layer chromatography on silica gel plates (ether/hexane, 3:1 vol/vol) and adjusted to 10 Ci/mmol specific activity with respective radioinert androgen. Sixty hours after cell transfection, medium was replaced with fresh medium containing substrate (50 nM final concentration) and aliquots taken at 2, 6, and 24 h of incubation. Cells were harvested after 24 h for determination of protein. Controls were mock-transfected cells and no-cell cultures. [^{3}H]Estrogen products were analyzed by procedures previously described and validated for goldfish brain (6). In brief, media were extracted with diethyl ether and steroids present in the ether residues were separated by thin-layer chromatography in the system above. Silica from areas corresponding to estrone (E_1) and estradiol (E_2) standards was scraped directly into vials for determination of radioactivity. To verify authenticity of estrogen products, methanolic eluates of E_1 and E_2 areas of representative samples were evaporated to dryness and partitioned twice between 0.05N NaOH and carbon tetrachloride. Results were expressed as counts per minute product formed per milligrams cell protein.

Results

Isolation and characterization of a cDNA encoding goldfish P450arom

A 1.9-kb cDNA encoding P450arom was isolated by RT-PCR from goldfish ovaries. During the first experiment, a ≈240-bp fragment was amplified with a degenerate pair of primers 1 and 2 (see Fig. 1 and *Materials and Methods*) designed to sequences in the highly conserved Ozols peptide and heme-binding regions. The isolated cDNA fragment was 75% homologous to the corresponding region of the goldfish P450arom derived from the brain cDNA library (8). In the second step of cloning, a ≈1-kb RT-PCR fragment was obtained using one degenerate and one sequence-specific primer (4 and 5). The sequence of degenerate primer 5 was chosen from the conservative region A described by Tanaka *et al.* (10). By means of 5′/3′-RACE and primers to newly identified sequences (6 and 7), the remaining sequence information was determined. The full-length cDNA was amplified with a pair of primers (8 and 9) targeted to the 5′- and 3′-ends. The products of four independent PCR reactions were combined and cloned into the pGEM vector. Three P450arom cDNA clones were fully sequenced, and two of them were identical (shown in Fig. 2). The third P450arom clone, when compared with the other two, had several nucleotide substitutions in the ORF: four synonymous (T293C, T380A, G1004A, and C1514A) and three nonsynonymous (C222T, T381A, and A807G). The latter resulted in the following amino acid substitutions: P68S, S121T, and R263G. Four more substitutions were observed in the 3′ untranslated region (UTR): A1687C, A1773C, A1780T, and T1812A. Some

```
TTAATACTCAATACATTCTTATGGCAGGTGAACTTCTCCAGCCCTGCGGAATGAAGCAGGTGCATCTTGGTGAGGCTGTTCTGGA 85
                    M  A  G  E  L  L  Q  P  C  G  M  K  Q  V  H  L  G  E  A  V  L  E

GCTTCTGATGCAAGGAGCCCATAACAGCAGTTACGGTGCTCAAGACAATGTGTGCGGAGCCATGGCCACACTACTGCTGCTGTTA 170
 L  L  M  Q  G  A  H  N  S  S  Y  G  A  Q  D  N  V  C  G  A  M  A  T  L  L  L  L  L

CTCTGCCTGCTGCTGGCCATCAGACACCATTGGACAGAGAAAGACCATGTGCCGGGTCCTTGTTTCTTGCTGGGTCTGGGTCCTC 255
L  C  L  L  L  A  I  R  H  H  W  T  E  K  D  H  V  P  G  P  C  F  L  L  G  L  G  P  L

TTCTCTCTTACTGTCGGTTCATCTGGTCTGGGATCGGTACGGCCAGCAACTACTACAACAGCAAATACGGAGACATTGTGCGGGT 340
  L  S  Y  C  R  F  I  W  S  G  I  G  T  A  S  N  Y  Y  N  S  K  Y  G  D  I  V  R  V

TTGGATCAATGGTGAGGAAACTCTCATCTTGAGCAGGTCTTCTGCTGTGTATCATGTGTTGAGGAAATCTCTGTACACCTCACGC 425
 W  I  N  G  E  E  T  L  I  L  S  R  S  S  A  V  Y  H  V  L  R  K  S  L  Y  T  S  R

TTTGGGAGCAAACTGGGTCTGCAGTGTATCGGAATGCATGAACAGGGCATTATATTCAACTCAAATGTGGCACTCTGGAAGAAAG 510
F  G  S  K  L  G  L  Q  C  I  G  M  H  E  Q  G  I  I  F  N  S  N  V  A  L  W  K  K  V

TCCGCTCCTTTTACGCTAAAGCTTTAACAGGTCCAGGACTTCAGAGAACATTGGAGATTTGCATCACTTCCACAAACACACACCT 595
  R  S  F  Y  A  K  A  L  T  G  P  G  L  Q  R  T  L  E  I  C  I  T  S  T  N  T  H  L

GGATAATTTGTCACACCTGATGGATGCCCGGGGACAGGTGGACATCCTCAACTTACTGCGGTGCATTGTAGTGGACATTTCCAAC 680
 D  N  L  S  H  L  M  D  A  R  G  Q  V  D  I  L  N  L  L  R  C  I  V  V  D  I  S  N

AGACTGTTTCTAGGAGTCCCTCTCAATGAGCATGATCTGCTTCAGAAGATTCATAAATATTTTGACACCTGGCAGACGGTACTAA 765
R  L  F  L  G  V  P  L  N  E  H  D  L  L  Q  K  I  H  K  Y  F  D  T  W  Q  T  V  L  I

TCAAACCTGATGTGTACTTCAGACTGGCCTGGTGGTTGCACAGGAAGCACAAGAGAGACGCTCAGGAGTTGCAGGATGCTATTGC 850
  K  P  D  V  Y  F  R  L  A  W  W  L  H  R  K  H  K  R  D  A  Q  E  L  Q  D  A  I  A

AGCTCTGATCGAACAGAAGAGAGTTCAGCTGACACGTGCAGAAAAATTCGACCAGCTCGACTTCACAGCAGAGCTGATATTTGCT 935
 A  L  I  E  Q  K  R  V  Q  L  T  R  A  E  K  F  D  Q  L  D  F  T  A  E  L  I  F  A

CAGAGCCACGGGGAGCTGAGCACTGAGAACGTCAGGCAGTGTGTGTTGGAGATGATAATCGCAGCTCCGGACACTCTCTCTATCA 1020
Q  S  H  G  E  L  S  T  E  N  V  R  Q  C  V  L  E  M  I  I  A  A  P  D  T  L  S  I  S

GTCTGTTCTTCATGTTGCTGTTGCTCAAACAGAATCCAGACGTCGAATTAAAGATCCTGCAGGAAATGAACGCTGTCCTAGCGGG 1105
  L  F  F  M  L  L  L  L  K  Q  N  P  D  V  E  L  K  I  L  Q  E  M  N  A  V  L  A  G

TCGGAGCCTGCAGCACTCACATCTGTCCGGGTTCCACATTCTGGAGAGTTTTATCAACGAGTCGCTCCGGTTCCATCCGGTCGTG 1190
 R  S  L  Q  H  S  H  L  S  G  F  H  I  L  E  S  F  I  N  E  S  L  R  F  H  P  V  V

GACTTCACCATGCGCCGGGCGCTGGATGATGATGTCATCGAAGGCTACAAAGTGAAAAGAGGGACAAACATCATCCTGAACGTGG 1275
D  F  T  M  R  R  A  L  D  D  D  V  I  E  G  Y  K  V  K  R  G  T  N  I  I  L  N  V  G

GTCGGATGCACAGATCGGAATTCTTCCCCAAGCCGAATGAGTTCAGTCTAGACAACTTCCAGAAAAACGTGCCGAGTCGTTTCTT 1360
  R  M  H  R  S  E  F  F  P  K  P  N  E  F  S  L  D  N  F  Q  K  N  V  P  S  R  F  F

CCAGCCGTTCGGATCCGGCCCGCGGTCGTGTGTGGGGAAGCACATGGCTATGGTGATGATGAAGTCCATTCTTGTGACGCTGCTG 1445
 Q  P  F  G  S  G  P  R  S  C  V  G  K  H  M  A  M  V  M  M  K  S  I  L  V  T  L  L

TCCCGTTTCTCTGTGTGTCCTGTGAAGGGCTGTACTGTAGACAGCATCCCCCAAACGAACGACCTGTCCCAGCAGCCGGTGGAGG 1530
S  R  F  S  V  C  P  V  K  G  C  T  V  D  S  I  P  Q  T  N  D  L  S  Q  Q  P  V  E  E

AGCCCTCGAGTCTCAGCGTGCAGCTCATCCTCAGAAACGCTCTCTGATTCACCTCCACCTGCTGCTCTGTCACTGTTAACGTATT 1615
  P  S  S  L  S  V  Q  L  I  L  R  N  A  L  *
TATCTACTGCTTTTAGTAGTCAGAGATATTTGTGTTTATATTTCGGTGTGAAATTACATTTATGCGTTTAGAAGACGCTAAAGCG 1700
ACTTACAGTGCATTCAGGCTATCAATTTTTACCTATCATGTGTTCCCGGGGAATCGAACCCCCAACCTAGCGATTGATAACGCAA 1785
TGCTCTACCAATTGAGCTACAGGAACTCTATGCAATGGCTGATATATGATGAATGTGGCTTTCTATGAATTACTGGTGTCTTCTA 1870
CTGTT**AATAAA**TGTC**AATAAA**CGTATTCTC                                                    1900
```

FIG. 2. Nucleotide and deduced amino acid sequence of goldfish P450arom cDNA derived from ovary. The 1554-bp ORF encodes a protein of 518 amino acids in length. Two ATGs and two polyadenylation signals are indicated with *underlining* and *boldface type*, respectively.

differences in the sequence among clones may be explained by allelic polymorphism (because starting material was RNA pooled from ovaries of ≈25 fish), whereas others may be due to PCR errors despite the use of a proofreading enzyme in the polymerase mix.

The amino acid sequence of P450arom represented by the two identical clones was derived from an ORF of 1554 bp, which starts at the first ATG (21 bp) and continues to the stop codon TGA (1575 bp) (Fig. 2). The ORF encoded a protein of the calculated mol wt of 58.7K. Similar to the reported fish ovarian aromatases (21–24), the goldfish P450arom had a second potential initiation site 30 bp downstream from the

first, but neither of the two ATGs had a nucleotide context that perfectly matched the proposed consensus sequence for initiation of translation (25). This differs from the brain P450arom, which has a single ATG that exactly corresponds to the initiation consensus sequence (8; Table 1).

The 5′UTR of the goldfish ovarian P450arom cDNA was 20 bp long and comparable in length to the reported sequences of the catfish (22) and tilapia (24) cDNAs, but much shorter than the 5′UTR of the goldfish brain-derived cDNA (Table 1) and other P450arom forms. The 3′UTR was 322 bp in length and contained two polyadenylation signals, 9 bp and 18 bp upstream from the poly(A) tail. It was the major feature accounting for size differences between the cloned ovarian and brain-derived cDNAs (1.9 *vs.* 3 kb; Table 1).

Figure 3 shows the deduced amino acid sequence of the goldfish ovary-derived P450arom aligned with other P450arom forms (8, 21–24, 26, 27). The goldfish ovarian P450arom shared 62/78% overall sequence identity/similarity with the goldfish brain aromatase; 68–72/82–85% with other fish aromatases; and 53/71% and 52/73% with chicken and human aromatases, respectively. The percent of identity/similarity was higher (64–97/79–100%) in the regions of high homology, including the I-helix, an aromatase-specific conserved region, and the heme-binding region, and was lowest at the termini of the different P450arom forms. Cross-comparison of the goldfish ovarian and brain sequences showed amino acid substitutions in all nine putative coding exons, indicating that the two P450aroms are more likely to be derived from different genes than from alternative exon splicing. The ovarian P450arom had an extended N-terminus and a shortened carboxy terminus relative to the brain form. Where overlapping, there were a total of 192 amino acid differences, of which ≈30% were nonconservative substitutions. Many of the differences were clustered at the N-terminus and the central part of molecule, including regions of putative exons II, V, and VII, and the N-terminus of exon IX. Based on mutational analysis and molecular modeling, amino acids known to be essential for catalytic functions in the human P450arom [I133 (28), E302, P308, D309, T310 (for review, see Ref. 29), and R435, C437 (30)] were identical in both goldfish forms. Many of the substitutions between goldfish ovarian and brain P450aroms were brain-specific in that they are conserved among all other fish ovarian forms: F72W, S94C, I218V, H262Y, H265Y, Q270K, L353V, R423K, and S452C. Others distinguish the brain P450arom from all non-neural aromatases, including chicken and human, and were located in putative functional domains: I-helix (E314D and N315D) and heme-binding domain (M467T) (based on numbering of the ovarian sequence). Of these, only the first was a conservative substitution. Also, a histidine residue at position 128 of the human P450arom, which may be involved in orientation of the substrate in the active site (29), corresponded to a histidine in the goldfish ovarian form but phenylalanine in the brain P450arom. Consistent with the glycosylation site described at the N terminus of human aromatase (31), a consensus N-glycosylation site (N-X-S/T) was identified in the amino terminal region of the ovarian form at N30, but the 5′-most glycosylation site in the brain form was at N42. Neither of these sequences was present in other fish aromatases. Further analysis with the Motifs program of the WI Package (GCG) indicated that the goldfish ovarian P450arom had consensus sequences for phosphorylation by cAMP-dependent protein kinase A at residue 409 (KRGT) and protein kinase C (PKC)-dependent sites at 133, 219, and 383 (S/T-X-R/K). The goldfish brain P450arom lacked the protein kinase A phosphorylation consensus sequence, but had all three PKC sites in corresponding positions (117, 198, and 361). The first two of these were conserved in all P450arom forms compared in Fig. 3, and the third was present in trout, chicken, and human, indicative of their possible functional importance. Each of the two goldfish forms had additional uniquely positioned PKC- and casein kinase-dependent phosphorylation sequences.

TABLE 1. Summary of differences between ovary- (A-) and brain- (B-) derived P450arom forms in goldfish

	Ovary	Brain[a]
cDNA (nt)	1990	2927
P450arom (aa)	518	510
ORF (nt)	1554	1530
5′ UTR (nt)	20	109
3′ UTR (nt)	322	1287
ATGs/consensus	2/None	1/1
poly(A) addition sites	2	5
Calculated mol wt	58.7K	58K
Measured mol wt	NM	56
Transcript(s) (kb)	1.9 ≫ 3.0	3.0

NM, not measured.
[a] Data from Ref. 8.

To illustrate the phylogenetic relatedness of the goldfish P450arom forms with other reported aromatases (8, 21–24, 26, 27, 32–36), an evolutionary tree was constructed using the Neighbor-Joining method of Clustal W (17) (Fig. 4). As expected, all fish ovarian aromatases were clustered together. However, the goldfish neural P450arom branched independently from the group comprising the fish aromatases, including the goldfish ovarian counterpart, and the calculated percent sequence divergence was indicative of a long evolutionary history from the branch point.

Ovary- and brain-derived P450arom are encoded by different genes loci

To determine whether ovary and brain P450arom derive from different gene loci, Southern analysis was carried out on genomic DNA of individual goldfish with discriminating brain- and ovary-specific cDNA probes (Fig. 5). Genomic DNA was digested with different restriction enzymes, electrophoresed on an 0.8% agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled probes, as described in *Materials and Methods*. Each probe gave a unique hybridization pattern with four different restriction digestions and with no evidence of cross-hybridization at the stringency conditions used (compare Fig. 5, A and B). The brain-derived probe demonstrated a single band with each of the four restriction enzymes tested (*Eco*RI, 1.7 kb; *Hin*dIII, 3.9 kb; *Pst*I, 2.8 kb; and *Xba*I, ≈10 kb); thus, it is encoded by a single gene locus. By contrast, the ovary-derived probe gave one to three bands. In all but *Hin*dIII digestions, results were identical for two individuals: *Eco*RI, 6.8, 2.4, and 1.65 kb (the latter band was seen on longer exposure); *Hin*dIII, 10 and

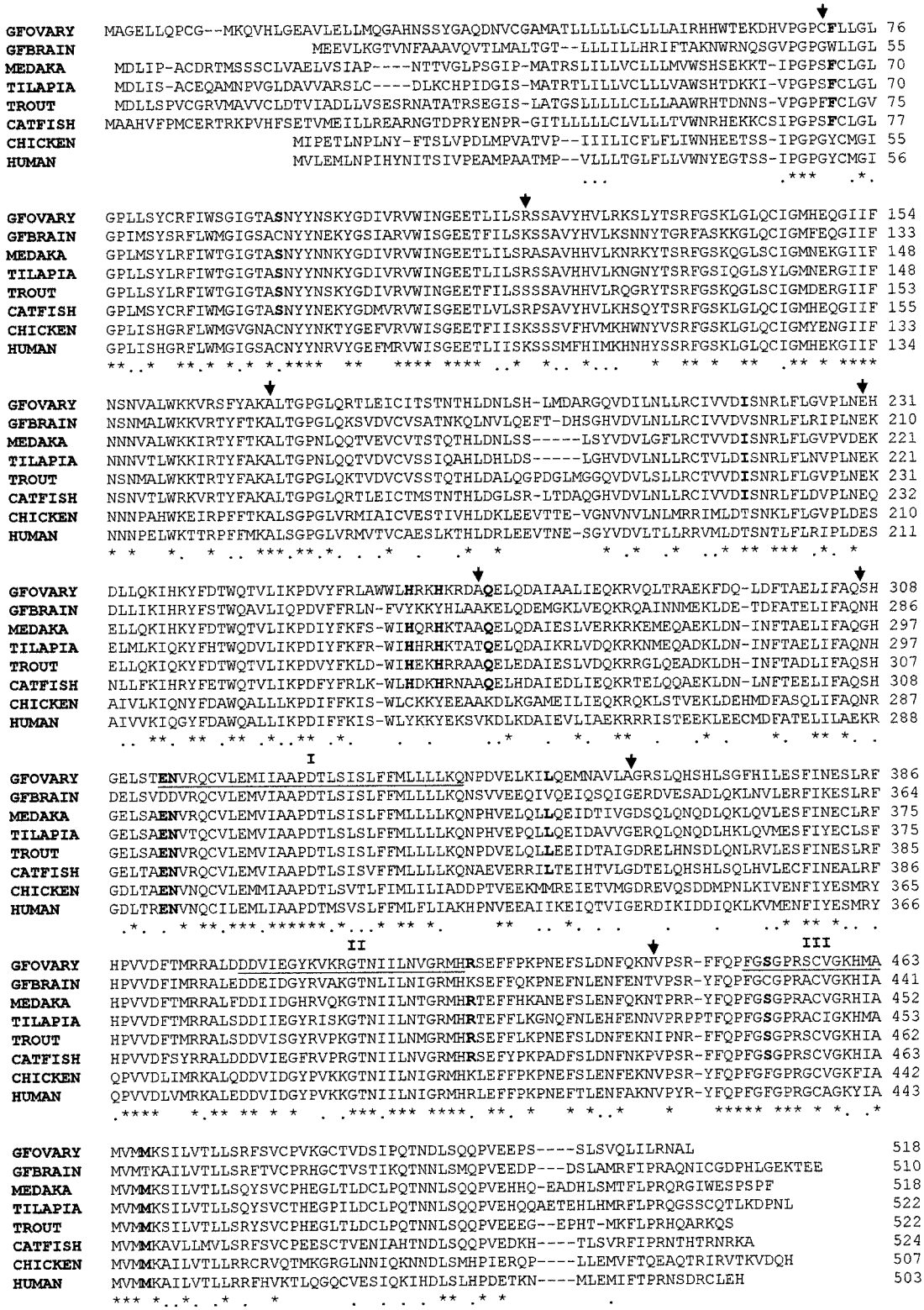

FIG. 3. Alignment of goldfish ovary-derived P450arom amino acid sequence with sequences of goldfish brain-derived P450arom and representative other vertebrates. Goldfish ovary-derived P450arom is compared with forms derived from goldfish brain (8), medaka ovary (23), trout ovary (21), catfish ovary (22), tilapia ovary (24), chicken ovary (26), and human placenta (27). Regions of high homology are *underlined* and indicated by *Roman numerals*: I-helix (I), an aromatase-specific conserved region (II), and heme-binding region (III) (1). Identical and similar amino acid residues are marked by *asterisks* and *dots*, respectively. Location of exon-intron boundaries in human and medaka genes (10, 11) is indicated by *arrows*. Residues where goldfish ovarian P450arom differs from goldfish brain P450arom but is identical to all other fish ovarian or to all other nonneural P450aroms are indicated in *boldface type*.

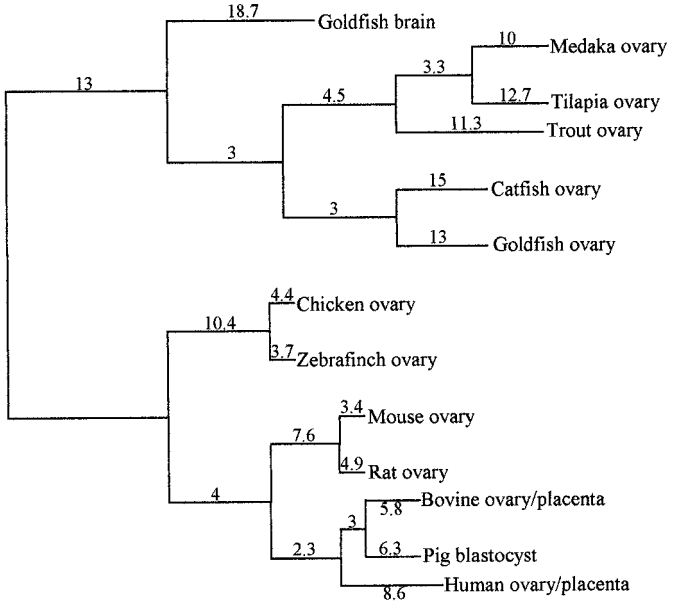

FIG. 4. Phylogenetic tree of P450arom proteins. Tree was constructed using Clustal W multiple sequence alignment (17) and deduced amino acid sequences of P450arom forms in goldfish brain, medaka ovary, trout ovary, catfish ovary, chicken ovary, human placenta (for references see legend to Fig. 3), zebra finch ovary (32), mouse ovary (33), rat ovary (34), bovine ovary/placenta (35), and pig blastocyst (36). *Numbers* indicate percentage sequence divergence. Bootstrap values (confidence figures for grouping) were higher than 97% for each internal branch.

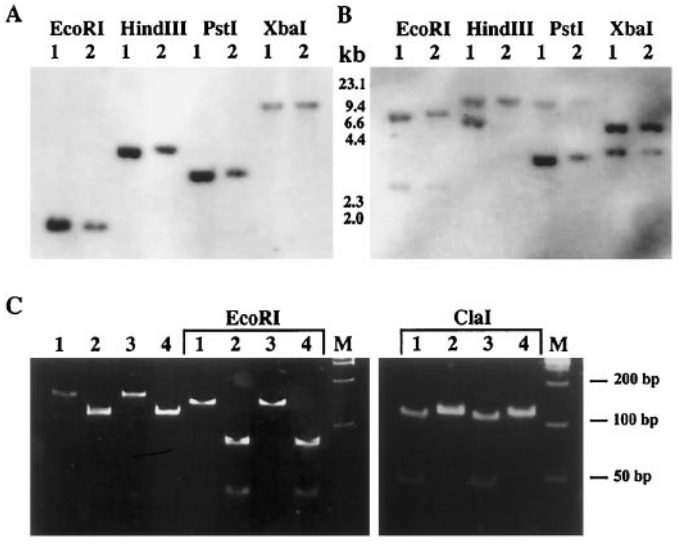

FIG. 5. Analysis of goldfish genomic DNA. Southern analysis was performed using genomic DNA from individual fish (shown here: 1 and 2) with brain- (A) and ovary-derived (B) P450arom cDNA probes. Probes were derived from a sequence within IXth exon of aromatase gene (based on structure of human and medaka CYP19 (10, 11) (see *Materials and Methods*). Positions of molecular size markers (λ DNA digested with *Hin*dIII) are indicated *between A* and *B*. Genomic DNA (200 ng) from two individuals was amplified with primers targeting sequences within presumptive exon IX (10, 11) but specific for brain- (15/16) or ovary-derived cDNAs (10/3)(C). Size of undigested PCR products generated with brain- (lanes 1 and 3) and ovary-type primers (lanes 2 and 4) was 153 bp and 116 bp, respectively. Digestion of brain product with *Eco*RI generated 131 bp and 22 bp fragments. The latter fragment was not visible on gel. Fragments of 74 bp and 42 bp in length were obtained with *Eco*RI cleavage of product amplified with ovary-specific primers. *Cla*I site was present in brain- but not in ovary-derived sequence, and generated 108 and 45 bp cleavage products.

5.8 kb (the latter was not seen in the second individual); *Pst*I, 10 and 3.4 kb; and *Xba*I, 5.6 and 3.8 kb. The third band obtained with the *Eco*RI restriction digestion is due to an internal *Eco*RI site in the ovarian probe. The identification of at least two fragments with most of the restriction digestions tested suggests that more than one CYP19 gene locus of the ovary-type sequence is present in the goldfish genome. Most importantly, the results of Southern analysis show the existence of two different gene loci in the goldfish genome encoding separate brain- and ovary-derived P450arom. This conclusion is supported by PCR analysis (Fig. 5C), in which regions of CYP19 genes corresponding to brain- and ovary-derived cDNAs were amplified from genomic DNA with type-specific primer pairs to give different-sized products. Amplified DNA products of both types were further analyzed by restriction digestion to give different-sized cleavage products for the two sequences. Thus, Southern and genomic PCR-restriction analyses both show that two P450arom mRNAs are transcribed from separate and distinct gene loci.

Tissue distribution of two P450arom mRNA variants

Northern blot analysis of goldfish poly(A) RNA was done to determine the size and number of ovary-derived aromatase mRNAs (Fig. 6). Two bands (3.0 and 1.9 kb) were observed with ovarian poly(A) RNA, and the 1.9-kb transcript was the most abundant. The size of the smaller transcript corresponds to the size of the P450arom cDNA isolated by RT-PCR from ovaries. The presence of the larger mRNA species may be explained by use of an alternative polyadenylation signal located downstream of the 3′most end of the cDNA shown in Fig. 2. Importantly, the 3.0-kb mRNA seen in goldfish ovaries cannot be ascribed to cross-hybridization with the brain-type mRNA, because forebrain poly(A) RNA failed to show a hybridization signal with the ovarian probe, although use of the brain probe in the same experiment (not shown) confirmed the presence of abundant brain-type message (8).

RT-PCR analysis was developed to estimate the ratio of expression of the two variants in a given tissue type by simultaneous amplification with two sets of primer pairs targeting ovary and brain sequences. Figure 7A illustrates representative EtBr-stained gels of RT-PCR products. Analysis of integral intensity of RT-PCR products by Bio Image's Low Light Scanning software demonstrated that in neural tissues, such as forebrain and mid/hindbrain (not shown), the brain variant was at least 14 times more abundant than the ovarian type, whereas in ovaries, only the ovary variant was detected. In pituitary, the brain variant predominated, but ratios were not determined (not shown). Interestingly, even though two types of mRNA were found in retinal tissue, similar to forebrain, their ratio was reversed (1:25). In Fig. 7B, results were validated by Southern analysis of forebrain and ovary RT-PCR products hybridized with internal oligonucleotides. Whereas the brain-type variant was much more

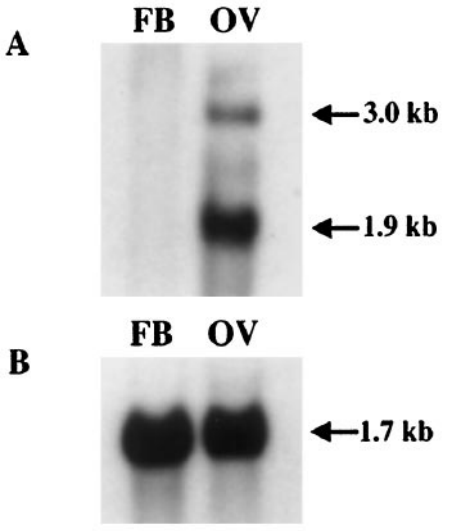

FIG. 6. Northern blot analysis of goldfish poly(A) RNA. Poly(A) RNA (5 μg) from forebrain (FB) and ovary (OV) was probed with a ^{32}P-labeled riboprobe derived from ovarian cDNA (A). The same blot was reprobed with ^{32}P-labeled actin cDNA to standardize for RNA loading (B).

abundant than the ovary type in forebrain, no brain-type P450arom cDNA variant was detected in ovaries.

Expression of P450arom cDNAs in COS 7 cells

Figure 8 shows that COS cells transfected with both pcDNA3.1-BRAROM and -OVAROM constructs transformed androgen to estrogen in a linear fashion over 24 h of incubation. Both A and T were aromatized; however, as previously reported, E_1 was the major estrogen produced from both substrates due to high constitutive expression of 17β-hydroxysteroid dehydrogenase in COS 7 cells (22). The radioactivity eluted from E_1 areas partitioned mainly into the phenolic fraction, indicating authentic estrogen. When expressed on the basis of total cellular protein (due to the lack of isoform-specific goldfish antibodies), estrogen yields were greater from A than from T and from cells expressing the brain construct compared with the ovarian construct. Mock-transfected cells and no-cell cultures produced no detectable E_1. Results were similar in a second experiment when cells were transfected with the PSGFLAG5-BRAROM and -OVAROM constructs, except that differences in estrogen yields from the brain- and ovarian-derived isozymes were negligible (from T) or reversed (from A) (not shown).

Discussion

In this article, we describe the cloning of a second goldfish aromatase cDNA encoding a protein of 58.7K calculated mol wt. This cDNA appears to be the product of a CYP19 gene, which is distinctly different from that encoding the previously described goldfish brain cDNA. Using the nomenclature suggested for the P450 gene superfamily (2), we designate these genes and their protein products CYP19A/P450aromA and CYP19B/P450aromB after the ovary- and brain-derived cDNAs, respectively. Moreover, results of Southern analysis, together with our unpublished sequence

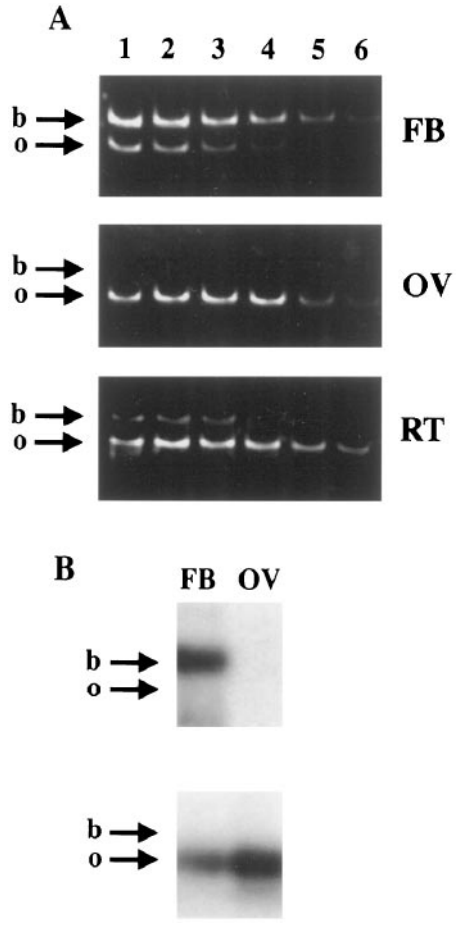

FIG. 7. RT-PCR analysis of brain- and ovary-type P450arom variants. A, RNA (5 μg) from forebrain (FB), ovary (OV), and retina (RT) was reverse transcribed and used in a dilution series (FB and OV, 1:1, 1:3, 1:9, 1:27, 1:81, 1:243, lanes 1–6, respectively; RT, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, lanes 1–6, respectively) for simultaneous amplification with brain- and ovary-specific primers. Brain-specific primers amplified a 142-bp fragment, whereas ovarian primers amplified a 116-bp fragment. EtBr- stained bands were quantified with Bio Image's Low Light Scanning software. B, RT-PCR products obtained with undiluted forebrain and ovary RT reactions shown above were resolved on an agarose gel, transferred to a nylon membrane, and hybridized with brain-specific (b), or ovary-specific (o) internal oligonucleotides.

of one of the ovarian 5′-RACE products, argue for two genes of the CYP19A type. Our findings are consistent with studies showing multiple genes encoding P450arom in pigs (13–15), and imply that the CYPl9 lineage in vertebrates comprises a multigene family of still undetermined size.

Alignment of the newly isolated P450aromA sequence with previously reported fish aromatases shows that it is more closely related to ovarian aromatases of other fish species than to the goldfish brain counterpart. Nonetheless, a uniformly high percentage of identity was observed among all fish aromatases when functionally important regions (I-helix, heme-binding) and specific amino acid residues were compared, and the overall percent of amino acid identity when compared with chicken and human aromatases was similar to other fish (21–24). Interestingly, all fish ovarian P450aroms reported so far are in a range of 518–524 amino

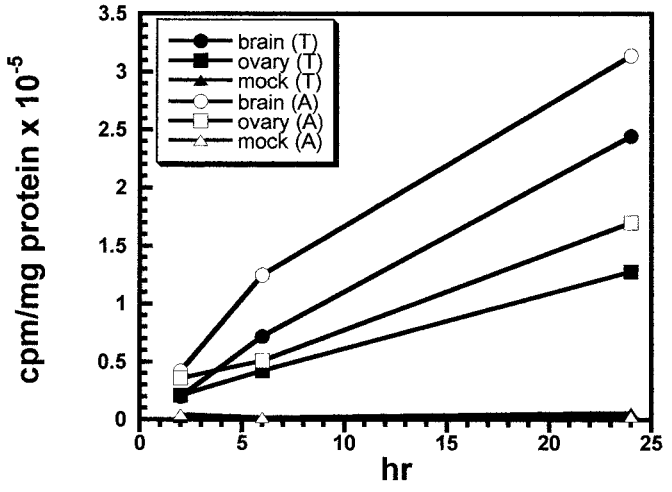

FIG. 8. Expression of functional brain- and ovary-derived P450arom isozymes in nonsteroidogenic COS cells. COS 7 cells were transiently transfected with pcDNA3.1-BRAROM (●), pcDNA3.1-OVAROM (■) or no DNA construct (▲) and incubated with [^{3}H]A (○, □, △) or -T (●, ■, ▲). [^{3}H]E_1 production was measured at 2, 6, and 24 h incubation and expressed as counts per minute per micrograms cell protein. Each point is mean of duplicate cultures.

acids, whereas goldfish brain, chicken, and mammalian aromatases have 503–510 amino acids. The difference in length is due mainly to the longer N-termini of ovary-derived fish sequences (Fig. 3) as previously noted (10). This characteristic separates the goldfish P450aromB from P450aromA and other teleostean P450aroms. The phylogenetic tree depicted in Fig. 4 further illustrates that the goldfish neural aromatase segregates from known ovarian sequences to form a distinct branch. Compared with the two goldfish isoforms, which have only 62% overall amino acid identity, the porcine isoforms (ovarian, blastocyst, and placental types) are much more homologous (87–93% identity) and virtually identical within certain exons (for review, see Ref. 15). Although a ≈700-bp P450arom fragment has been isolated from quail brain (37), and 5′ cDNA variants have been identified in rodent and primate brain (38, 39), to our knowledge no full-length P450arom cDNA has been characterized in the brain of any species. Efforts to obtain additional brain P450arom sequences will help to resolve evolutionary relationships within the CYPl9 family and test the predictive value of a separate brain-type isoform in goldfish.

Tetraploidization, or genomic duplication, occurred at several points during early vertebrate evolution (>500 million years ago), and is believed to be the genetic basis of subsequent rapid evolutionary change and the origin of multigene families (40). A more recent genome duplication event (15–20 million years ago) led to tetraploidy in present-day goldfish, which have multiple loci for many single-copy mammalian genes (41). However, tetraploidy *per se* cannot account for multiple CYPl9 genes in goldfish because corresponding P450aromA and P450aromB sequences of putative exon IX were identified by PCR cloning in the genome of the zebrafish, a closely related diploid species (16). The presence of multiple CYPl9 genes in pigs (13–15) and a CYPl9 pseudogene in the cow (42) signifies that CYPl9 gene duplication originated in a common ancestor of fish and mammals or, more likely, is the end result of two or more independent duplication events during the course of vertebrate evolution. The idea that CYPl9 gene duplication followed by diversification and/or convergence was a common occurrence at many points in evolution is further indicated by the presence of alternatively spliced 5′-exons and promoters in the CYPl9 genes of several mammals (1, 38, 39).

The two goldfish CYP19 genes are nonequivalent in their tissue-specific expression, indicating distinct promoters and regulatory mechanisms. RT-PCR analysis shows that expression of the brain-derived P450aromB is restricted to brain and retina, which have high levels of mRNA and enzyme activity, whereas P450aromA is the only form expressed in ovary, which has relatively low aromatase activity (6). Based on our initial results from ribonuclease protection assays (unpublished studies), transcript levels in ovary are low, reflecting low enzyme levels. Interestingly, the CYPl9A and CYPl9B genes have overlapping expression in neural tissues, but the B/A ratios differ regionally: brain, 14:1 and retina, 1:25. Tissue-specific and coexpression of different P450arom isoforms has been seen in pigs (43). An important question is whether the two isoforms expressed in goldfish brain are colocalized in the same cell populations or have unique distributions in different subsets of cells. Although human placental P450arom antibody immunoreacts with a single 56-kDa protein in goldfish brain membranes, and recognizes specific P450arom-labeled cells in sections of goldfish brain and retina (7, 44), further studies using variant-specific antibodies are required to determine whether earlier work may have underestimated the amount of immunolabeling per cell or failed to recognize the full population of P450arom-expressing cells. Recently, it has been shown that two P450arom mRNA variants are present in the rat brain, but only one is a full-length transcript that correlates neuroanatomically with enzyme levels (45, 46). Multiple brain forms, if present in mammals and birds, might also account for reported discrepancies between immunoreactivity and enzyme activity (for review, see Ref. 47).

In addition to sequence analysis, formal proof that each of the two CYPl9 genes encodes an authentic P450arom isozyme is based on the ability of the recombinant proteins to catalyze the transformation of androgen to estrogen. Due to high constitutive expression of l7β-hydroxysteroid dehydrogenase activity in COS cells, and resulting conversion of added T to A (22), functional differences between the two isozymes with respect to substrate preference or turnover cannot be tested in this cell line, nor can we compare product yields without antibodies to quantify aromatase-specific protein. Nonetheless, it is interesting that the goldfish P450aromB isoform differs from the ovarian P450aromA by having phenylalanine instead of histidine at a position corresponding to H128 of the human P450arom. Mutations at this position (H to A or Q) resulted in an approximately 30% increase in the Michaelis-Menten constant (K_m) with A, but not T, as substrate, but maximun velocity (V_{max}) was reduced to only 20% of wild-type with both substrates (for review, see Ref. 29). Based on the three-dimensional model for aromatase, it has been suggested that this residue may affect the reaction indirectly by stabilizing the substrate in the active site (29). To explain a surplus of l9-hydroxylating activity

relative to aromatase in rat brain membranes, and differences between brain and placental aromatases in their sensitivity to selective P450 inhibitors, Fishman and associates (48) speculated that aromatases of neural and nonneural tissues may differ. Further work is required to determine whether the goldfish isozymes differ in their basic catalytic properties, or whether sequence differences are indicative of regulation at pre- or posttranslational levels.

We conclude that CYPl9A and CYPl9B have had a long evolutionary history as separate functional genes, and that sequence divergence affected both transcribed and regulatory regions. Analysis of the gene promoter regions is in progress (49). Taken together, these observations and the differential expression of P450aromA and P450aromB in neural and gonadal tissues, signifies that retention of multiple gene loci and isozymes in the teleost lineage has had some adaptive advantage. Whether this is a cause or consequence of high neural estrogen biosynthesis remains unknown.

Acknowledgments

We are grateful to Dr. Tom Gilmore of this department, Dr. Stephen Charnock-Jones, Cambridge University, and Dr. Sandra Graham-Lorence, University of Texas Southwestern Medical Center at Dallas, for their invaluable technical expertise and inspiration. Dr. Gilmore kindly provided the pSGFLAG5 vector and COS 7 cells. We thank Dr. John Trant, Center of Marine Biotechnology, Baltimore, for actin primers and pcDNA3.1 vector, and Danielle Gelinas and Helen Krasnow for technical help.

References

1. **Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, Amarneh B, Ito Y, Fisher CR, Michael MD, Mendelson CR, Bulun SE** 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 15:342–354
2. **Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW** 1996 P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6:1–42
3. **Callard GV, Petro Z, Ryan KJ** 1978 Phylogenetic distribution of aromatase and other androgen converting enzymes in the central nervous system. Endocrinology 10:2283–2290
4. **Callard GV, Pudney JA, Kendall SL, Reinboth R** 1984 *In vitro* conversion of androgen to estrogen in amphioxus gonadal tissue. Gen Comp Endocrinol 56:53–58
5. **Callard GV, Petro Z, Ryan KJ, Claiborne JB** 1981 Estrogen synthesis *in vivo* and *in vitro* in the brain of a marine teleost (Myoxocephalus). Gen Comp Endocrinol 43:243–255
6. **Pasmanik M, Callard GV** 1988 Changes in brain aromatase and 5α-reductase activities correlate significantly with seasonal reproductive cycles in goldfish (*Carassius auratus*). Endocrinology 122:1349–1356
7. **Gelinas D, Callard GV** 1993 Immunocytochemical and biochemical evidence for aromatase in neurons of the retina, optic tectum and retinotectal pathways in goldfish. J Neuroendocrinol 5:635–641
8. **Gelinas D, Pitoc GA, Callard GV,** Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment. Mol Cell Endocrinol, in press
9. **Matsumine H, Herbst MA, Ou SH, Wilson JD, McPhaul MJ** 1991 Aromatase mRNA in the extragonadal tissues of chickens with the henny-feathering trait is derived from a distinctive promoter structure that contains a segment of a retroviral long terminal repeat. J Biol Chem 266:19900–19907
10. **Tanaka M, Fukada S, Matsuyama M, Nagahama Y** 1995 Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (*Oryzias latipes*). J Biochem 117:719–725
11. **Means GD, Mahendroo MS, Corbin CJ, Mathis JM, Powell FE, Mendelson CR, Simpson ER** 1989 Structural analysis of the gene encoding human aromatase cytochrome P-450, the enzyme responsible for estrogen biosynthesis. J Biol Chem 264:19385–19391
12. **Harada N, Yamada K, Saito K, Kibe N, Dohmae S, Takagi Y** 1990 Structural characterization of the human estrogen synthetase (aromatase) gene. Biochem Biophys Res Commun 166:365–372
13. **Choi I, Troyer DL, Cornwell DL, Kirby-Dobbels KR, Collante WR, Simmen FA** 1997 Closely related genes encode developmental and tissue isoforms of porcine cytochrome P450 aromatase. DNA Cell Biol 16:769–777
14. **Lahbib-Mansais Y, Barbosa A, Yerle M, Parma P, Milan D, Pailhoux E, Gellin J, Cotinot C** 1997 Mapping in pig of genes involved in sexual differentiation: AMH, WT1, FTZF1, SOX2, SOX9, AHC, and placental and embryonic CYP19. Cytogenet Cell Genet 76:109–114
15. **Conley A, Corbin J, Smith T, Hinshelwood M, Liu Z, Simpson E** 1997 Porcine aromatases: studies on tissue-specific, functionally distinct isozymes from a single gene? J Steroid Biochem Mol Biol 61:407–413
16. **Callard GV, Tchoudakova AV** 1997 Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish. J Steroid Biochem Mol Biol 61:387–392
17. **Higgins DG, Bleasby AJ, Fuchs R** 1992 Clustal V: improved software for multiple sequence alignment. Comput Appl Biosci 8:189–91
18. **Sambrook J, Fritsch EF, Maniatis T** 1989 Molecular Cloning: a Laboratory Manual, ed. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 1.1–11.61
19. **Hatzivassiliou E, Cardot P, Zannis VI, Mitsialis SA** 1997 Ultraspiracle, a Drosophila retinoid X receptor α homolog can mobilize the human thyroid hormone receptor to transactivate a human promoter. Biochemistry 36:9221–9231
20. **Kawai S, Nishizawa M** 1984 New procedure for DNA transfection with polycation and dimethyl sulfoxide. Mol Cell Biol 4:1172–1174
21. **Tanaka M, Telecky TM, Fukada S, Adachi S, Chen S, Nagahama Y** 1992 Cloning and sequence analysis of the cDNA encoding P-450 aromatase (P450arom) from a rainbow trout (*Oncorhynchus mykiss*) ovary; relationship between the amount of P450arom mRNA and the production of oestradiol-17B in the ovary. J Mol Endocrinol 8:53–61
22. **Trant JM** 1994 Isolation and characterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450arom. Gen Comp Endocrinol 95:155–168
23. **Fukada S, Tanaka M, Matsuyama M, Kobayashi D, Nagahama Y** 1996 Isolation, characterization, and expression of cDNA's encoding the medaka (*Oryzias latipes*) ovarian follicle cytochrome P-450 aromatase. Mol Reprod Dev 45:285–290
24. **Chang XT, Kobayashi T, Kajiura H, Nakamura M, Nagahama Y** 1997 Isolation and characterization of the cDNA encoding the tilapia (*Oreochromis niloticus*) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis. J Mol Endocrinol 18:57–66
25. **Kozak M** 1987 An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res 15:8125–8148
26. **McPhaul MJ, Noble JF, Simpson ER, Mendelson CR, Wilson JD** 1988 The expression of a functional cDNA encoding the chicken cytochrome P-450arom (aromatase) that catalyzes the formation of estrogen from androgen. J Biol Chem 263:16358–16363
27. **Corbin CJ, Graham-Lorence S, McPhaul M, Mason JI, Mendelson CR, Simpson ER** 1988 Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells. Proc Natl Acad Sci USA 85:8948–8952
28. **Kao YC, Cam LL, Laughton CA, Zhou D, Chen S** 1996 Binding characteristics of seven inhibitors of human aromatase: a site-directed mutagenesis study. Cancer Res 56:3451–3460
29. **Graham-Lorence S, Amarneh B, White R, Peterson J, Simpson E** 1995 A three-dimensional model of aromatase cytochrome P450. Protein Science 4:1065–1080
30. **Ito Y, Fisher CR, Conte FA, Grumbach MM, Simpson ER** 1993 Molecular basis of aromatase deficiency in an adult female with sexual infantilism and polycystic ovaries. Proc Natl Acad Sci USA 90:11673–11677
31. **Shimozawa O, Sakaguchi M, Ogawa H, Harada N, Mihara K, Omura T** 1993 Core glycosylation of cytochrome P-450 (arom). Evidence for localization of N terminus of microsomal cytochrome P-450 in the lumen. J Biol Chem 268:21399–21402
32. **Shen P, Campagnoni CW, Kampf K, Schlinger BA, Arnold AP, Campagnoni AT** 1994 Isolation and characterization of a zebra finch aromatase cDNA: *in situ* hybridization reveals high aromatase expression in brain. Mol Brain Res 24:227–237
33. **Terashima M, Toda K, Kawamoto T, Kuribayashi I, Ogawa Y, Maeda T, Shizuta Y** 1991 Isolation of a full-length cDNA encoding mouse aromatase P450. Arch Biochem Biophys 285:231–237
34. **Hickey GJ, Krasnow JS, Beattie WG, Richards JS** 1990 Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3′, 5′-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5′ genomic DNA. Mol Endocrinol 4:3–12
35. **Hinshelwood MM, Corbin CJ, Tsang PCW, Simpson ER** 1993 Isolation and characterization of a complementary deoxyribonucleic acid insert encoding bovine aromatase cytochrome P450. Endocrinology 133:1971–1977
36. **Choi I, Simmen RCM, Simmen FA** 1996 Molecular cloning of cytochrome P450 aromatase complementary deoxyribonucleic acid from periimplantation porcine and equine blastocysts identifies multiple novel 5′-untranslated exons expressed in embryos, endometrium, and placenta. Endocrinology 137:1457–1467
37. **Harada N, Yamada K, Foidart A, Balthazart J** 1992 Regulation of aromatase

cytochrome P450 (estrogen synthetase) transcripts in the quail by testosterone. Mol Brain Res 15:19–26
38. **Honda S, Harada N, Takagi Y** 1994 Novel exon I of the aromatase gene specific for aromatase transcripts in the human brain. Biochem Biophys Res Commun 198:1153–1160
39. **Yamada-Mouri N, Hirata S, Kato J** 1996 Existence and expression of the untranslated first exon of aromatase mRNA in the rat brain. J Steroid Biochem Mol Biol 58:163–166
40. **Ohno S** 1970 Evolution by Gene Duplication. Springer-Verlag, Berlin/New York, pp 59–88
41. **Risinger C, Larhammar D** 1993 Multiple loci for synapse protein SNAP-25 in the tetraploid goldfish. Proc Natl Acad Sci USA 90:10598–10602
42. **Furbass R, Vanselow J** 1995 Aromatase pseudogene is transcribed in the bovine placenta. Gene 154:287–291
43. **Choi I, Collante WR, Simmen RCM, Simmen FA** 1997 A developmental switch in expression from blastocyst to endometrial/placental type cytochrome P450 aromatase genes in the pig and horse. Biol Reprod 56:688–696
44. **Gelinas D, Callard GV** 1997 Immunolocalization of aromatase- and androgen receptor-positive neurons in the goldfish brain. Gen Comp Endocrinol 106:155–168
45. **Abdelgadir SE, Resko JA, Ojeda SR, Lephart ED, McPhaul MJ, Rosseli CE** 1994 Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain. Endocrinology 135:395–401
46. **Kato J, Yamada-Mouri N, Hirata S** 1997 Structure of aromatase mRNA in the rat brain. J Steroid Biochem Mol Biol 61:381–385
47. **Lephart ED** 1996 A review of brain aromatase cytochrome P450. Brain Res Rev 22:1–26
48. **Michnovicz JJ, Hahn EF, Fishman J** 1987 19-Hydroxylation and aromatization of androgens in the developing rat brain. Endocrinology 121:1209–1214
49. **Tchoudakova AV, Callard GV.** 1996 Molecular cloning of the 5′-flanking region of the goldfish brain aromatase gene. Program of the 26th Annual Meeting of the Society for Neuroscience, Washington, DC, p 325 (Abstract 133.12)