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Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells

(cDNA/COS-1 cells)

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ABSTRACT **The isolation and cloning of a full-length cDNA insert complementary to mRNA encoding human aromatase system cytochrome P-450 is reported. The insert contains an open reading frame encoding a protein of 503 amino acids. This gene is clearly a member of the cytochrome P-450 gene superfamily, because the sequence contains regions of marked homology to those of other members, notably a putative membrane-spanning region, I helix, Ozols, and heme-binding regions. The cDNA was inserted into a modified pCMV vector and expressed in COS-1 monkey kidney tumor cells. The expressed protein was similar in size to human placental aromatase system cytochrome P-450, as detected by immunoblot analysis, and catalyzed the aromatization of androstenedione, testosterone, and 16α-hydroxyandrostenedione. This activity was inhibited by the known aromatase inhibitors, 4-hydroxyandrostenedione and econazole. Thus the several steps involved in the aromatization reaction appear to be catalyzed by a single polypeptide chain, which can metabolize the three major physiological substrates.**

The conversion of androgens to estrogens is catalyzed by an enzyme complex named aromatase. This complex is comprised of a specific form of cytochrome P-450, aromatase-system cytochrome P-450 ($P\text{-}450_{Arom}$), and the flavoprotein NADPH-cytochrome P-450 reductase (1–3). The components of this enzyme complex are located in the endoplasmic reticulum of the tissue sites of expression, including the syncytiotrophoblast of the placenta, granulosa cells of the ovary (4), testicular Sertoli cells (5), adipose tissue (6), hypothalamus, hippocampus, and amygdala of the brain (7–9), and the preimplantation blastocyst (10). In these tissue sites the enzyme is subject to complex multifactorial regulation by a diverse group of factors including gonadotropins and cyclic AMP, glucocorticoids, phorbol esters, and a number of growth factors such as transforming growth factor β, epidermal growth factor, fibroblast growth factor, as well as tumor necrosis factor (11). Aromatization of the A ring of Δ^4-3-one steroids to form the phenolic A ring characteristic of estrogens has been shown to require 3 mol of oxygen and 3 mol of NADPH for every mol of steroid biosynthesized (12). Utilization of the first two oxygen molecules is believed to result in sequential hydroxylation of the C_{19} angular methyl group with its subsequent loss. The site of attack by the third oxygen molecule has not been determined but has been suggested to result in the formation of a 2β-hydroxyl group with concomitant nonenzymatic collapse of the resultant intermediate, giving rise to the phenolic A ring (13).

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In addition to defining the mechanisms underlying the multifactorial and tissue-specific regulation of this enzyme, a number of important questions remain unanswered. (*i*) Because different products are formed in different tissue sites—namely, estriol in the placenta, estradiol in the ovary, and estrone in the adipose tissue—it has been suggested that there exist two or more forms of the aromatase enzyme with different substrate specificities (14). Alternatively, these various products could result from the nature of the substrates that are presented to the enzyme in each of these tissues—namely, 16α-hydroxylated C_{19} steroids in the placenta, testosterone in the ovary, and androstenedione in adipose tissue. (*ii*) Because the production of 19-hydroxy-, 19-oxo-, and 19-norsteroids has been reported, as well as the aromatization of these compounds, a separate polypeptide could catalyze each step involved in the overall aromatase reaction (15). (*iii*) If indeed all three steps of the aromatization reaction are catalyzed by the same polypeptide, then clearly one active site must be capable of the sequential insertion of several oxygen atoms at different positions within the steroid molecule, and this activity presents an interesting problem in terms of the structure–function relationships involved in this reaction.

To answer these questions as well as to provide a basis for studies on the relationship of catalytic activity to primary sequence involving site-directed mutagenesis, we have isolated and cloned a full-length cDNA insert complementary to the mRNA encoding human cytochrome $P\text{-}450_{Arom}$.† Comparisons have been made between the derived amino acid sequences of human $P\text{-}450_{Arom}$ and other cytochrome P-450 species. By inserting this full-length cDNA into a eukaryotic expression vector and using this to transfect COS-1 monkey kidney tumor cells, we have sought to express and characterize this enzyme activity in cells that do not normally have steroidogenic capacity.

MATERIALS AND METHODS

Preparation and Screening of Primer-Extended Library. $Poly(A)^+$ RNA was prepared from total human placental RNA by two passes over an oligo(dT)-cellulose column (16). A 20-base pair (bp) oligonucleotide was used to prime first-strand cDNA synthesis under the modified condition described by Gubler and Hoffman (17). Second-strand cDNA synthesis was accomplished using RNase H and *Escherichia coli* DNA polymerase I (17). The termini of the double-stranded cDNA molecules were blunted with T4 DNA poly-

Abbreviation: $P\text{-}450_{Arom}$, aromatase system cytochrome P-450 [the product of the CYP19 or P450XIX gene (27)].

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†The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04127).

merase I (17). The termini of the double-stranded cDNA molecules were blunted with T4 DNA polymerase (18) and then methylated with *Eco*RI methylase. Synthetic *Eco*RI linkers were attached, and the cDNA (≈100 ng) was then ligated into phosphatased λgt10 arms (Promega Biotec, Madison, WI) and packaged *in vitro*. After plating of the library (7×10^4 plaque-forming units) on the restrictive host, C600 Hfl$^-$, the library was screened using a cDNA fragment priming toward the 5′ terminus. Positive clones were purified to homogeneity, and the inserts were characterized as described below.

Double-Stranded Sequencing. Amplified plugs from the sixth screening of a human placental λgt10 cDNA library (screened using the 2.5-kb cDNA insert previously isolated from a human placental λgt11 library; ref. 19) were cut with *Hin*dIII and *Bgl* II. The resulting 4.0-kilobase (kb) insert, which was flanked by some vector sequence, was subcloned into pUC19. Double-stranded sequencing was performed using oligonucleotides priming to the 5′ end of the 2.5-kb insert and subsequently to a site ≈160 bp upstream from this end.

Expression in COS-1 Cells. P-450$_{Arom}$ cDNA was cleaved from pUC19 by first digesting with *Stu* I, which cleaves in the 3′ untranslated region of the cDNA, 301 bp from the TAG stop codon, and then partially digested with *Eco*RI. The resultant 1.9-kb fragment was isolated from a 1.0% low-melting-point agarose gel and ligated into the *Eco*RI/*Sma* I sites of the eukaryotic expression vector pCMV2 to produce pCMV$_{Arom}$. pCMV2, which contains a simian virus 40 origin of replication, a cytomegalovirus promoter, and the human growth hormone termination and polyadenylylation signals, was obtained from David Russell (Department of Molecular Genetics, University of Texas Southwestern Medical School), and modified by Matthew Lorence (Department of Biochemistry, University of Texas Southwestern Medical School) (D. Russell and M. Lorence, personal communication). pCMV$_{Arom}$ was then transfected into COS-1 monkey tumor kidney cells (obtained from Y. Gluzman, Cold Spring Harbor Laboratory) with a DEAE-dextran method as described (20).

Northern (RNA) and Western (Immunologic) Analyses of RNA and Proteins in Transfected COS-1 Cells. These analyses were conducted as described (20). ^{125}I-labeled protein A was used instead of a second antibody.

Assay of Aromatase Activity. Aromatase activity was determined by assaying the incorporation of tritium into [^{3}H]water from [1β-^{3}H]androstenedione, [1,2-^{3}H]testosterone, and [1β-^{3}H]16α-hydroxyandrostenedione as described (21). Nontransfected cells or else cells transfected with a modified pCMV vector containing a full-length bovine P-450$_{C21}$ cDNA (provided by M. Lorence), served as controls; these yielded values no different from medium blanks. Characterization of the steroid products was accomplished in parallel experiments using [4-^{14}C]-labeled substrates. After extraction with methylene chloride, radiolabeled steroids were separated by TLC with the solvent system chloroform/ethyl acetate, 8:2 for experiments in which the substrate was testosterone and with chloroform/ethyl acetate, 7:3 for experiments in which the substrate was androstenedione. The regions of the plates corresponding to authentic standards were excised, and radioactivity was determined by liquid scintillation spectrometry.

Hydropathy Analysis. Hydropathy measurements were performed by the method of Kyte and Doolittle using a window of six residues (22). The output of these analyses was transferred to the LOTUS 1-2-3 program, the values were then aligned (where necessary, gaps were placed between data points), and plots were made using the SIGMA-PLOT program.

RESULTS AND DISCUSSION

Isolation and Characterization of a Full-Length cDNA Encoding Human P-450$_{Arom}$. The present report documents the isolation and characterization of a full-length cDNA insert complementary to human P-450$_{Arom}$. The sequence of the full-length cDNA insert encoding P-450$_{Arom}$ is shown in Fig. 1; this insert is 2736 bp in length. We previously reported the sequencing of an ≈2.5-kb insert, isolated from a human placental λgt11 library, in which the sequence commenced at the site indicated by the second arrow (19, 23). A similar sequence was recently reported by Chen *et al.* (24), which also started at this site. To obtain sequence upstream from this site we prepared a primer-extended library using poly(A)$^+$ RNA from human placenta as indicated in *Materials and Methods*. Twelve clones were isolated from the primer-extended library. Of these, a single clone was found to extend beyond the internal *Eco*RI restriction site indicated in Fig. 1. This clone was sequenced. A second clone was isolated from a λgt10 library prepared from human placental RNA (donated by P. Seeburg, Genentech) and screened using the 2.5-kb cDNA insert. This latter insert was 4.0 kb in length and was ligated into pUC19. The 5′ end was sequenced by the double-stranded method and was found to have a sequence identical to the clone from the primer-extended library.

Primary and Secondary Structure of Human P-450$_{Arom}$. The sequence of the full-length cDNA is shown in Fig. 1. The amino acid sequence of human P-450$_{Arom}$ was derived from the open reading frame and compared with that of other forms of cytochrome P-450. There is an open reading frame starting with an ATG that is 39 bp from the 5′ end of the cDNA and that continues for another 1509 bases—i.e., 503 amino acids—to a TAG stop codon. We consider this ATG to be the start of translation because the following 10 amino acids, beginning with valine and ending with tyrosine, are identical to the amino-terminal sequence of the purified P-450$_{Arom}$ reported by Chen *et al.* (2). This latter sequence lacks the initiating methionine. Furthermore, there are no ATG sequences upstream of this site in the cDNA, and the corresponding ATG in the sequence of a full-length chicken cytochrome P-450$_{Arom}$ cDNA, which has recently been characterized and expressed by McPhaul *et al.* (18), is the start of the open reading frame of that insert. The present derived sequence diverges markedly from that of Chen *et al.* (2) after Tyr-11 but retains pronounced homology with that of chicken P-450$_{Arom}$ (18).

The derived coding region of P-450$_{Arom}$ clearly indicates that this polypeptide belongs to a member of the cytochrome P-450 superfamily of genes. Generally, no greater than 30% sequence homology was found between P-450$_{Arom}$ and other forms of cytochrome P-450, indicating that cytochrome P-450$_{Arom}$, in common with other steroidogenic P-450 species, belongs to a separate gene family within the overall superfamily, designated cytochrome P-450XIX (25). The most highly conserved region is the carboxy-terminal 200 residues that contain Ozols (residues 349–371), aromatic (residues 407–418), and heme-binding (residues 430–443) regions, and the I helix (residues 290–324).

Near the carboxy-terminal end is the region believed to be the heme-binding region. Within this sequence is a cysteine, indicated by underlining, which is common to all cytochrome P-450s, and is believed to be the fifth coordinating ligand of the heme iron (Fig. 1). The amino acid sequence of this heme-binding region shows marked homology with that of the other steroidogenic forms of P-450 so far determined (19).

The Ozols region, the second most conserved region in all P-450 species, has been suggested to be involved in substrate binding (26). In human P-450$_{Arom}$, both Glu-362 and Arg-365 are conserved. Also Pro-368 and Val-369 appear to be conserved among the steroidogenic P-450s.

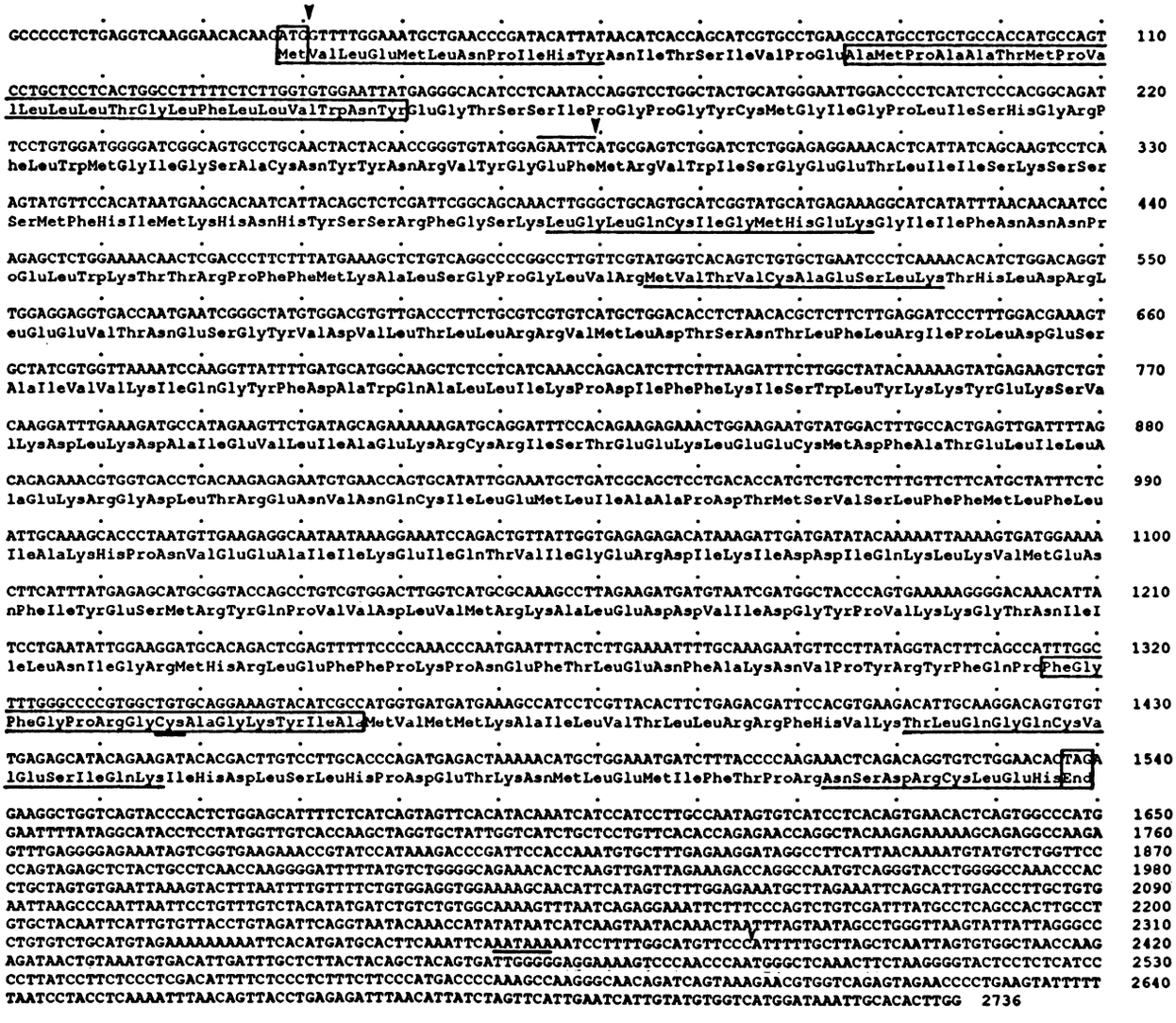

FIG. 1. Sequence of the full-length cDNA encoding human cytochrome P-450$_{Arom}$. The beginning and end of the open reading frame are indicated by boxes. Extending from the second amino acid (Val) for 10 amino acids and indicated by underlining is a sequence identical to that of the amino terminus of the purified P-450$_{Arom}$ protein published by Chen *et al.* (2). The start of a previously characterized 2.5-kb cDNA clone (19) is indicated by the second arrow and just upstream from this is indicated an internal *Eco*RI restriction site. The other four underlined sequences are identical to the sequences of cysteine-containing tryptic peptides, reported by Chen *et al.* (2). Indicated by the boxed areas are the putative membrane-spanning domain and heme-binding region. Within the 3′ untranslated region a polyadenylylation signal is indicated, and 20 bp downstream a third arrow indicates the start of the poly(A)$^+$ tail of another cDNA clone that otherwise appears identical to this one (19, 23).

In addition, near the amino-terminal end there is a highly hydrophobic region (residues 23–39), present in all microsomal P-450 species and believed to be the membrane-spanning domain.

From the hydropathy plots of human P-450$_{Arom}$, human P-450$_{17\alpha}$, human P-450$_{c21}$, and P-450$_{Cam}$ shown in Fig. 2, the region corresponding to the I helix (indicated by I) shows a characteristic hydrophobic double peak. In P-450$_{Cam}$, in which the three-dimensional structure has been determined by x-ray crystallography (27), the I helix is a hydrophobic backbone through the center of the molecule, which along with the L helix and heme-binding region, interacts with the heme molecule (27). Also in these plots one can identify the putative membrane-spanning region (indicated by M) at the amino-terminal end. When comparing the sequence of this region in P-450$_{Arom}$ with those of the other P-450s, an additional 22 residues are found between the initiating methionine and the membrane-spanning domain of P-450$_{Arom}$ (Fig. 1); these residues are also present in some hydrocarbon-metabolizing forms of cytochrome P-450, such as P-450$_c$ of rat (28).

The sequence of the cDNA insert shown in Fig. 1 does not contain a poly(A)$^+$ tail, but terminates in a guanine, which possibly is the start of another *Eco*RI restriction site. However, within the sequence of the 3′ untranslated region and indicated by underlining, there is a polyadenylylation signal. Twenty-one bases downstream from that and indicated by the third arrow is the start of the poly(A)$^+$ tail of another cDNA insert complementary to P-450$_{Arom}$, which we isolated from the same λgt11 library as the 2.5-kb insert (19, 23). These clones then appear to differ by the alternative use of more than one polyadenylylation signal but are otherwise identical. The existence, therefore, of two mRNA species of different size would be anticipated, and this is indeed what we have found in human placenta, granulosa cells, and adipose stromal cells (19, 29, 30).

Expression of Human P-450$_{Arom}$ in COS-1 Cells. To determine whether this cDNA encodes a protein possessing aromatase activity, the insert was ligated into a modified pCMV vector and used to transfect COS-1 monkey kidney tumor cells. Three days after transfection, RNA was extracted and subjected to Northern analysis with the 2.5-kb

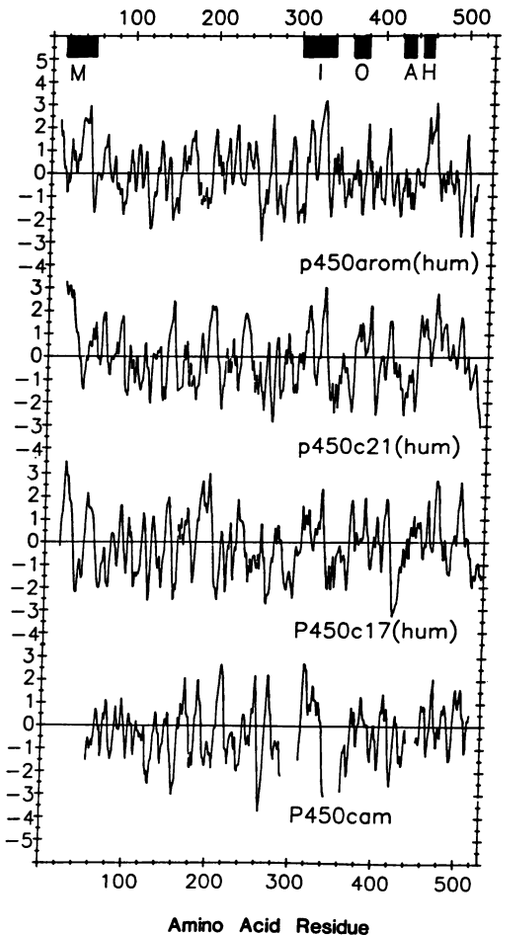

FIG. 2. Hydropathy profiles of human (hum) P-450$_{Arom}$, P-450$_{c21}$, P-450$_{17\alpha}$, as well as P-450$_{Cam}$. The hydropathy index is shown on the left, with negative numbers indicating hydrophilicity and positive numbers indicating hydrophobicity. The amino terminus of each protein is on the left. The procedure of Kyte and Doolittle (22) was used with a window of six residues. M, membrane-spanning region; I, I helix; O, Ozols region; A, aromatic region; and H, heme-binding region.

insert as hybridization probe. As shown in Fig. 3, a single hybridizing species of RNA of 2.1 kb was seen, consistent with the anticipated size of the expressed construct. To determine whether this RNA was translated into protein, whole cell protein extract was subjected to Western analysis, with use of the polyclonal anti-P-450 IgG (1). As shown in Fig. 3, the antibody reacted with a protein of 55 kDa, similar in size to purified human placental P-450$_{Arom}$, that was not present in nontransfected cells.

In cells from parallel dishes, aromatase activity was assayed by measuring the incorporation of tritium into [^{3}H]water from [1β-^{3}H]androstenedione, [1,2-^{3}H]testosterone, and [1β-^{3}H]16α-hydroxyandrostenedione. From the results shown in Table 1, an apparent K_m of 50 nM and a V_{max} of 102 pmol/hr per mg of protein were obtained using [1β-^{3}H]androstenedione as substrate, and an apparent K_m of 55 nM and a V_{max} of 148 pmol/hr per mg of protein were obtained using [1,2-^{3}H]testosterone as substrate. When [1β-^{3}H]16α-hydroxyandrostenedione was used as substrate, the values obtained for the K_m and V_{max} were 99 nM and 11 pmol/hr per mg, respectively. These results are similar to those reported for purified reconstituted P-450$_{Arom}$ (3, 31, ‡)

‡Yoshida, N. & Osawa, Y., Abstracts of the Eighth International Congress on Endocrinology, July 1988, Kyoto, Japan, p. 389.

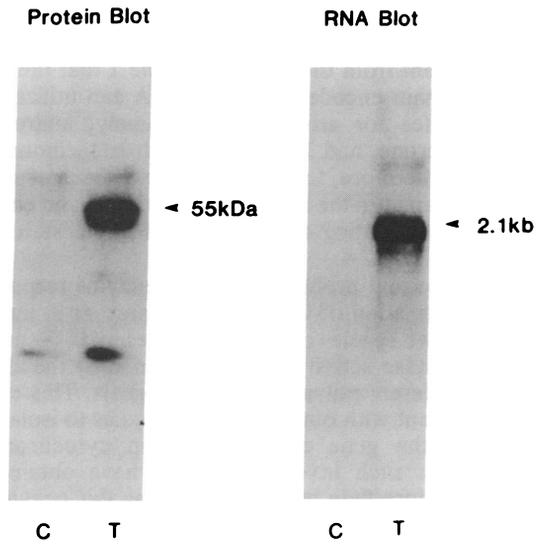

FIG. 3. Expression of human cytochrome P-450$_{Arom}$ in COS-1 cells. pCMV$_{Arom}$ was used to transfect COS-1 cells as described. (*Left*) Protein blot obtained using anti-human P-450$_{Arom}$ polyclonal IgG. (*Right*) RNA blot obtained using the 2.5-kb cDNA insert encoding P-450$_{Arom}$ as hybridization probe. Lanes: C, nontransfected cells; T, cells transfected with pCMV$_{Arom}$.

and indicate that the V_{max} for the 16α-hydroxylated substrate is less than the corresponding values for the other two substrates. The ability of two well-characterized aromatase inhibitors—namely, the "suicide" substrate 4-hydroxyandrostenedione and the imidazole antimycotic econazole, (32, 33) to inhibit the activity of expressed P-450$_{Arom}$ in COS-1 cells was examined, and the results are also presented in Table 1. Both of these were effective inhibitors of the expressed enzyme with [1β-^{3}H]androstenedione as substrate.

When [4-^{14}C]androstenedione was used as substrate and the products were analyzed by TLC, the principal product migrated with an R_f similar to that of estrone. Small amounts of material with polarity similar to estradiol and testosterone also were seen. When [4-^{14}C]testosterone was substrate, the principal products migrated with R_f values similar to those of estradiol and estrone. In addition, conversion to material with R_f similar to androstenedione was seen. Thus in these transfected cells the expected C_{18} steroid products are formed as a result of expressed aromatase activity. In addition, it is apparent that endogenous 17β-hydroxysteroid dehydrogenase is present in COS-1 cells.

Expression of the cDNA in COS-1 cells allows one to draw the following conclusions: (*i*) A single polypeptide chain clearly can catalyze the entire sequence of steps involved in

Table 1. Kinetic properties of P-450$_{Arom}$ expressed in transfected COS-1 cells

	K_m, nM	V_{max}*	IC_{50}, nM†
Substrate			
[1β-^{3}H]Androstenedione	50 ± 18‡	102 ± 59‡	
[1,2-^{3}H]Testosterone	55 ± 7‡	148 ± 58‡	
[1β-^{3}H]16α-hydroxyandrostenedione	99§	11	
Inhibitor			
4-Hydroxyandrostenedione			30
Econazole			40

*pmol/hr per mg of protein.
†Concentration of inhibitor required to cause 50% inhibition of aromatase activity with [1β-^{3}H]androstenedione (150 nM) as substrate.
‡Mean ± SEM (three determinations).
§Mean of two determinations.

the overall aromatase reaction, resulting in the formation of a phenolic A ring from the Δ^4-3-one structure of the substrate. (*ii*) It is apparent from the results in Table 1 that the single polypeptide chain encoded by this cDNA can utilize three major substrates for aromatization—namely, androstenedione, testosterone, and 16α-hydroxyandrostenedione. It is unnecessary, therefore, to postulate the existence of several forms of aromatase in the ovary, adipose tissue, and placenta responsible for forming the different phenolic steroids in these tissues.

We conclude that probably only one enzyme responsible for the aromatization of C_{19} steroids is present in humans. The fact that two species of mRNA are present in tissues that express aromatase activity can be explained by the alternative use of different polyadenylylation signals. This conclusion is consistent with our preliminary studies to isolate and characterize the gene encoding human cytochrome P-450_{Arom}. From such investigations we have obtained no evidence for more than one gene encoding this enzyme.[§]

[§]Means, G., Mathis, J. M. & Simpson, E. R., Proceedings of the 70th Meeting of the Endocrine Society, June 1988, New Orleans, p. 300 (abstr.).

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