The Expression of a Functional cDNA Encoding the Chicken Cytochrome P-450<sub>arom</sub> (Aromatase) That Catalyzes the Formation of Estrogen from Androgen*

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A complementary DNA (cDNA) copy of the aromatase P-450 has been isolated from a chicken ovary library using as probe a partial cDNA believed to encode the human placental aromatase. The predicted amino acid sequence of the chicken aromatase cDNA possesses regions of homology to that of its human counterpart, but only limited homology to other cytochrome P-450 enzymes. The introduction of the cDNA clone into COS-1 cells results in the production of high levels of aromatase activity. The chicken enzyme is targeted to the appropriate subcellular fraction in the transfected COS cells, and the apparent K<sub>m</sub> of the chicken aromatase activity, measured in microsomes prepared from the transfected cells, is similar to that of the enzyme prepared from chicken ovary microsomes. These findings establish that the cDNA clone encodes chicken ovarian aromatase and demonstrate that this protein can catalyze the three successive oxidation reactions necessary to form estrogen from androgen.

The formation of estrogens from androgens is believed to proceed via three successive hydroxylation reactions, followed by C-19 decarboxylation and aromatization of the A-ring of the steroid hormone nucleus (1-4). Each of the three hydroxylation reactions requires the consumption of 1 mol of NADPH and 1 mol of molecular oxygen (1). The enzymatic machinery that catalyzes these reactions is believed to include a specific cytochrome P-450 (P-450<sub>arom</sub>) and a flavoprotein, NADPH-cytochrome P-450 reductase (2). A partial cDNA from the P-450<sub>arom</sub> of human placenta has been described by Evans et al. (5).

In this paper, we describe studies in which the partial cDNA for the human enzyme was utilized for the isolation and characterization of a full length cDNA clone encoding the chicken ovary aromatase P-450. Transfection of this chicken cDNA into COS-1 cells results in the expression of high levels of aromatase activity with kinetic properties similar to those of the native chicken ovary enzyme. Furthermore, in these transfected cells, aromatase activity is targeted to a dense microsomal fraction characteristic of human and chicken aromatase. The nucleotide sequence predicts a protein with limited homology to other cytochrome P-450 species, but with high homology to a putative human placental aromatase P-450.

EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes were purchased from New England Biolabs. *Escherichia coli* DNA polymerase I was from Boehringer Mannheim. RNase H and E. coli (NAD<sup>T</sup>) DNA ligase were from Pharmacia LKB Biotechnology Inc. Oligo(dT)-cellulose was from Collaborative Research. White Leghorn chickens were obtained from Pilgrims Pride Poultry Farms (Dallas, TX). Minimal essential medium (MEM)<sup>1</sup> was obtained from Grand Island Biological Co. All steroid radioisotopes were from Du Pont-New England Nuclear. [1<sup>14</sup>Cl]-Testosterone (18.2 Ci/mmol) was prepared from [12,23,3H]-Testosterone (50 Ci/mmol) and purified by celfite chromatography as described before (6). [α-<sup>32</sup>P]Uridine triphosphate (800 Ci/mmol) was from Amersham. NADPH-cytochrome P-450 reductase was purified from chicken liver as described before (7). COS-1 cells were kindly provided by J. L. Goldenstein (University of Texas Southwestern Medical Center, Dallas, TX).

RNA Isolation—Ovaries from 9-week-old chickens were solubilized in guanidium isothiocyanate, and total RNA was prepared by centrifugation over a 5.7 M cesium chloride step gradient (8). Poly(A)<sup>+</sup> RNA was isolated by oligo(dT)-cellulose chromatography (9).

Okayama and Berg cDNA Library Construction—A cDNA library was prepared in the Okayama and Berg eukaryotic plasmid expression vectors (10). First strand synthesis, linker piece ligation, and second strand synthesis were performed as described (11, 12). Following transformation into *E. coli* strain MC1061, a library of 3 × 10<sup>8</sup> independent colonies was obtained.

Construction of the Primer Extended Library—A 21-base oligonucleotide (5' AGCATATGAGATGTCACG 3') was synthesized on an Applied Biosciences DNA synthesizer and purified on a 20% polyacrylamide gel. 0.5 μg of this oligonucleotide was annealed to 8 μg of polyadenylated RNA prepared from chicken ovary. First strand synthesis was initiated by the addition of reverse transcriptase (11, 12). Second strand synthesis was accomplished by the procedure of Gubler and Hoffman (13) with modifications. Following methylation with EcoRI methylase, the ends of the double-stranded cDNA molecules were blunted with T<sub>4</sub> DNA polymerase (14). The cDNA was then ligated to EcoRI linkers and ligated into xgtI10 arms (15). Following in vitro packaging, a library of 2 × 10<sup>9</sup> independent recombinants was obtained.

Screening the Okayama and Berg cDNA Library—A sample of the original unamplified library was plated at a density of 10<sup>4</sup> colonies/15 cm plate. Lifts onto nitrocellulose filters and processing were performed as described (12). After baking, the filters were washed in 6 × SSC,<sup>2</sup> 0.1% sodium dodecyl sulfate at 42 °C for 12 h. They were then prehybridized in 25% formamide, 5 × SSC, 5 × Denhardt's<sup>3</sup>, 50 mM sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate, 100 μg/ml salmon sperm DNA, and 50 μg/ml polyguanosine and polythymidine at 42 °C. Hybridization was performed for 12 h under the same conditions in buffer containing 1 × 10<sup>6</sup> cpm/ml of probe. The probe used was [α-<sup>32</sup>P]UTP-labeled single-stranded RNA (specific activity > 2 × 10<sup>9</sup> cpm/μg) synthesized from a 1.8 kilobase partial DNA.

<sup>1</sup>The abbreviations used are: MEM; minimal essential medium; Hepes; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

<sup>2</sup>1 × SSC contains 0.15 M sodium chloride, 0.15 M sodium citrate (pH 7.5).

<sup>3</sup>5 × Denhardt's solution contains 10 μg/ml bovine serum albumin, 10 μg/ml polyvinylpyrrolidone, and 10 μg/ml Ficoll.

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The restriction sites are abbreviated in the text. Six h following addition of the purified DNA to the appropriate M13 vector (mp18 or mp19) and screening, the Sanger dideoxy termination method (18) was used to generate overlapping clones. The EcoRI linkers were subcloned into the appropriate M13 vector (mp18 or mp19) and screened for inserts. The estrogen formation experiments were performed as described (18). Hybridization was performed in 50% formamide, 50% sodium phosphate (pH 7.0), 0.1% sodium dodecyl sulfate, 100 pg/ml denatured salmon sperm DNA, and 50 pg/ml polyguanosine and polyadenosine for 12 h. Positive clones were chosen using a 1.8-kilobase pair partial cDNA insert as probe.

**RESULTS**

**Screening the Okayama and Berg Library**—The Okayama and Berg library was prepared from chicken ovary mRNA. The screening of approximately 1 x 10^6 clones identified a single recombinant that hybridized strongly at reduced stringency. After purification, the recombinant plasmid was found to contain an insert of approximately 3.2 kilobases (pcDVaram 3.2), as shown in Fig. 2A.

**RNA Blot**—To identify the length of the mRNA encoding the chicken aromatase and to assess the degree of homology between the chicken and human aromatase enzymes, we prepared RNA blots containing human placental total RNA and mRNA from chicken ovaries. In Fig. 3, a single Northern blot was hybridized sequentially to probes for the human placental and chicken ovary aromatase enzymes. In lanes 2-4, the blot has been hybridized to the human placental probe at reduced stringency. Although the signal is apparent in the lane corresponding to the human placental aromatase, the signal is not as strong as in the lane corresponding to the chicken ovary aromatase. This suggests that the chicken ovary aromatase is less closely related to the human aromatase than the human placental aromatase.
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**Fig. 3.** Northern blot analysis of RNA isolated from chicken ovary and human placenta. The same blot is shown hybridized to the human placental aromatase (lanes 2–4) and the chicken ovary aromatase probes (lanes 5–9). Lane 1 contains DNA size markers labeled with [α-32P]dCTP. Lanes 2 and 5 contain 20 μg of total RNA from human placenta. Lanes 3, 6, and 8 contain 10 μg of poly(A)+ RNA from chicken ovary enriched twice by oligo(dT) selection. Lanes 4, 7, and 9 contain 10 μg of poly(A)+ RNA prepared from chicken ovary RNA. Lanes 8 and 9 have an apparent size of approximately 7 kilobases. (lanes 5).

**Fig. 4.** Reconstruction of a functional chicken aromatase cDNA. The A and B fragments resulting from the digestion of pcDVaram 3.2 with PstI and BglII were purified and ligated to the C fragment isolated from the BglII/PstI digestion of BSpeAromI (see text). The thick lines correspond to the cDNA portions of the plasmids.

**Table I.** Enzymatic activity expressed by the transfected cells. The enzymatic activity expressed by the transfected cells was compared to the activity of the native protein present in chicken ovary. Microsomes were prepared from COS cells transfected with the full length cDNA and from chick ovary. In preliminary experiments, aromatase activity in transfected COS cell microsomes was measured in the presence and absence of added chicken liver NADPH-cytochrome P-450 reductase. As shown in Table I, COS-1 cells produce large amounts of aromatase activity following transfection with pcDVaram 4.0. By contrast, mock transfection of the cells or transfection with the pcDVaram 3.2 partial cDNA clone produces no detectable activity.

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TABLE I

Expression of Functional Chicken Aromatase cDNA

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>Total protein per dish</th>
<th>Aromatase activity</th>
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<tr>
<td></td>
<td></td>
<td>mg</td>
<td>pmol H₂O formed/mg protein/h</td>
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<tr>
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<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td></td>
<td>pcDVaram 4.0</td>
<td>0.98</td>
<td>17</td>
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Table 1. After 48 h, the cells were scraped, rinsed twice with Tris chloride (pH 7.4), containing 20% glycerol, and stored in liquid nitrogen. These preparations were thawed and reconstituted at 100,000 × g, and the pellets were resuspended in 0.2 ml of 10 mM Tris-Cl (pH 7.4). Aliquots of ovarian microsomes (3.5 mg of protein) and COS cell microsomes (4.7 mg of protein) were then mixed with 0.1 M Tris citrate (pH 6.5), 1 mM NADPH, 0.2 mg of bovine serum albumin, and varying concentrations of [18-3H]testosterone as indicated in a total volume of 0.2 ml. After incubation for 1 h at 25 °C, H₂O generation was measured as described in the text.

Fig. 5, although the V₅₀ for the ovarian aromatase is 10 times that of the transfected COS cells, the apparent Kₐ values of the two enzymes were similar (0.15 μM and 0.17 μM for chicken ovary microsomes and transfected COS cell microsomes, respectively) and are also similar to values previously reported for the chicken enzyme (6). When the experiment shown in Fig. 5 was performed in parallel with [1,2,6,7-3H]testosterone and the estrogen products were purified by thin layer chromatography (29) (Table II), comparable rates of estrogen formation were obtained with the two methods.

The Chicken Aromatase Is Correctly Targeted to the Microsomal Fraction of Transfected COS Cells—To examine the intracellular distribution of aromatase activity, COS cells transfected with the aromatase cDNA were lysed, and the lysate was subjected to differential centrifugation. As shown in Table III, most of the aromatase activity is found in the microsomal fraction. The distribution is similar to that previously reported for human placental aromatase activity (24, 25) and for chicken ovary aromatase (7). It is interesting to note that the levels of activity seen with broken cell assays is similar to that seen in the monolayer assay.

Nucleotide and Protein Sequence Analysis—The nucleotide sequence and predicted amino acid sequence of the chicken ovary P-450 aromatase are shown in Fig. 6. Examination of the sequence shows two potential translation start sites (underlined with thin bars in Fig. 6). Utilization of the first would yield a protein of 507 amino acids with a predicted molecular weight of 58,146. Initiation at the second potential initiator methionine would yield a protein of 507 amino acids with a predicted molecular weight of 58,146. Examination of the nucleotide sequences surrounding these two potential initiator
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The predicted amino acid sequence was also examined for homologies to other cytochrome P-450 enzymes and to the protein sequence data bases. Comparison of the protein sequence with those of the other known species of cytochrome P-450 enzymes identified only one homologous sequence, that of a phenobarbital-induced cytochrome P-450 (cytochrome P-450 111) from the rabbit (28). The overall conservation is 33% over an 89-amino-acid internal segment (residues 305–394), but this takes into account only exact matches.

A second protein that contains sequences of homology to the chicken aromatase P-450 is the bovine mitochondrial cytochrome c oxidase (residues 71–90) (29). This segment, which extends from residues 313 to 322 in the chicken aromatase, is conserved exactly. Furthermore, many of the mismatches that occur are in fact conservative changes.

We describe the isolation and expression of a cDNA clone that encodes a functional aromatase enzyme. These experiments conclusively demonstrate the identity of this protein as the chicken ovary P-450 aromatase. Furthermore, these experiments are the first to demonstrate that the expression of this single enzyme in eukaryotic cells is sufficient to catalyze the complex series of reactions resulting in the conversion of androgen to estrogen.

The aromatase activity that is expressed in the transfected COS cells possesses many of the properties characteristic of the chicken ovary aromatase. While the levels of enzymatic activity are less than in chicken ovary, the apparent $K_m$ of the enzyme produced in the transfected COS cells is similar to that in ovary. Furthermore, the bulk of the aromatase activity in the transfected cells is in subcellular fractions enriched for dense microsomes. As this distribution is similar to that described for aromatase activity from other tissues (7, 24, 25), it is likely that the signals encoding the intracellular targeting of the enzyme are also conserved between different species and allow for the correct insertion and localization of the chicken enzyme to the microsomal fraction of monkey cells.

Several points can be made regarding the relationship between the chicken aromatase and what is believed to be its human homologue. First, the aromatase enzyme in chicken ovary appears to be encoded by a single mRNA species, whereas in human placenta and adipose tissue, numerous forms are seen that are probably the product of differential polyadenylation (5). It is presumed that on further analysis the chicken transcript(s) will be found to lack the alternative

FIG. 6. Nucleotide sequence and predicted amino acid sequence of the chicken ovary P-450 aromatase. The two potential initiator methionine codons are underlined. The presumed amino-terminal transmembrane region is underlined with a thick bar. The sequence of the oligonucleotide used to construct the primer extended library is enclosed in a box. Amino acid residues that are identical between the chicken aromatase and the partial cDNA for the human placental aromatase (23) are indicated by dots placed below the corresponding residue.

Additional text continues...
polyadenylation sites proposed for the human placental aromatase transcripts.

The chicken and human enzymes are highly conserved. In particular, in the region surrounding the presumed heme binding domain, the sequences of the two proteins are virtually identical. While other regions are not as highly related, the overall structure of the functional domains must be remarkable, as the expression of this chicken enzyme in monkey cells appears not to be stimulated by the addition of exogenous chicken cytochrome reductase. Thus, the interaction of the chicken enzyme with the monkey cytochrome reductase in COS cells must be quite efficient.

The chicken ovary aromatase enzyme is not highly related to other known species of cytochrome P-450. It is interesting to note that the limited area of homology that can be demonstrated between the chicken aromatase and rabbit cytochrome P-450 (III) and cytochrome oxidase (polypeptide II) is situated on a hydrophobic helical segment that in P-450 (camphor) has been shown to interact with the heme molecule and substrate (30). Whether these homologies represent conservation of functionally important structures remains to be tested.

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REFERENCES