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Structure–function studies of human aromatase by site-directed mutagenesis: Kinetic properties of mutants Pro-308 → Phe, Tyr-361 → Phe, Tyr-361 → Leu, and Phe-406 → Arg

(stable expression/cytochrome P-450)

DUJIN ZHOU*, DENIS POMPON†, AND SHIUAN CHEN*‡

*Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA 91010; and †Centre de Genetique Moleculaire du Centre National de la Recherche Scientifique, Laboratoire Propre Associe a l'Universite Pierre et Marie Curie, 91190 Gif-sur-Yvette, France

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ABSTRACT **Aromatase, a cytochrome P450, catalyzes the formation of aromatic C-18 estrogenic steroids from C-19 androgens. Four mutants of human aromatase have been expressed in Chinese hamster ovary cells using a stable expression method. The activities of these mutants were determined using [1β,2β-³H]androstenedione, [19-¹⁴C]androstenedione, and [1β,2β-³H]testosterone as substrates. The mutant Phe-406 → Arg was completely inactive. Since there were only small changes in the K_m and V_{max} values for all substrates for mutants Tyr-361 → Phe and Tyr-361 → Leu, the residue Tyr-361 appears not to be directly involved in the substrate binding. The mutant Pro-308 → Phe had altered catalytic properties; the K_m values for androstenedione, but not testosterone, decreased significantly. These results, along with those obtained from inhibition studies with aromatase inhibitors, 4-hydroxyandrostenedione and aminoglutethimide, suggest that Pro-308 is probably situated in the active site of the enzyme and may be interacting with the D ring of the steroids.**

Aromatase, a cytochrome P450, catalyzes the formation of aromatic C-18 estrogenic steroids from C-19 androgens. This enzyme has received considerable attention because of the central importance of estrogens in many reproductive and metabolic processes. The synthesis of estrogens is required for the normal expression of secondary sexual characteristics and establishment and maintenance of pregnancy. Fetal expression in the brain is believed to determine male or female metabolic patterns expressed during adult development (1). Moreover, the abnormal expression of aromatase in a significant number of breast tumors (2–5) and the inhibition of the enzyme as part of a therapeutic approach to this disease make the study of aromatase of paramount importance. This enzyme is also of interest to a number of investigators (e.g., refs. 6–8) because of the complexity of the reaction it catalyzes. Although it has been shown that three molecules of molecular oxygen and six reducing equivalents of NADPH are consumed during estrogen formation (9), the reaction mechanism is not yet completely understood. Site-directed mutagenesis may help to unravel the reaction mechanism of aromatase and provide structural information useful in the design of more effective aromatase inhibitor(s) for the treatment of breast cancer.

Although the human placental aromatase cDNA has been cloned and the complete amino acid sequence of the enzyme has been deduced from the nucleotide sequence (10–13), the structure–function relationship of the enzyme has yet to be established. A mammalian cell expression plasmid, pH β-Aro, containing the human placenta aromatase cDNA, was constructed recently, and this enzyme was expressed in Chinese hamster ovary (CHO) cells by a stable expression method using this plasmid (14). Site-directed mutagenesis experiments were performed using this expression system. Four mutants, P308F (Pro-308 → Phe), Y361F (Tyr-361 → Phe), Y361L (Tyr-361 → Leu), and F406R (Phe-406 → Arg), were prepared, and their kinetic properties were examined.

MATERIALS AND METHODS

Chemicals. T4 kinase, T4 DNA ligase, Klenow fragment, and restriction endonucleases were obtained from Boehringer Mannheim Biochemicals and Bethesda Research Laboratories. Radiolabeled nucleotides, radiolabeled androgens, and [³⁵S]methionine were from New England Nuclear. DNA sequencing kits were from United States Biochemical. The site-directed mutagenesis kits were from Bio-Rad.

Aromatase Expression in Mammalian Cells. Aromatase was expressed in CHO cells through transfection using the expression plasmid pH β-Aro (14). The transfection experiments were done using Lipofectin following the manufacturer's protocol (BRL). The cells were incubated at 37°C in 5% CO_2 in air for 24 hr in Ham's F12 medium, after which medium containing 10% fetal calf serum was added. After an additional 48 hr, the cells were transferred to selective medium (Ham's F12) containing G418 (600 μg/ml). After 2 weeks of selection, the cells were screened for aromatase expression by Southern blot analysis, Northern blot analysis, immunoprecipitation analysis, and enzyme assay.

Southern and Northern Blot Analyses. The genomic DNA was isolated from cultured cells according to Davis *et al.* (15), digested with restriction enzymes, electrophoresed in a 0.8% agarose gel, and then transferred to a Zeta-probe membrane using the method provided by Bio-Rad. The bound DNA was hybridized using the aromatase cDNA 2.4-kilobase (kb) fragment as the probe. The rest of the procedure for Southern blot analysis is identical to that described previously (16).

The Northern blot analysis was performed according to the procedure described by Pompon *et al.* (13). The probe was the aromatase cDNA 2.4-kb fragment.

Detection of Expressed Aromatase Protein by Immunoprecipitation Analysis. After 4 hr of incubation with culture medium containing [³⁵S]methionine (50 μCi/ml; specific activity, 1164.9 Ci/mmol; 1 Ci = 37 GBq), transfected CHO cells were lysed using a triple-detergent lysis buffer [50 mM Tris·HCl, pH 8.0/150 mM NaCl/0.02% sodium azide/0.1% sodium dodecyl sulfate (SDS)/100 μg of phenylmethylsulfonyl fluoride per ml/1 μg of aprotinin per ml/1% Nonidet P-40/0.5% sodium deoxycholate]. The cell lysates were

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Abbreviations: P308F, mutant Pro-308 → Phe; Y361F, mutant Tyr-361 → Phe; Y361L, mutant Tyr-361 → Leu; F406R, mutant Phe-406 → Arg; CHO, Chinese hamster ovary.
‡To whom reprint requests should be addressed.

centrifuged at 3000 × g to remove nuclei, followed by a preclearing step of treating the supernatant with normal rabbit serum and protein A-Sepharose. The treated supernatant was centrifuged to remove protein A-Sepharose, and a rabbit polyclonal antibody against human placental aromatase (kindly provided by Peter F. Hall, University of New South Wales, Australia) was added. The antibody-antigen (i.e., the expressed human aromatase protein) complexes were collected using protein A-Sepharose. SDS gel electrophoresis sample buffer was added to the precipitate to release the antigen from the complex. After centrifugation, the supernatant was analyzed by SDS/12% polyacrylamide gel electrophoresis according to the method of Laemmli (17). Detection of ^{35}S-labeled antigen was carried out by autoradiography.

"In-Cell" Aromatase Assay. The enzyme assay in transfected cultured cells was performed without purifying the enzyme, using a modification of a previously reported method (9, 18). Cells were grown to confluence on six-well cell culture plates and washed twice with serum-free cell culture medium. The substrate, [1β,2β-^{3}H(N)]androst-4-ene-3,17-dione (specific activity, 41.8 Ci/mmol; specific activity at the 1β position, 18 Ci/mmol), [19-^{14}C]androst-4-ene-3,17-dione (specific activity, 53.5 mCi/mmol), or [1β,2β-^{3}H(N)]testosterone (specific activity, 53.5 Ci/mmol; specific activity at the 1β position, 23 Ci/mmol), was dissolved in serum-free cell culture medium, filter-sterilized, and added to each well. Progestrone (1 μM) was included in the assay mixture to inhibit 5α-reductase present in the cells. This latter enzyme utilizes the same substrates as aromatase. After a 30-min incubation at 37°C, followed by a 5-min incubation on ice, 1 ml of culture medium was withdrawn from each well. The culture medium was mixed with an equal volume of chloroform to extract unused substrate. The aqueous phase was treated with dextran-treated charcoal and centrifuged, and the amount of the product, ^3H_2O or [^{14}C]formic acid, was determined using a scintillation counter. The protein concentration was determined using the method of Bradford (19) after dissolving cells with 0.5 M NaOH. The ^3H_2O release assay for human aromatase expressed in CHO cells has been previously validated by the product isolation assay (14).

Site-Directed Mutagenesis Experiments. The introduction of specific base changes in the aromatase cDNA was accomplished by site-directed mutagenesis using the phagemid mutagenesis kit from Bio-Rad. Four oligonucleotide primers were synthesized, and their sequences are shown in Fig. 1. These are reverse primers. In addition to changes at the specific bases, a silent mutation was introduced to destroy a unique restriction site in each primer. The procedure for mutagenesis is described briefly as follows. Appropriate aromatase cDNA fragments were generated through digestion of plasmid pH β-Aro using proper restriction enzymes. Bluescript phagemids containing these cDNA fragments were constructed and transferred into *dut ung Escherichia coli* strain CJ236 so that phagemids were synthesized with uracils in thymine positions. Positive transformants were

Primer for Y361F	5'- TGGTATCGCATGCTCTCAAAAATGA -3' (Kpn I)
Primer for Y361L	5'- TGGTATCGCATGCTCTCCAAAATGA -3' (Kpn I)
Primer for P309F	5'- ATGGTGTCAAAAGCAGCGAT -3' (Alu I)
Primer for F406R	5'- GGGGAAACGCTCAAGTCTGT -3' (Xho I)

FIG. 1. Nucleotide sequences of primers used to generate the aromatase mutants. The mutated residues are highlighted by using a different font. The impaired restriction sites are underlined and annotated.

cultured in the presence of helper phages. For each transformant, uracil-containing single-stranded DNA was isolated from the supernatant and annealed to the mutagenic primer. A second strand was synthesized with T4 DNA polymerase and ligated with T4 DNA ligase. The product was transferred into *E. coli* strain MC 1061 so that the uracil-containing strand was destroyed. The DNA was harvested and the mutant was identified by the disappearance of the unique restriction enzyme site. The mutated cDNA fragments were ligated back into the previously digested pH β-Aro plasmids. The transfection experiments were carried out with these mutated pH β-Aro plasmids. All mutated cDNA fragments were sequenced to confirm that there were no other mutations except those designed.

RESULTS AND DISCUSSION

Design and Constructions of Aromatase Mutant Expression Plasmids. A large quantity of the transfected cells was needed for kinetic analysis of the mutants using different substrates. A transient expression method would not have provided a sufficient quantity of transfected cells. Therefore, a stable expression system was used. A mammalian cell expression plasmid containing the human placenta aromatase cDNA, pH β-Aro, was constructed, and aromatase was successfully expressed in three types of mammalian cells through transfection experiments using this plasmid (14). Among these three cell types, transfected CHO cells expressed the highest level of aromatase (14). Because the K_m values for the expressed enzyme were very similar to that reported for the enzyme in human placenta, the expressed aromatase enzyme probably had the same conformation as the enzyme present in human placenta.

Because aromatase exhibits no more than 20–30% sequence homology with other cytochromes P450 (11, 13, 16), aromatase represents a unique cytochrome P450 gene family. Through sequence comparison with other cytochromes P450, several homologous regions have been identified and thought to be important for the substrate binding. Because the substrates and products of aromatase are steroids, aromatic amino acid residue(s) in the active site of the enzyme could be involved in steroid binding. A region, ^{353}L–^{365}R, has been suggested to be a part of the steroid substrate binding site (20). Therefore, the two mutants, Y361F and Y361L, were designed to test the importance of Tyr-361 for steroid binding. The mutant P308F was prepared to determine whether the binding affinity for the substrate would be changed by introducing an aromatic amino acid residue at position 308. This residue is in an α-helical region according to a secondary structure analysis using the method of Chou and Fasman (21). By sequence homology, this region should correspond to the I helix in cytochrome P450cam (22). The I helix region of cytochrome P450cam is located adjacent to the heme prosthetic group and is a part of the substrate binding site. Proline tends to break ordered helical structures, and Pro-308 might cause a slight bend in this proposed helix in aromatase. Changing Pro to Phe may induce a conformational change, affecting enzyme properties. For the last mutant, the Phe-406 residue in the "aromatic" region was changed to Arg. The aromatic region is a highly conserved region throughout many cytochromes P450, and it is thought to be involved in substrate binding. Mutants were generated as described in *Materials and Methods*. To facilitate the selection of mutants, a silent mutation that impaired an existing restriction site was introduced in each mutant oligonucleotide. Therefore, the mutant could be easily detected upon restriction of the plasmid DNA using the proper restriction enzyme.

Characterization of Aromatase Mutants. The presence of pH β-Aro plasmid DNA in transfected cells was demonstrated by Southern blot analysis, and the presence of a RNA

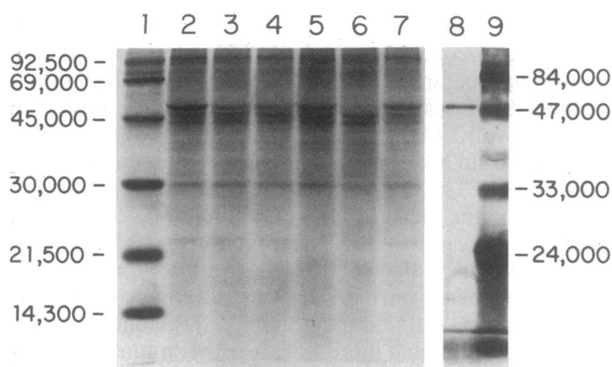
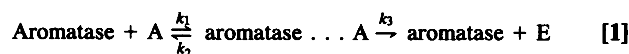


FIG. 2. Immunoprecipitation analysis of the cells expressing aromatase and its mutants. (*Left*) Autoradiogram. Lane 1, ^{14}C-labeled molecular weight standards; lane 2, cells expressing mutant Y361F; lane 3, cells expressing mutant Y361L; lane 4, cells expressing mutant P308F; lane 5, cells expressing mutant F406R; lane 6, cells transfected with the expression vector; and lane 7, cells expressing the wild-type enzyme. (*Right*) Silver-stained gel. Lane 8, aromatase purified from human placenta (5 μg); and lane 9, molecular weight standards.

transcript for human aromatase was determined by Northern blot analysis (results not shown). The expression of aromatase protein and its mutants in CHO cells was indicated by immunoprecipitation analysis. Fig. 2 shows that the aromatase antibody reacts with a protein whose molecular weight corresponds to that of aromatase in human placental microsomes. Aromatase protein was not detected in cells transfected with the expression vector only. The results obtained from immunoprecipitation analysis indicate that all four aromatase mutant proteins are synthesized and remain stable in CHO cells. Additional protein bands were found in all samples, including cells transfected with the expression vector only (see Fig. 2). Although the nature of these proteins is not known, they are thought to be proteins in CHO cells reacting with our antibody against human placental aromatase. It has been demonstrated, by Western blot analysis, that the antibody reacts exclusively with the aromatase protein in human placental microsomes (J. M. Esteban, Z. Warsi, M. Haniu, S.-H. Zhao, A. Baily, P. F. Hall, J. E. Shively & S.C., unpublished data).

Enzyme assays were carried out to demonstrate the expression of active aromatase protein. Enzyme kinetic analyses and inhibition studies with aromatase inhibitors were performed on the CHO cells expressing the wild-type enzyme and the four enzyme mutants. The simple in-cell assay method, without the need for purification of the enzyme or its mutants, was performed in this study. [$1\beta,2\beta$-^{3}H]Androstenedione, [19-^{14}C]androstenedione, and [$1\beta,2\beta$-^{3}H]testosterone were used as substrates in the kinetic analysis of the expressed proteins. All of the kinetic measurements were performed at least three times. One mole of ^3H_2O is formed when 1 mol of [$1\beta,2\beta$-^{3}H]androstenedione or [$1\beta,2\beta$-^{3}H]testosterone is converted to estrogenic products (9, 23). Because only the ^{3}H at the 1β position is involved in the formation of ^3H_2O (24), the activity of the enzyme was calculated based on the specific radioactivity at the 1β position of these substrates. Androstenedione and testosterone are two major substrates of aromatase. It was intended to determine the reactivities of the expressed wild-type enzyme and the four mutants toward these two substrates. One mole of [^{14}C]HCOOH is formed when 1 mol of [19-^{14}C]androstenedione is converted to estrone by aromatase (25). The results obtained using [19-^{14}C]androstenedione as substrate should be similar to those obtained using [$1\beta,2\beta$-^{3}H]androstenedione as substrate.

As shown in Table 1, the K_m and V_{max} values obtained with all of the substrates for the mutants Y361F and Y361L were slightly smaller than those for the wild-type enzyme. The K_m values are in the range determined for the enzyme existing in human placenta (26). Assuming the rate-limiting step of the enzyme reaction to be formation of the products (E, estrogens) rather than substrate (A, androgens) binding (see Eq. 1), the rate constant, k_3, would be significantly smaller than k_1 and k_2.



$$\text{Aromatase} + \text{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{aromatase} \ldots \text{A} \xrightarrow{k_3} \text{aromatase} + \text{E} \quad [1]$$

In this situation, the K_m value can be considered to be the dissociation constant for the substrate–enzyme complex, and the V_{max} value should correlate directly with k_3. Accordingly, the mutation at Tyr-361 has little effect on substrate binding, and the small changes in K_m and V_{max} values probably result from an indirect effect. The smaller V_{max} value for the mutant Y361L compared to the mutant Y361F may be due to a lower level of expression for mutant Y361L as shown by the immunoprecipitation analysis (Fig. 2). As a test of the specificity of the assay for aromatase activity, the effects of two known aromatase inhibitors, 4-hydroxyandrostenedione and

Table 1. Kinetic constants of human aromatase and its mutants

Enzyme	K_m, nM	n	% of WT K_m	V_{max}, pmol/mg per hr	% of WT V_{max}
[$1\beta,2\beta$-^{3}H(N)]Androstenedione					
WT	50.2 ± 4.2	4	100	144.0 ± 24.2	100
Y361F	44.0 ± 8.0	3	87.6	109.5 ± 11.5	76.0
Y361L	40.0 ± 8.0	3	79.7	97.3 ± 12.4	67.6
P308F	16.1 ± 1.6	3	25.9	13.1 ± 3.9	9.1
F406R				0	0
[$1\beta,2\beta$-^{3}H(N)]Testosterone					
WT	67.5 ± 8.3	3	100	116.2 ± 9.5	100
Y361F	58.0 ± 6.0	3	85.9	104.4 ± 4.6	89.8
Y361L	67.0 ± 8.0	3	99.3	80.0 ± 7.0	68.8
P308F	53.9 ± 4.8	3	79.9	8.6 ± 3.7	7.4
F406R				0	0
[19-^{14}C]Androstenedione					
WT	34.2 ± 4.5	4	100	83.9 ± 14.0	100
Y361F	25.6 ± 2.8	3	74.9	58.3 ± 4.5	69.5
Y361L	23.4 ± 2.4	3	68.4	48.5 ± 14.2	57.8
P308F	13.2 ± 4.4	3	38.6	9.6 ± 2.7	11.4
F406R				0	0

WT, wild type; n, number of measurements.

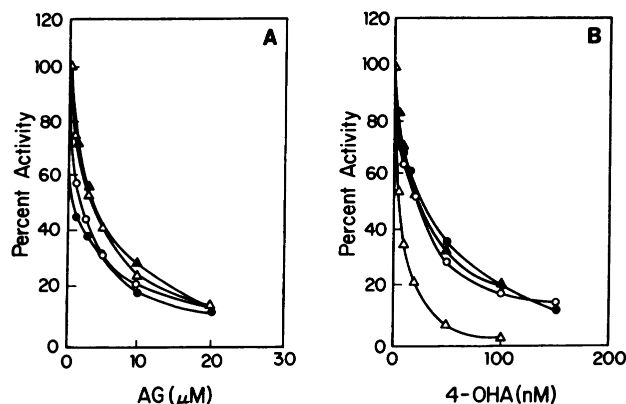


FIG. 3. Inhibition of the expressed aromatase and its mutants by D-(+)-aminoglutethimide (AG) (*A*) and 4-hydroxyandrostenedione (4-OHA) (*B*). The concentration of the substrate, [1β,2β-^{3}H]androstenedione, was 100 nM. ○, Y361F; ●, Y361L; △, P308F; ▲, wild type.

aminoglutethimide, were determined using [1β,2β-^{3}H]androstenedione as the substrate. Inhibition profiles for the mutants Y361F and Y361L by the two inhibitors were very similar to that of the wild-type enzyme (Fig. 3*B*), especially in the case of inhibition by 4-hydroxyandrostenedione, a substrate analogue. These results are consistent with the conclusion that these mutations did not significantly alter the binding affinities of the substrates or their analogues.

The mutant F406R was completely inactive. This indicates either that the residue Phe-406 is essential for the enzyme activity or that the mutation causes vital conformational changes, resulting in loss of enzyme activity.

The fourth mutant, P308F, has unusual enzyme properties. The mutation caused a major reduction in V_{max} values for all substrates tested. This suggests that residue Pro-308 may be near the active site of the enzyme and may interact with steroids directly. This is not unexpected, because Pro-308 is in a proposed helical region positioned next to the heme prosthetic group. However, it was surprising to find that although the K_m values for androstenedione substrates (both [1β,2β-^{3}H]androstenedione and [19-^{14}C]androstenedione) decreased significantly, the K_m value for testosterone was reduced only slightly. This suggests that the binding affinity for androstenedione was increased, whereas that for testosterone was affected to a lesser extent (see Table 1). Androstenedione and testosterone differ in that androstenedione has a keto group whereas testosterone has a hydroxyl group at the C-17 position. These results suggest that Pro-308 may be situated in the site interacting with the D ring of the steroids. On the other hand, it is possible that the introduction of a phenylalanine residue at position 308 may change the conformation of the proposed helical region and that the presence of this aromatic amino acid residue enhances or stabilizes the androstenedione binding. Our kinetic analyses indicate that androstenedione may bind a bit more strongly to wild-type aromatase than testosterone [i.e., the K_m values for two androstenedione substrates are slightly lower than that for testosterone (see Table 1)].

Further evidence that the mutant P308F has a higher binding affinity for androstenedione was obtained through inhibition studies. With [1β,2β-^{3}H]androstenedione (100 nM) as substrate, 4-hydroxyandrostenedione inhibited mutant P308F with an IC_{50} value of 3 nM and the wild-type enzyme and the two other mutants with IC_{50} values of ≈22 nM (Fig. 3). The IC_{50} values for 4-hydroxyandrostenedione were much smaller when enzyme assays were performed using [1β,2β-^{3}H]testosterone as the substrate (results not shown). These latter results are consistent with a weaker binding of testosterone to aromatase than androstenedione (as discussed above). Since aminoglutethimide is not a substrate analogue, it is not surprising that the inhibition profile for mutant P308F is not much different from those for other mutants and the wild-type enzyme. The binding of aminoglutethimide to aromatase elicits a type II cytochrome P450 spectrum, suggesting that this compound binds the heme prosthetic group of the enzyme and binds differently from substrate (27). Studies using aromatase inhibitors are important in that they confirm conclusions obtained from the kinetic analyses. In addition, the inhibition analyses are not functions of enzyme concentrations. The results would not be influenced by differences in the level of the enzyme or its mutants in the expressed cells because the inhibitor concentrations are in excess of the enzyme concentrations.

In conclusion, four mutants of aromatase have been prepared using a stable expression system. The enzyme properties of mutants Y361F and Y361L are similar to those of the wild-type enzyme. The mutant F406R is completely inactive. The mutant P308F has a higher affinity for androstenedione substrates but not for testosterone by comparison to the wild-type enzyme and other mutants. This present study provides a strong basis for further structure–function studies of aromatase by site-directed mutagenesis experiments.

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1. MacLusky, N. J., Clark, A. S., Naftolin, F. & Goldman-Rakic, P. S. (1987) *Steroids* **50**, 459–474.
2. Killinger, D. W., Perel, E., Daiilescu, D., Kharlip, L. & Blackstein, M. E. (1987) *Steroids* **50**, 523–535.
3. Miller, W. R. & O'Neill, J. (1987) *Steroids* **50**, 537–548.
4. Silva, M. C., Rowlands, M. G., Dowsett, M., Gusterson, B., McKinna, J. A., Fryatt, I. & Coombes, R. C. (1989) *Cancer Res.* **49**, 2588–2591.
5. Reed, M. J., Owen, A. M., Lai, L. C., Coldham, N. G., Ghilchik, M. W., Shaikh, N. A. & James, V. H. T. (1989) *Int. J. Cancer* **44**, 233–237.
6. Townsley, J. D. & Brodie, H. J. (1968) *Biochemistry* **7**, 33–40.
7. Akhtar, M., Calder, M. R., Corina, D. L. & Wright, J. N. (1981) *J. Chem. Soc., Chem. Comm.* 129–130.
8. Cole, P. A. & Robinson, C. H. (1988) *J. Amer. Chem. Soc.* **110**, 1284–1285.
9. Thompson, E. A., Jr., & Siiteri, P. K. (1974) *J. Biol. Chem.* **249**, 5364–5372.
10. Harada, N. (1988) *Biochem. Biophys. Res. Commun.* **156**, 725–732.
11. Crobin, C. J., Graham-Lorence, S., McPhaul, M., Mason, J. I., Mendelson, C. R. & Simpson, E. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8948–8952.
12. Toda, K., Terashima, M., Mitsuuchi, Y., Yamasaki, Y., Yokoyama, Y., Nojima, S., Ushiro, H., Maeda, T., Yamamoto, Y., Sagara, Y. & Shizuta, Y. (1989) *FEBS Lett.* **247**, 371–376.
13. Pompon, D., Liu, R. Y.-K., Besman, M. J., Wang, P.-L., Shively, J. E. & Chen, S. (1989) *Mol. Endocrinol.* **3**, 1477–1487.
14. Zhou, D., Pompon, D. & Chen, S. (1990) *Cancer Res.* **50**, 6949–6954.
15. Davis, L. G., Dibner, M. D. & Battey, J. F. (1986) in *Basic Methods in Molecular Biology* (Elsevier, New York), pp. 44–46.
16. Chen, S., Besman, M. J., Sparkes, R. S., Zollman, S., Klisak, I., Mohandas, T., Hall, P. F. & Shively, J. E. (1988) *DNA* **7**, 27–38.
17. Laemmli, V. K. (1970) *Nature (London)* **227**, 680–685.
18. Ackerman, G. E., Smith, M. E., Mendelson, C. R., MacDonald, P. C. & Simpson, E. R. (1981) *J. Clin. Endocrinol. Metab.* **53**, 412–417.
19. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.

20. Picardo-Leonard, J. & Miller, W. L. (1988) *Mol. Endocrinol.* **2,** 1145–1150.
21. Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol.* **47,** 45–148.
22. Poulos, T. L., Finzel, B. C. & Howard, A. J. (1987) *J. Mol. Biol.* **195,** 687–700.
23. Osawa, Y., Higashiyama, T., Fronckowiak, M., Yoshida, N. & Yarborough, C. (1987) *J. Steroid Biochem.* **27,** 781–789.
24. Osawa, Y., Fronckowiak, M., Yoshida, N. & Higashiyama, T. (1987) *Steroids* **50,** 649–650.
25. Covey, D. F., McMullan, P. C., Wixler, L. L. & Cabell, M. (1988) *Biochem. Biophys. Res. Commun.* **157,** 81–86.
26. Kellis, T. T., Jr., & Vichery, L. E. (1987) *J. Biol. Chem.* **262,** 4413–4420.
27. Salhanick, H. A. (1982) *Cancer Res.* **42,** Suppl., 3315s–3321s.