THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 15, Issue of May 25, pp. 8893-8900, 1990
Printed in U.S.A.

A Single Base Mutation in the Androgen Receptor Gene Causes Androgen Insensitivity in the Testicular Feminized Rat*

(Received for publication, December 12, 1989)

Wendell G. Yarbrough‡§¶, Valerie E. Quarmby‡§‖, Jorge A. Simental‡§, David R. Joseph‡§, Madhabananda Sar‡ ‡‡, Dennis B. Lubahn‡§ §§, Kathie L. Olsen¶¶, Frank S. French‡§, and Elizabeth M. Wilson‡§‖‖**

*From the ‡Laboratories for Reproductive Biology and the Departments of §Pediatrics, **Physiology, ‡‡Cell Biology and Anatomy, and §§Pathology, University of North Carolina, Chapel Hill, North Carolina 27599 and the ¶¶National Science Foundation, National Institutes of Health, Washington, D. C. 20550*

The complete form of androgen insensitivity is an inherited X-linked syndrome in which genetic males fail to undergo masculinization *in utero* due to defective functioning of the androgen receptor (AR). The molecular basis of androgen insensitivity was investigated in the testicular feminized (*Tfm*) rat with this syndrome. AR mRNA size and amount, as well as nuclear AR protein revealed by immunocytochemistry, suggested normal expression of the AR gene in the *Tfm* rat. Sequence analysis of the AR coding region from *Tfm* and wild-type littermate male rats revealed a single transition mutation, guanine to adenine, within exon E, changing arginine 734 to glutamine within the steroid-binding domain of the AR. This arginine is highly conserved among the family of nuclear receptors and may be part of a phosphorylation recognition site. A recreated mutant AR ($Arg^{734} \rightarrow Gln$) expressed in COS cells had only 10–15% of the androgen-binding capacity of wild-type AR; the reduced androgen-binding capacity was similar to that of AR in tissue extracts of the *Tfm* rat. Stimulation of transcriptional activity by the recreated mutant AR was reduced relative to wild-type AR in cotransfection assays in CV1 cells using as reporter plasmid the mouse mammary tumor virus promoter linked to the chloramphenicol acetyltransferase gene. Thus, arginine 734 appears essential for normal AR function both in androgen binding and transcriptional activation. Absence of these functions results in androgen insensitivity and lack of male sexual development.

The X-linked syndrome of androgen insensitivity (known also as testicular feminization (*Tfm*)[1]) results from a failure of tissue response to androgen. In the human, phenotypic expression ranges from partial forms, in which 46,XY phenotypic males are infertile, to the complete syndrome, in which the phenotype is female (1). Androgen insensitivity in a strain of King/Holtzman rats was discovered by Stanley and Gumbreck and propagated as a substrain (2, 3). Affected genetic male rats retain the female phenotype although abdominal testes produce normal to elevated male levels of testosterone, and there is a normal rate of conversion of testosterone to dihydrotestosterone (1). In affected genetic males, Müllerian ducts are absent because of the effect of testicular Müllerian-inhibiting substance, but androgen-dependent male accessory sex glands fail to develop.

Cloning of human (4–9) and rat (10) androgen receptor (AR) complementary DNA (cDNA) has facilitated the study of molecular defects associated with androgen insensitivity. A partial deletion (11) and more recently a single base mutation (12) in the human AR steroid-binding domain have been described in families with the complete syndrome. Naturally occurring mutations that result in obvious physiological disturbances offer the opportunity to discover important structure/function relationships in the receptor. In the present report, the molecular defect in the androgen-insensitive *Tfm* rat was investigated at the level of the AR gene and messenger RNA (mRNA) expression, subcellular localization of the AR protein, and androgen binding and functional properties of AR recreated by site-directed mutagenesis. It is demonstrated that a single base alteration in the AR gene leads to replacement of an arginine that is highly conserved within the family of nuclear receptors. Androgen binding and functional activities of the recreated mutant AR are similar to the activities of the *Tfm* rat AR.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were obtained: T4 DNA polymerase and Muta-Gene M13 *in vitro* mutagenesis kit from Bio-Rad; *Taq* polymerase (3.75 units/reaction) from Perkin-Elmer Cetus; nylon membranes (Biodyne, 0.2-μm pore size) from ICN, Irvine, CA; Immobilon transfer membrane from Millipore; ^{125}I-protein A and [^{3}H] methyltrienolone (17α-[*methyl*-^{3}H]R1881), 80 Ci/mmol) from Du Pont-New England Nuclear; Amplify, 1,2,4,5,6,7-5α-[^{3}H]dihydrotestosterone (120 Ci/mmol), ^{32}P-dCTP, and ^{14}C-labeled protein molecular weight standards from Amersham Corp.; T4 polynucleotide kinase, T4 DNA ligase, and M13 bacteriophage from New England

* This work was supported by Grants HD16910, HD04466, and P30-HD18968 (recombinant DNA and histochemistry cores) from the National Institute of Child Health and Human Development Center for Population Research; National Institutes of Health Grant NS17479; the Andrew W. Mellon Foundation; and the Pew Scholars Program in the Biomedical Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05454.

¶ Present address: Dept. of Surgery, University of North Carolina, Chapel Hill, NC 27599.

‖ Present address: Endocrine Sciences, Tarzana, CA 91356.

‖‖ To whom correspondence should be sent: Laboratories for Reproductive Biology, CB 7500 MacNider Bldg., University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-5159; Fax: 919-966-2423.

[1] The abbreviations used are: *Tfm*, testicular feminization; AR, androgen receptor; MMTV-CAT, mouse mammary tumor virus promotor linked to the chloramphenicol acetyltransferase gene; R1881, methyltrienolone; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; AA, amino acid(s).

BioLabs; avian myeloblastosis virus reverse transcriptase from Life Sciences; oligo(dT) from Collaborative Research; isopropyl β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside and most restriction enzymes from Bethesda Research Laboratories; glyoxal from Fluka Chemical Corp.; W-Omat-AR diagnostic x-ray film from Kodak; buffers and chemicals from Fisher or Sigma.

Vectors and Cell Lines—The wild-type AR expression vector was prepared by cloning the full AR coding sequence into the *Eco*RI site of pCMV1. pCMV1 is a eukaryotic expression vector that contains the human cytomegalovirus major immediate early gene promoter-regulatory region, SV40 origin of replication, and the human growth hormone transcription and polyadenylation signals as described previously (13). The pCMV vector was kindly provided by Dr. David W. Russell, Dept. of Molecular Genetics, University of Texas Health Science Center at Dallas. The *Tfm* rat AR construct was prepared by site-directed mutagenesis as described below. The mouse mammary tumor virus-chloramphenicol acetyltransferase reporter plasmid (MMTV-CAT) was kindly provided by Dr. Keith R. Yamamoto, Dept. of Biochemistry and Biophysics, University of California, San Francisco, and has been described (14).

COS-7 cells are African green monkey kidney cells derived from a clone of SV40-transformed CV1 cells (15). CV1 cells were obtained from the American Type Culture Collection.

Animals—King/Holtzman *Tfm* rats were provided by Dr. Kathie L. Olsen, who maintains a breeding colony derived from the originally described *Tfm* Stanley/Gumbreck rat (2). The genetic male *Tfm* rat lacks Müllerian duct derivatives, epididymides, and male accessory sex glands. Small abdominal testes produce elevated levels of testosterone (16). For androgen-binding studies, wild-type and *Tfm* rats were castrated and tissues removed as described previously (17). For RNA isolation, intact rats were killed by decapitation and the tissue removed and immediately frozen in liquid N_2. Care of the animals was in accordance with institutional guidelines.

DNA Amplification and Sequencing—Oligonucleotide primers (24–33 nucleotide residues in length) homologous to rat AR were synthesized by Dr. Dana Fowlkes, Dept. of Pathology, University of North Carolina at Chapel Hill, using the phosphoramidite method and an Applied Biosystems model 380A DNA synthesizer. The polymerase chain reaction (PCR) in 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 10 mM Tris, pH 8.3 (18), was carried out with a Perkin-Elmer Cetus thermal cycler using *Taq* polymerase (3.75 units/reaction). Primer and deoxynucleotide triphosphate concentrations were 0.2 mM. The first coding exon and a portion of the 5′-noncoding region (1561 bases in rat AR, intron-exon splice position based on human gene sequence; Refs. 5 and 12) were amplified as four overlapping fragments from 1 μg of *Tfm* and wild-type male sibling genomic DNA. In four separate reactions, four pairs of oligonucleotides were used as primers and 30 cycles of PCR performed with an extension time of 6 min + 3 s/cycle at either 67 or 69 °C followed by denaturation for 30 s at 95 °C. The remaining portion of the AR coding region and part of the 3′-noncoding region (1274 bases) were amplified from cDNA reverse transcribed (avian myeloblastosis virus reverse transcriptase) from 10 μg of *Tfm* and wild-type testis poly(A) RNA using oligo(dT) as primer (19). Alkaline hydrolysis of the RNA was carried out in 0.17 M NaOH for 20 min at 65 °C after the addition of EDTA to a concentration of 20 mM. The solution was neutralized with Tris, pH 7.5, ethanol precipitated, and resuspended in a solution of 0.2 mM EDTA, 10 mM Tris, pH 8.0. Forty cycles of PCR (denatured at 95 °C for 2 min, annealed at 55 °C for 2 min, and extended at 72 °C for 3 min 10 s + 3 s/cycle) with buffers and reagents as described above were performed on 0.5 μg of single-stranded cDNA to produce a 1.2-kilobase fragment that encompassed both the DNA and ligand-binding domains. The 1.2-kilobase fragment was electroeluted after agarose gel electrophoresis and, in separate reactions, 100 ng subjected to 30 cycles of the PCR (denatured at 95 °C for 30 s, annealed at 55 °C for 1 min, extended at 72 °C for 6 min + 3 s/cycle) to amplify the DNA and ligand binding domains. The amplified fragments from wild-type and *Tfm* rats were blunt end ligated into M13 bacteriophage, and at least two independent clones were sequenced using the dideoxy method. In areas in which the sequence diverged from that of the known Sprague-Dawley or King/Holtzman wild-type sequence, both strands were sequenced.

Site-directed Mutagenesis—A full-length cDNA clone was mutated using the oligonucleotide-directed single base mutagenesis method described by Kunkel (20). A full-length AR cDNA was cloned into M13 bacteriophage and grown in dut⁻ung⁻ *Escherichia coli* strain CJ236, and single-stranded phage DNA was isolated. An oligonucleotide 26 bases in length (GCCATGGGTTGGCAGTCCTTCACTAA) containing the single base mutation of the *Tfm* rat AR (underlined) was phosphorylated and annealed to the single-stranded dUTP-containing AR clone in M13 as described in the Bio-Rad Muta-Gene M13 *in vitro* mutagenesis kit. The complementary strand was synthesized *in vitro* using T4 DNA polymerase and ligated using T4 DNA ligase. Double-stranded circular M13 DNA was transfected into competent DH5 *E. coli*, and the replicative plaque-forming bacteriophage M13 clones containing AR were isolated after selection using isopropyl β-D-thio-galactoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside. The mutation was confirmed by direct sequencing of double-stranded DNA before cloning into the expression vector pCMV5 (13).

Northern Blot Analysis—Poly(A) RNA was isolated from wild-type and *Tfm* rat kidney (21, 22), denatured with dimethyl sulfoxide and glyoxal for 1 h at 50 °C, and fractionated on 1% agarose gels in 10 mM sodium phosphate, pH 6.8 (23). RNA was transferred to nylon membranes (0.2-μm pore size), cross-linked by exposure to UV light for 30 s, and vacuum baked for 1 h at 80 °C. The 5′ *Eco*RI fragment of rat AR cDNA clone rARep1 (10) was labeled with ^{32}P-dCTP by nick translation (24). Filters were hybridized as described previously (24, 25) at 42 °C in the presence of 50% formamide and 0.1% SDS. Filters were washed at high stringency (0.1 × SSC; 0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0, containing 0.1% SDS at 60 °C), blotted dry, exposed to Kodak XAR x-ray film for 1–4 days at −70 °C, and processed according to manufacturer's recommendations.

Transient Transfection Assays—For androgen binding studies, COS-7 cells at 50–75% of confluence were transfected with 1 μg of DNA/6-cm dish using the DEAE-dextran method (26). Cells were cleared of serum 20 h after transfection and 18 h later were incubated with increasing concentrations of [^{3}H]methyltrienolone (R1881) in duplicate dishes in the presence and absence of 100-fold excess unlabeled R1881 for 2 h at 37 °C. [^{3}H]R1881 was used rather than [^{3}H]dihydrotestosterone to minimize steroid metabolism during the incubation. Cells were washed twice in cold phosphate-buffered saline and harvested in 2% SDS, 10% glycerol, and 10 mM Tris, pH 6.8. Aliquots were taken for determination of radioactivity and for Western blot analysis using an antibody specific for AR (10). Specific binding was estimated from the difference in radioactivity determined in the presence and absence of unlabeled hormone.

For photoaffinity labeling studies, about 1×10^6 COS-7 cells (60% confluence) in 10-cm dishes were treated with 5 μg of vector DNA using the DEAE-dextran method (26). Twenty-four h after transfection, the medium was changed to serum-free, phenol red-free Dulbecco's modified Eagle's medium. Eighteen h later, the medium was removed and the cells washed and labeled for 2 h at 37 °C with 12 nM R1881 with or without a 100-fold excess unlabeled R1881 to assess nonspecific binding. Cells were washed in phosphate-buffered saline and harvested in SDS sample buffer. For the fluorograph, half of the gel was fixed for 30 min in isopropyl alcohol/H_2O/acetic acid (25:65:10) and immersed in Amplify for 1 h. The antibody IgG (10) concentration was 11 μg/ml, and 4 μg peptide/ml was used in preadsorption studies. ^{14}C-Labeled molecular weight markers were myosin (200,000), phosphorylase *b* (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

For measurement of AR functional activity, CV1 cells were cotransfected with pCMV expression vector containing either wild-type or mutant AR full coding sequence cloned in the *Eco*RI site and an MMTV-CAT reporter plasmid described previously (14). CV1 cells (1.5×10^6 cells/10-cm dish) in 5% fetal calf serum and Dulbecco's modified Eagle's medium were plated to yield approximately 80% of confluence for use in transfection 18 h later. Each plasmid (5 μg) was transfected using the calcium phosphate transfection procedure as described previously allowing the DNA to incubate with the cells for 4 h at 37 °C (27). Cells were washed twice with Tris-buffered saline (28) and maintained in 0.2% fetal calf serum in Dulbecco's modified Eagle's medium with or without the addition of R1881 as indicated. Forty-eight h after transfection, cells were harvested in phosphate-buffered saline and analyzed for chloramphenicol acetyltransferase activity as described previously (29).

Gel Electrophoresis and Immunoblot Analysis—Cell lysates were prepared in SDS sample buffer (30) with 3% 2-mercaptoethanol and boiled for 5 min prior to electrophoresis. Polyacrylamide separating gels were 6% acrylamide with a 4% stacking gel as described previously (30). Gels were electrophoresed for 700 volt h and transferred to Immobilon (0.45 μm) by electroblotting at 4 °C for 480 volt h in 20% methanol, 0.19 M glycine, and 24 mM Tris, pH 8.3 (31). Filters were air dried and incubated with blocking buffer (10% Carnation

nonfat dry milk, 0.05% Tween 20, 0.9% NaCl, 10 mM Tris, pH 7.5) for 4–6 h with shaking before addition of the IgG AR antibody (AR1–52, 2.8 mg/ml) at a 1:250 dilution (11 μg/ml in 5 ml) in blocking buffer. For preadsorption with immunogen, 10 μg of peptide (10) was incubated with 20 μl of undiluted IgG for 4–6 h on ice followed by dilution in blocking buffer as above. Filters were incubated with antibody overnight at 4 °C with shaking, washed three times for 5 min in blocking buffer at room temperature, and incubated with 5 μCi of ^{125}I-protein A in 5 ml of blocking buffer for 90 min at room temperature. The filters were washed two times for 5 min in blocking buffer, five times for 5 min in blocking buffer without Carnation milk, and air dried and exposed to x-ray film. The IgG fractions of immune and preimmune sera were obtained by chromatography on protein A-agarose as described previously (10). For fluorography, the gel was fixed in isopropyl alcohol/H_2O/acetic acid (25:65:10) for 30 min at room temperature and immersed in Amplify for 1.5 h at room temperature. The gel was dried and exposed to x-ray film at −70 °C for 110 days.

RESULTS

AR Gene Structure—The possibility of a major AR gene deletion associated with androgen insensitivity in the *Tfm* rat was investigated by Southern blot analysis. Genomic DNA isolated from wild-type and *Tfm* rat liver was digested with each of a group of restriction enzymes. The AR gene fragments obtained with each enzyme (three shown in Fig. 1) were identical electrophoretically using DNA from androgen-insensitive *Tfm* and normal male littermate rats. Thus, there was no indication of a major deletion or rearrangement of the AR gene in the *Tfm* rat.

Northern blot analysis of poly(A) RNA revealed a 10-kilobase AR mRNA in the *Tfm* rat which was indistinguishable in size and intensity from wild type (shown in Fig. 2 for kidney poly(A) RNA). Smaller size bands were attributed to nonspecific interaction of the cDNA probe with ribosomal RNA; the ribosomal RNA signal intensity and that observed with an actin cDNA probe (not shown) indicated nearly equivalent loading of RNA in the two lanes. The characteristic 10-kilobase AR mRNA was also observed on Northern blot hybridization of RNA extracts from *Tfm* rat liver, brain, submandibular gland, testis, and skeletal muscle (results not shown). Thus, AR mRNA abundance and size were consistent with normal AR gene transcription and RNA processing in the *Tfm* rat.

AR Androgen Binding Activity—AR binding of [^{3}H]dihydrotestosterone was measured in kidney cytosol fractions of *Tfm* and wild-type rats using a charcoal adsorption assay (32).

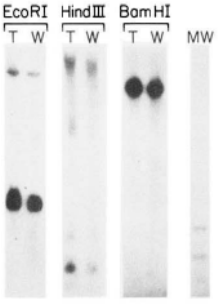

FIG. 1. **Southern blot analysis of genomic DNA from *Tfm* and wild-type littermates.** DNA was isolated from liver of *Tfm* (*T*) rats and wild-type (*W*) siblings. Aliquots of DNA (10 μg) were cleaved with *Eco*RI, *Hin*dIII, or *Bam*HI and analyzed on Southern blots as described under "Experimental Procedures" using as probe a ^{32}P-labeled rat AR 5′ *Eco*RI fragment of the cDNA clone rARep1 (10). Additional enzymes used to digest genomic DNA which revealed no alteration in fragment sizes between *Tfm* and wild-type included *Sac*I, *Kpn*I, *Hae*III, *Pst*I, and *Sma*I (not shown). Molecular weight markers (*MW*) were ^{32}P-labeled *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 DNA.

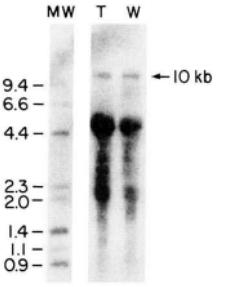

FIG. 2. **Northern blot analysis of kidney RNA from *Tfm* rat and wild-type male siblings.** Poly(A) RNA was isolated from kidney of *Tfm* and wild-type sibling rats, and 10-μg aliquots were denatured and analyzed by Northern blot hybridization as described under "Experimental Procedures." Shown are Northern blots of poly(A) RNA from *Tfm* (*T*) and wild-type littermates (*W*). Not shown is actin cDNA hybridization, which indicated nearly equivalent loading of RNA in the two *lanes*. Molecular weight markers (*MW*) were ^{32}P-labeled *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 DNA. *kb*, kilobases.

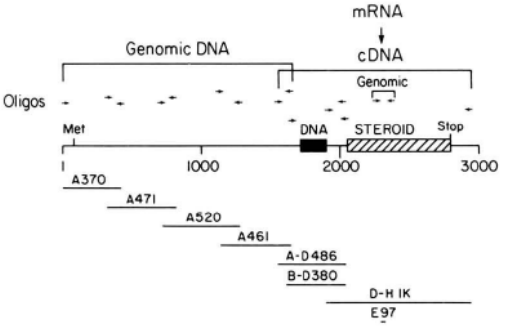

FIG. 3. **Strategy for the polymerase chain reaction and DNA sequence analysis of AR DNA.** The 3000-base pair AR cDNA is shown schematically to include the predicted initiation methionine (*Met*), central DNA binding domain (*black box*), carboxyl-terminal steroid binding region (*hatched box*), and the termination signal (*Stop*). Wild-type and *Tfm* rat AR DNAs were amplified using PCR. The first exon was amplified from genomic DNA using four sets of oligonucleotide primers (*small arrows*) yielding amplified fragments A370, A471, A520, and A461, where the *letter* designates the exon, and the *number* is the length of the fragment in base pairs. The DNA and steroid binding domains and surrounding regions were amplified from cDNA that was reverse transcribed from testis poly(A) RNA using three sets of oligonucleotides as indicated, yielding amplified fragments A-D486, B-D380, and D-H1K. The DNA sequence in the region of the *Tfm* rat AR single base mutation was confirmed by amplification of genomic DNA and direct sequencing (fragment E97). At least two independent isolates for each fragment were sequenced.

Specific binding was detectable (1–3 fmol/mg of protein), but was only 10–15% of that observed in wild-type siblings and too low to determine apparent binding affinity. Thus, the *Tfm* rat AR shows a major reduction in androgen binding.

Identification of a Single Base Mutation—To identify a possible single base mutation or small gene deletion, synthetic oligonucleotides homologous to rat AR cDNA (10) were used in the polymerase chain reaction to prime the amplification of wild-type and *Tfm* rat AR DNA and generate overlapping fragments within the coding region. Genomic DNA was used as template to amplify four fragments from exon A (Fig. 3); the remaining coding sequence was amplified from single-stranded cDNA obtained by reverse transcription of poly(A) RNA. All fragments were cloned into M13 bacteriophage and sequenced by the dideoxy chain termination method (33).

Since the *Tfm* rat derives from the King/Holtzman strain, it was necessary to identify first any polymorphic differences from the known AR cDNA sequence of the Sprague-Dawley

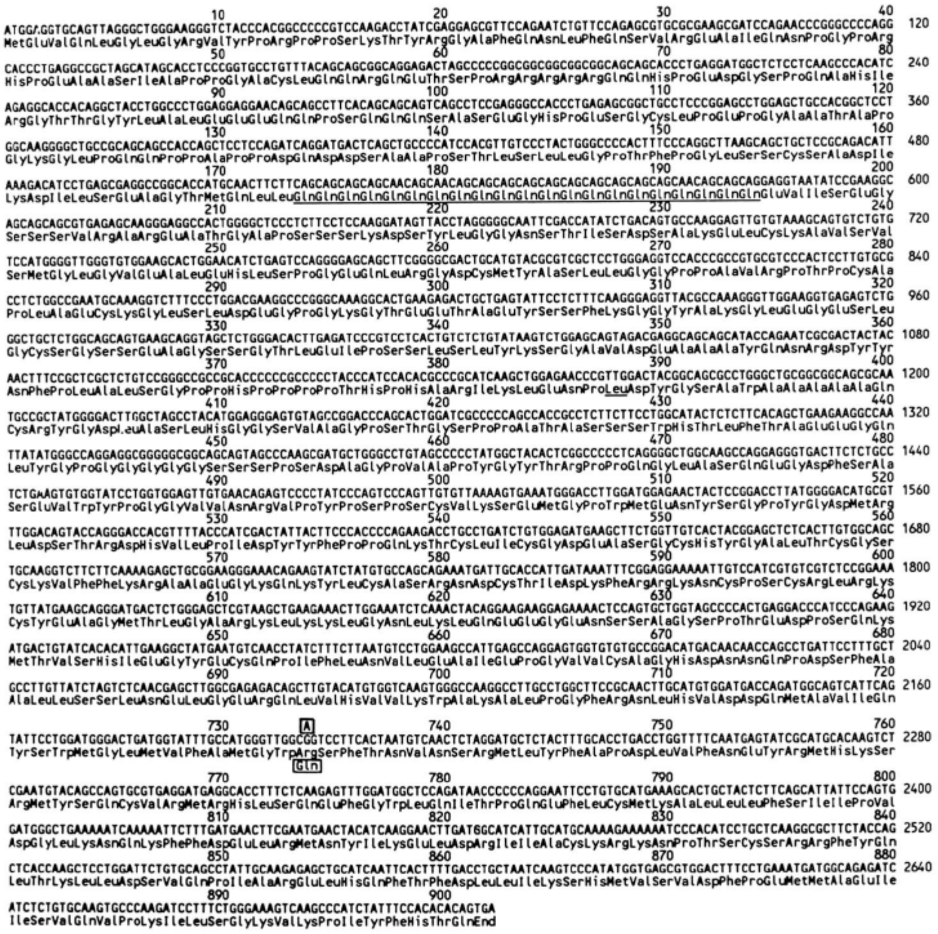

FIG. 4. **Full-length coding DNA sequence of wild-type King/Holtzman and *Tfm* rat AR.** DNA sequence was determined from amplified DNA fragments isolated by electroelution from 1.4% (w/v) agarose gels. The DNA fragments were phosphorylated (T4 polynucleotide kinase) and blunt end ligated (T4 DNA ligase) into M13mp18 DNA cleaved with *Sma*I. Competent DH5 *E. coli* were transformed with the M13mp18 DNA, combined with 50 μl of DH5 *E. coli* cells, and grown overnight at 37 °C in media containing 0.83 mM isopropyl β-D-thio-galactoside and 0.02% 5-bromo-4-chloro-3-indolyl β-D-galactoside. DNA was isolated from 10 randomly selected clear viral plaques for each fragment. At least two independent isolates for each fragment were sequenced using the dideoxy chain termination method and ^{35}S-labeled dCTP (28). Shown is the full coding sequence of the King/Holtzman wild-type rat AR and the single nucleotide difference in the *Tfm* rat AR sequence. The glutamine repeat that differs in length by 1 residue and a polymorphic leucine residue that differs from serine in the Sprague-Dawley wild-type rat AR sequence are *underlined*. Enclosed in a *box* is the single base and amino acid difference between King/Holtzman wild-type and *Tfm* rat AR sequence. Amino acid residues are *numbered* above and nucleotides on the *right*, both starting at the predicted initiating methionine.

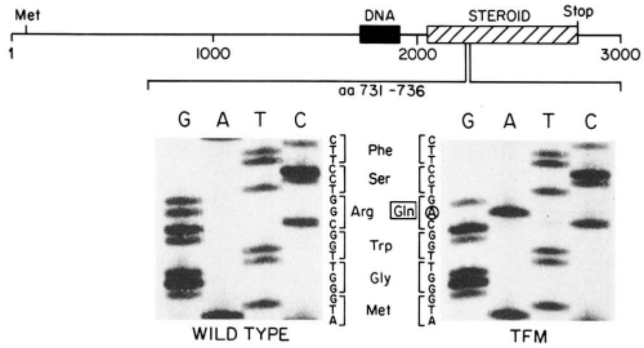

FIG. 5. **Single base mutation in *Tfm* rat AR.** Shown schematically above are the AR functional domains as described in Fig. 4. Below are autoradiograms of DNA sequencing gels from coding strands of normal male sibling (wild-type) and *Tfm* male animals with the sequencing reactions, guanine (*G*), adenine (*A*), thymine (*T*), and cytosine (*C*). The DNA sequence shown codes for amino acid residues 731–736 of rat AR starting from the *lower portion* of the gel. A single base change from guanine in wild-type DNA to adenine (*encircled*) in *Tfm* rat DNA on the coding strand changes arginine at position 734 in wild-type rat AR to glutamine (enclosed in *box*) in the *Tfm* rat AR.

RECEPTOR	CONSERVED REGION OF THE LIGAND BINDING DOMAIN						
Androgen (rat)	Met	Gly	Trp	Arg	Ser	Phe	Thr
Androgen (human)	*	*	*	*	*	*	*
Androgen (rat Tfm)	*	*	*	Gln	*	*	*
Progesterone	Leu	*	*	*	*	Tyr	Lys
Glucocorticoid	Leu	*	*	*	*	Tyr	Arg
Mineralocorticoid	Leu	Ser	*	*	*	Tyr	Lys
Estrogen	Leu	Val	*	*	*	Met	Glu
Retinoic acid	Ile	Cys	Thr	*	-	Tyr	*
Thyroid	Ala	Ala	Val	*	-	Tyr	Asp

FIG. 6. **Partial amino acid sequence comparisons of nuclear receptors within the region of the single base mutation in the *Tfm* AR steroid binding domain.** The amino acid (AA) sequence of rat AR (AA 731–737) is compared with the corresponding sequence of human AR (AA 749–755), *Tfm* rat AR (AA 730–737), and receptors for human progesterone (AA 763–769), glucocorticoid (AA 608–614), mineralocorticoid (AA 814–820), estrogen (AA 391–397), retinoic acid (AA 243–249), and thyroid hormone (AA 263–269) (Refs. 4–10, 55–62). Identical (*) and missing (–) amino acids are indicated relative to wild-type rat AR.

rat (10). The full coding sequence of the wild-type King/Holtzman rat AR was determined as shown in Fig. 4. Comparison with the Sprague-Dawley sequence revealed two strain differences. At nucleotide residue 1163, a thymine in codon TTG differs from cytosine in the corresponding Sprague-Dawley codon TGG, changing serine to leucine at amino acid residue 388 in the King/Holtzman rat (shown *underlined* in Fig. 4). This polymorphic change is of no apparent functional significance since both wild-type strains produce normal genetic males. Furthermore, like the King/Holtzman strain, leucine occurs at a corresponding position in human AR (4–9). Another strain difference was one less glutamine in the King/Holtzman rat AR glutamine repeat beginning at amino acid residue 174 (shown *underlined* in Fig. 4). The lengths of these repeated amino acid motifs in the N-terminal region of AR typically vary (4–9), possibly because of misincorporation errors intrinsic to cloning.

Comparison of AR cDNA coding sequences of *Tfm* and wild-type genetic male siblings showed sequence identity throughout, with one exception (Figs. 4 and 5). A single base change was observed at nucleotide residue 2201, approximately one-third the distance (5′–3′) into the steroid-binding

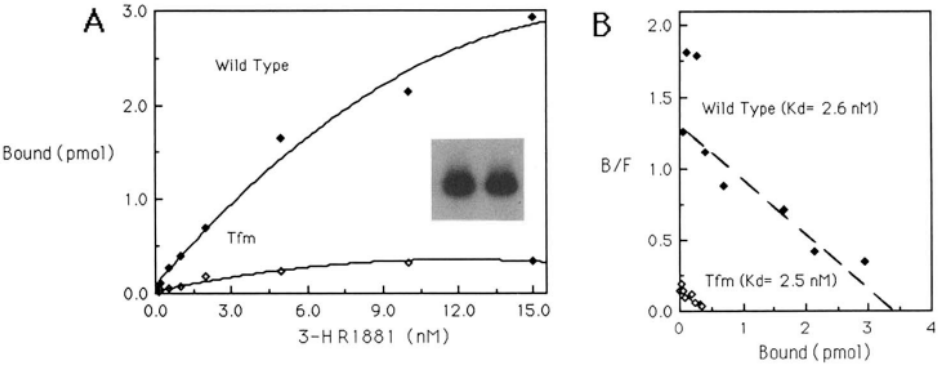

FIG. 7. **Saturation binding analysis of wild-type and mutant AR created by site-directed mutagenesis in M13.** *A*, saturation binding curves for wild-type (♦) and *Tfm* (◇) rat AR; binding of [^{3}H]R1881 was determined as described under "Experimental Procedures." *Inset*, immunoblot of equivalent aliquots of cells transfected with wild-type (*left*) and *Tfm* (*right*) rat AR DNA expression vectors performed as described under "Experimental Procedures." *B*, Scatchard plot of wild-type (♦) and *Tfm* (◇) rat AR binding. Standard deviation of the binding constants was ±1.3 nM.

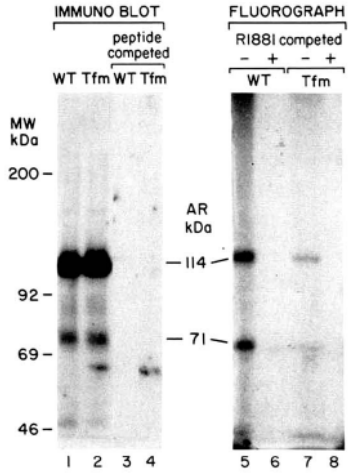

FIG. 8. **Photoaffinity labeling of transiently expressed AR with sequence of wild-type (*WT*) and *Tfm* rat AR.** COS-7 cells were transiently transfected with pCMV expression vector containing either wild-type or *Tfm* rat AR full-length coding sequence as described under "Experimental Procedures." Approximately 125 μg of protein was applied to each *lane* for the fluorograph and 65 μg of protein to each *lane* for the immunoblot. The gel for fluorography was exposed to XAR-5 x-ray film for 110 days at −80 °C and the immunoblot for 3 days. Electrophoretic procedures and molecular weight markers are as described under "Experimental Procedures." Specificity was demonstrated in the immunoblot by peptide adsorption of the IgG antibody faction (*peptide competed*) and in the fluorograph, by labeling in the presence of a 100-fold M excess of unlabeled R1881 (*R1881 competed*) as described under "Experimental Procedures."

domain. The G (wild type) to A (*Tfm*) mutation resulted in conversion of highly basic arginine (codon CGG) to neutral glutamine (codon CAG) at amino acid residue 734 (Fig. 5). Confirmation of the mutation was obtained by PCR amplification and sequencing genomic DNA from individual *Tfm* male rats as shown schematically in Fig. 3. The single base mutation occurs within exon E of the AR gene based on sequence homology and exon positioning of the human AR gene (12).

Sequence Homologies—Arginine 734, which is replaced by glutamine in the *Tfm* rat AR, is positionally conserved throughout the nuclear receptor family (Fig. 6). Arginine at this position appears critical therefore to the function of this group of nuclear proteins. Interestingly, it is adjacent to an almost equally conserved serine residue (Fig. 6); together, they may form a recognition sequence for phosphorylation. The conserved arginine is probably not a determinant of ligand binding specificity since it occurs among receptors that bind diverse ligands. However, the low level of androgen binding by the *Tfm* rat AR suggests that the arginine is essential for normal steroid binding capacity.

Recreation and Expression of the Mutant Receptor—As proof that the G to A transition mutation at nucleotide 2201 caused the functional defect in AR, site-directed mutagenesis was performed in M13 bacteriophage using wild-type rat AR cDNA as template for DNA synthesis, and as primer, a single-stranded oligonucleotide containing the mutant sequence. Mutant and wild-type AR cDNAs were cloned into pCMV and expressed in COS-7 cells. A striking reduction in binding capacity (about 10-fold) was observed with the mutant AR (Fig. 7, *A* and *B*), even though the amount of mutant AR protein expressed in COS cells was equivalent to wild type as determined by immunoblot analysis (see *inset*, Fig. 7*A*). Interestingly, the mutant AR had an apparent affinity for R1881, a synthetic androgen, equivalent to that of the wild-type receptor (Fig. 7*B*) despite its low binding capacity. No specific binding was measured in mock transfected COS cells.

Photoaffinity [^{3}H]R1881 labeling of wild-type and recreated *Tfm* AR showed a striking reduction in labeling of *Tfm* rat AR (Fig. 8, *fluorograph*), even though transfected COS cells contained similar levels of expressed wild-type and mutant AR protein, respectively (Fig. 8, *immunoblot*). Preadsorption of the IgG fraction with the peptide immunogen (10) eliminated antibody reactivity with the 114,000 and 70,000 molecular weight protein bands. Similarly, a 100-fold excess of unlabeled R1881 eliminated the binding of [^{3}H]R1881 to both protein bands. Thus, the 114,000 and 70,000 protein bands likely represent AR, the smaller probably resulting from either proteolytic degradation or from an alternate translation initiation site.

The reduced photoaffinity labeling of the recreated *Tfm* rat AR is consistent with the low binding capacity observed by Scatchard plot analysis (see Fig. 7) and the low level of androgen binding observed in kidney cytosol of the *Tfm* rat. The reduced binding capacity of the recreated *Tfm* rat AR is, in fact, similar to the low or undetectable binding reported previously for endogenous *Tfm* male rat AR (34–36). The rat pituitary gland AR of the *Tfm* rat was found to have low binding capacity but to retain normal binding affinity for [^{3}H] testosterone (35). Thus, both the *Tfm* rat AR and the recreated mutant AR have reduced androgen binding capacity with affinity equivalent to that of wild-type AR.

Immunocytochemical Localization—Subcellular localization of the *Tfm* rat AR was examined by immunocytochemical staining in pituitary, testis, and brain using an AR-specific antibody described previously (10). As in wild-type rat tissues, AR in the *Tfm* rat was present in nuclei (illustrated by the pituitary in Fig. 9). Staining intensity of AR was indistinguishable from wild-type littermate males (not shown). The finding of nuclear AR protein in the *Tfm* rat would appear to contradict previous biochemical evidence that [^{3}H]testoster-

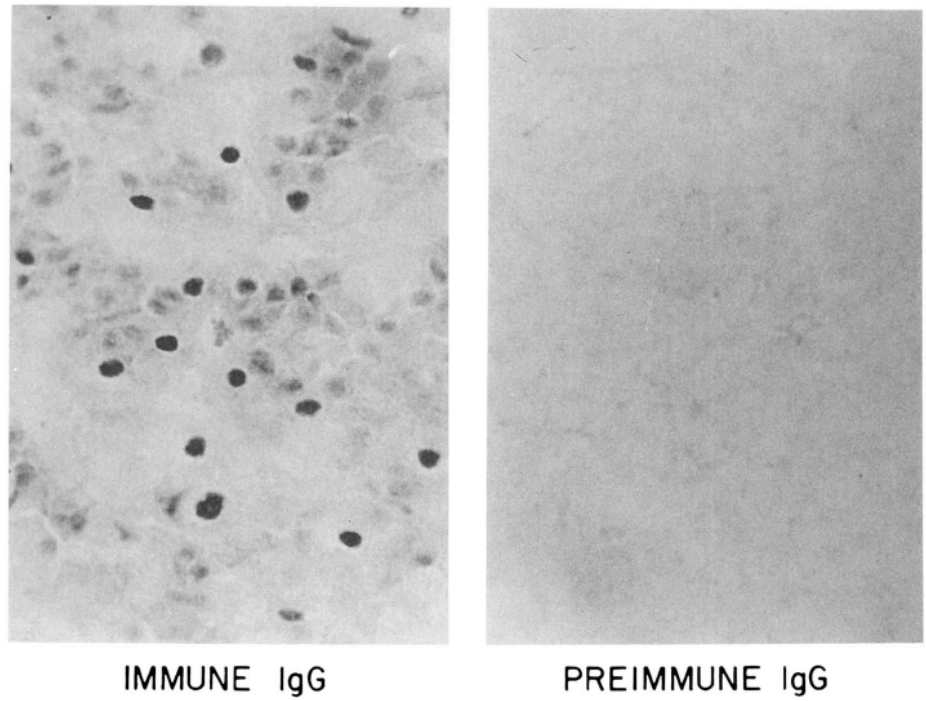

FIG. 9. **Immunocytochemical localization of AR in *Tfm* male rat pituitary gland.** Adult *Tfm* rats received a 15-mg injection of testosterone propionate in sesame oil intramuscularly 16 h prior to tissue removal and rapid freezing. Treatment with the high dose of testosterone propionate was to enhance AR occupancy and recognition by the antibody. Frozen sections (6 μm) of pituitary were incubated with the IgG fraction of androgen receptor antibody (*left*) or preimmune serum (*right*) and stained by the avidin-biotin-peroxidase method (63). The antibody AR1-52 was raised against a synthetic peptide with sequence just 5′ of the AR DNA binding domain (10) which is not in the region of the *Tfm* rat AR mutation. The IgG fraction was prepared (10) and used at a dilution of 5 μg/ml of protein. Magnification, × 690.

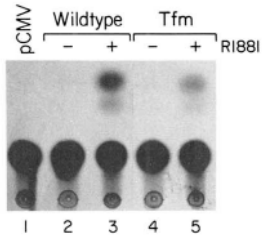

FIG. 10. **A functional assay for AR by cotransfection with the MMTV-CAT reporter plasmid.** CV1 cells were transfected with wild-type and the recreated mutant AR expression vectors in pCMV together with the MMTV-CAT reporter plasmid as described under "Experimental Procedures." After transfection, cells were maintained in 0.2% fetal calf serum in Dulbecco's modified Eagle's medium with or without the addition of 50 nM R1881. Shown is the fluorogram of chloramphenicol acetyltransferase activity separated into its acetylated forms by silica plate chromatography for *lane 1*, pCMV parent plasmid lacking AR sequence; *lane 2*, wild-type AR without androgen; *lane 3*, wild-type AR with androgen treatment; *lane 4*, *Tfm* mutant AR without androgen; and *lane 5*, *Tfm* mutant AR with androgen treatment. The results are representative of four independent experiments.

one does not accumulate in nuclei of the *Tfm* male rat *in vivo* (35–39); however, the results can be explained by a mutant AR with low androgen binding capacity.

Functional Activity—The transcriptional activation function of the *Tfm* rat AR was tested by cotransfection into CV1 cells of the parent vector or expression vectors containing the mutant or wild-type AR together with an MMTV-CAT reporter plasmid. It was shown previously that AR in the presence of androgen stimulates transcriptional activity of the enhancer/promoter of the MMTV long terminal repeat (40–43). As shown in Fig. 10, CV1 cells transfected with wild-type AR respond to androgen with an increase in chloramphenicol acetyltransferase activity. In CV1 cells expressing the recreated mutant receptor, chloramphenicol acetyltransferase activity following androgen stimulation was lower than wild type. Thus, the arginine to glutamine mutation of the *Tfm* rat reduces both androgen binding capacity and functional activity, although AR is localized in the nucleus.

DISCUSSION

This study has established the molecular basis of androgen insensitivity in the Stanley/Gumbreck *Tfm* rat and has thereby revealed the functional importance of an amino acid within the steroid binding domain that is absolutely conserved among the family of nuclear receptors. Recreation of the *Tfm* rat AR by site-directed mutagenesis resulted in a receptor with identical properties, including greatly reduced androgen binding capacity and reduced function as a transcriptional activator.

Previous reports describing the hormonal responses of the *Tfm* rat present somewhat contradictory findings. *Tfm* male rats are unresponsive to physiological concentrations of androgen (34), and hence the androgen insensitivity syndrome develops. However, pharmacological doses of testosterone (44) and dihydrotestosterone (35) can induce detectable biological effects. For example, serum luteinizing hormone levels were decreased in *Tfm* males in response to high doses of androgen in a manner similar to the normal feedback effect in the wild-type rat at physiological androgen levels (35). Similarly, high doses of androgen induced preputial gland growth in the *Tfm* rat (44). In the present report, the *Tfm* rat AR is localized in the nucleus in the presence of androgen at high levels, and a high androgen concentration in CV1 cells induced a low level of chloramphenicol acetyltransferase activity in cotransfection assays.

These observations could be accounted for by the low amount of high affinity binding by AR which persists in the *Tfm* rat. An AR with reduced androgen binding capacity may be insufficient to elicit androgen responses at physiological hormone concentrations, whereas a high concentration of androgen may stabilize nuclear interactions of the defective AR, perhaps including that fraction of AR with binding activity below the level of detection in our assay. Thus, even with their reduced binding capacity, the *Tfm* rat AR (35) and the recreated mutant AR are functionally active in the presence of pharmacological levels of androgen.

On the other hand, androgen withdrawal by castration does not increase AR mRNA in *Tfm* rat kidney as observed in the

wild-type rat, and treatment with high doses of testosterone propionate (10 mg intramuscularly) did not down-regulate AR mRNA (16). It is possible that the inhibitory function of the receptor involves different mechanisms that cannot be compensated for by high dose androgen. Testosterone effects on sexual behavior in the *Tfm* rat have been attributed to the conversion of testosterone to estrogen in the brain; male type sexual behavior occurs to a limited extent in response to estrogen or testosterone, but not to dihydrotestosterone, a nonaromatizable androgen (45, 46).

Why the recreated *Tfm* rat AR mutation is expressed as two populations of receptors, *i.e.* a small amount of receptor that retains high affinity androgen binding and a larger amount with undetectable binding, is not presently understood. However, adjacent to arginine 734 in AR are potential phosphorylation sites (47) on serine or threonine. In addition, a partially conserved tyrosine occurs in the region in other members of the nuclear receptor family (see Fig. 6). Arginine 734 may be a necessary basic amino acid in a phosphorylation recognition sequence, and its loss may result in inefficient phosphorylation, possibly on the highly conserved serine 735 or threonine 737. It was demonstrated recently that a decrease in tyrosine phosphorylation in the steroid binding domain of the estrogen receptor greatly decreases estradiol binding capacity without changing binding affinity (48). The difference in estradiol binding of nonphosphorylated and phosphorylated estrogen receptors as shown by Scatchard plot analysis (48) was quite similar to the androgen binding of mutant and wild-type rat AR observed in this report. Thus, a hypothesis to be tested further is that the arginine 734 to glutamine mutation in the *Tfm* rat AR prevents phosphorylation at one or more critical sites in the steroid-binding domain. Other possible consequences of the single base mutation include distortion of protein folding and/or loss of dimer formation or interactions with other proteins (49–51).

Most steroid binding domain mutations disrupt steroid binding activity and/or transcriptional activity, indicating that much of the carboxyl-terminal region is important for receptor function (52–54). The present study indicates that a single base mutation within the AR steroid binding domain greatly reduces androgen binding capacity. Arginine at position 734 is involved in one or more mechanisms required for normal functioning of the androgen binding domain. Naturally occurring AR mutations associated with androgen insensitivity provide experimental models that serve as guides in correlating structural, physiological, developmental, and behavioral aspects of receptor function.

Acknowledgments—We are grateful to Malcolm Lane, Jon Kemppainen, and Rachel McNeil for excellent technical assistance. We thank Dr. David W. Russell for providing the expression vector pCMV.

REFERENCES

1. Wilson, J. D., Griffin, J. E., Leshin, M., and MacDonald, P. C. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., and Brown, M. S., eds) pp. 1001–1026, McGraw-Hill, New York
2. Stanley, A. J., Gumbreck, L. G., and Allison, J. E. (1973) *Recent Prog. Horm. Res.* **29,** 43–109
3. Bardin, C. W., and Wright, W. (1980) *Ann. Clin. Res.* **12,** 236–242
4. Lubahn, D. B., Joseph, D. R., Sullivan, P. M., Willard, H. F., French, F. S., and Wilson, E. M. (1988) *Science* **240,** 327–330
5. Lubahn, D. B., Joseph, D. R., Sar, M., Tan, J. A., Higgs, H. N., Larson, R. E., French, F. S., and Wilson, E. M. (1988) *Mol. Endocrinol.* **2,** 1265–1275
6. Chang, C., Kokontis, J., and Liao, S. (1988) *Science* **240,** 324–326
7. Chang, C., Kokontis, J., and Liao, S. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7211–7215
8. Trapman, J., Klaassen, P., Kuiper, G. G. J. M., van der Korput, J. A. G. M., Faber, P. W., van Rooij, H. C. J., Geurts van Kessel, A., Voorhorst, M. M., Mulder, E., and Brinkmann, A. O. (1988) *Biochem. Biophys. Res. Commun.* **153,** 241–248
9. Tilley, W. D., Marcelli, M., Wilson, J. D., and McPhaul, M. J. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 327–331
10. Tan, J. A., Joseph, D. R., Quarmby, V. E., Lubahn, D. B., Sar, M., French, F. S., and Wilson, E. M. (1988) *Mol. Endocrinol.* **2,** 1276–1285
11. Brown, T. R., Lubahn, D. B., Wilson, E. M., Joseph, D. R., French, F. S., and Migeon, C. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8151–8155
12. Lubahn, D. B., Brown, T. R., Simental, J. A., Higgs, H. N., Migeon, C. J., Wilson, E. M., and French, F. S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9534–9538
13. Andersson, S., Davis, D. L., Dahlbäck, H., Jörnvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264,** 8222–8229
14. Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B. A., Okret, S., Wikstrom, A.-C., Gustafsson, J.-A., and Yamamoto, K. R. (1986) *Cell* **46,** 389–399
15. Gluzman, Y. (1981) *Cell* **23,** 175–182
16. Quarmby, V. E., Yarbrough, W. G., Lubahn, D. B., French, F. S., and Wilson, E. M. (1990) *Mol. Endocrinol.* **4,** 22–28
17. Wilson, E. M. (1985) *J. Biol. Chem.* **260,** 8683–8689
18. Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1986) *Cold Spring Harbor Symp. Quant. Biol.* **51,** 263–273
19. Gubler, U., and Hoffman, B. J. (1983) *Gene (Amst.)* **25,** 263–269
20. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 488–492
21. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299
22. Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1408–1412
23. Carmichael, G. G., and McMaster, G. K. (1980) *Methods Enzymol.* **65,** 380–391
24. Quarmby, V. E., Beckman, W. C., Wilson, E. M., and French, F. S. (1987) *Mol. Endocrinol.* **1,** 865–874
25. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5201–5205
26. Lopata, M. A., Cleveland, D. W., and Sollner-Webb, B. (1984) *Nucleic Acids Res.* **12,** 5707–5717
27. Banerji, J., Rusconi, S., and Schaffner, W. (1981) *Cell* **27,** 299–308
28. Kimura, G., and Dulbecco, R. (1972) *Virology* **49,** 394–403
29. Gorman, C. M., Moffat, L. F., and Mavard, B. M. (1982) *Mol. Cell. Biol.* **2,** 1044–1051
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
32. Wilson, E. M., and French, F. S. (1976) *J. Biol. Chem.* **251,** 5620–5629
33. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **64,** 5463–5467
34. Bardin, C. W., Bullock, L. P., Sherins, R. J., Mowszowicz, I., and Blackburn, W. R. (1973) *Recent Prog. Horm. Res.* **29,** 65–109
35. Naess, O., Haug, E., Attramadal, A., Aakvaag, A., Hansson, V., and French, F. (1976) *Endocrinology* **99,** 1295–1303
36. Smith, A. A., McLean, W. S., Nayfeh, S. N., French, F. S., Hansson, V., and Ritzen, E. M. (1975) *Curr. Top. Mol. Endocrinol.* **2,** 257–280
37. Ritzen, E. M., Nayfeh, S. N., French, F. S., and Aronin, P. A. (1972) *Endocrinology* **91,** 116–124
38. Max, S. R. (1981) *Biochem. Biophys. Res. Commun.* **101,** 792–799
39. Wieland, S. J., and Fox, T. O. (1981) *J. Steroid Biochem.* **14,** 409–414
40. Darbre, P. D., Moriarty, A., Curtis, S. A., and King, R. J. B. (1985) *J. Steroid Biochem.* **23,** 379–384
41. Darbre, P., Page, M., and King, R. J. B. (1986) *Mol. Cell. Biol.* **6,** 2847–2854
42. Cato, A. C. B., Henderson, D., and Ponta, H. (1987) *EMBO J.* **6,** 363–368
43. Otten, A. D., Sanders, M. M., and McKnight, G. S. (1988) *Mol. Endocrinol.* **2,** 143–147
44. Sherins, R. J., and Bardin, C. W. (1971) *Endocrinology* **89,** 835–841
45. Olsen, K. L. (1979) *Horm. Behav.* **13,** 66–84

46. Olsen, K. L. (1990) in *Handbook of Behavioral Neurobiology, Vol. 10: Sexual Differentiation: A Lifespan Approach* (Gerall, A. A., Mottz, H., and Ward, I. C., eds) Plenum Publishing Corp., New York, in press
47. Blackshear, P. J., Nairn, A. C., and Kuo, J. F. (1988) *FASEB J.* **2,** 2957–2969
48. Migliaccio, A., Di Domenico, M., Green, S., de Falco, A., Kajtaniak, E. L., Blasi, F., Chambon, P., and Auricchio, F. (1989) *Mol. Endocrinol.* **3,** 1061–1069
49. Bresnick, E. H., Dalman, F. C., Sanchez, E. R., and Pratt, W. B. (1989) *J. Biol. Chem.* **264,** 4992–4997
50. Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer-Groyer, G., Catelli, M.-G., Evans, R. M., and Baulieu, E.-E. (1988) *J. Biol. Chem.* **263,** 267–273
51. Colvard, D. S., and Wilson, E. M. (1984) *Biochemistry* **23,** 3479–3486
52. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R., and Chambon, P. (1987) *Cell* **51,** 941–951
53. Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) *Cell* **46,** 645–652
54. Green, S., and Chambon, P. (1988) *Trends Genet.* **4,** 309–314
55. Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Meriel, C., Findlansky, F., Guiochon-Mantel, A., Galibert, F., and Milgrom, E. (1977) *Biochem. Biophys. Res. Commun.* **143,** 740–748
56. Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985) *Nature* **318,** 635–641
57. Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987) *Science* **237,** 268–275
58. Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986) *Science* **231,** 1150–1154
59. Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J., Argos, P., and Chambon, P. (1986) *Nature* **320,** 134–139
60. Petkovich, M., Brand, N., Krust, A., and Chambon, P. (1987) *Nature* **330,** 444–450
61. Giguere, V., Ong, E. S., Segui, P., and Evans, R. M. (1987) *Nature* **330,** 624–629
62. Evans, R. M. (1988) *Science* **240,** 889–895
63. Sar, M. (1985) in *Techniques in Immunocytochemistry* (Bullock, G. R., and Petrusz, P., eds) Vol. 3, pp. 43–54, Academic Press, New York