

Cloning of Human Androgen Receptor Complementary DNA and Localization to the X Chromosome

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The androgen receptor (AR) mediates the actions of male sex steroids. Human AR genomic DNA was cloned from a flow-sorted human X chromosome library by using a consensus nucleotide sequence from the DNA-binding domain of the family of nuclear receptors. The AR gene was localized on the human X chromosome between the centromere and q13. Cloned complementary DNA, selected with an AR-specific oligonucleotide probe, was expressed in monkey kidney (COS) cells and yielded a high-affinity androgen-binding protein with steroid-binding specificity corresponding to that of native AR. A predominant messenger RNA species of 9.6 kilobases was identified in human, rat, and mouse tissues known to contain AR and was undetectable in tissues lacking AR androgen-binding activity, including kidney and liver from androgen-insensitive mice. The deduced amino acid sequence of AR within the DNA-binding domain has highest sequence identity with the progesterone receptor.

DEVELOPMENT OF MALE EXTERNAL genitalia in the human embryo and virilization of the pubertal male are dependent on androgen binding to its receptor and subsequent activation of gene expression. Male sex differentiation fails to occur in the absence of androgen, as in the normal female fetus, or without a functioning androgen receptor (AR), as in the genetic male that develops female external genitalia because of androgen insensitivity. Thus androgen, acting through its receptor, functions as a morphogen to direct formation of the male phenotype during a critical period of early fetal development. At puberty the AR complex functions as a growth and differentiation factor acting in concert with other hormones and growth factors to stimulate reproductive functions that characterize the fully virilized male.

Studies on the androgen insensitivity syndrome [referred to also as testicular feminization (Tfm)] in rats, mice, and man have established that this disorder is linked to the X chromosome (1, 2). Androgen insensitivity is characterized by lack of target cell response to androgen (testosterone and its 5- α -reduced metabolite, dihydrotestosterone) (3). The disorder is usually associated with abnormal AR androgen-binding activity and absence of nuclear localization

of androgen (4). To further localize the AR gene, Migeon and co-workers (1) produced a series of Tfm mouse-human cell hybrids containing X:autosome translocation chromosomes lacking specific segments of the human X chromosome. Expression of AR androgen binding indicated that either the AR locus or a factor controlling AR expression is located on the human X chromosome near the centromere between Xq13 and Xp11. We demonstrate here that it is the AR structural gene that occupies this locus on the X chromosome.

The androgen receptor belongs to the subfamily of steroid hormone receptors within a larger family of nuclear proteins that likely evolved from a common ancestral gene. Each contains an amino-terminal region, variable in length, that may have a role in transcriptional activation, a central cysteine-rich DNA-binding domain, and a carboxyl-terminal ligand-binding domain (5-10). Highest sequence identity occurs in the DNA-binding domain, including the conserved positioning of cysteines resembling the zinc-binding motif (finger structure) described for *Xenopus laevis* 5S RNA gene transcription factor IIIA (11).

Our strategy for isolating AR DNA was based on evidence that the AR gene is X-linked and that no other steroid receptor gene is located on the X chromosome. In addition, we assumed that AR would resemble other members of the steroid receptor family in the conserved DNA-binding domain. A consensus oligonucleotide probe [oligonucleotide A (oligo A)] was synthesized (Fig. 1A) from homologous sequences within the DNA-binding domains of human progesterone, glucocorticoid, thyroid hormone, and estrogen receptors (5, 7-9). Screening an X chromosome library with

oligo A (12) resulted in several recombinants whose inserts were cloned into bacteriophage M13 DNA and sequenced. One recombinant clone (Charon 35 X05AR) contained a sequence similar to, yet distinct from, the DNA-binding domains of other steroid receptors (Fig. 1A). It had 84% sequence identity with oligo A, whereas other receptor DNAs were 78% to 91% homologous with oligo A.

The nucleotide sequence immediately upstream from the putative DNA-binding domain of the genomic isolate was synthesized [oligo B (13)] for use in screening bacteriophage λ gt11 complementary DNA (cDNA) libraries from human epididymis and cultured human foreskin fibroblasts (14). Recombinant phage (unamplified) screened with oligo B by in situ hybridization revealed one positive clone in each library. The epididymal clone (gt11 ARHEL1) contained the complete DNA-binding domain and approximately 1.5 kb of upstream sequence, whereas the fibroblast clone (gt11 ARHFL1) contained the DNA-binding domain and 1.5 kb of downstream sequence. The DNA-binding domains of the cDNA isolates were identical to the DNA-binding domain of the genomic exon sequence. On the basis of the structure of other steroid receptors, the fibroblast cDNA clone (structure shown in Fig. 1B) should contain the complete steroid-binding domain.

The deduced amino acid sequence in the DNA-binding domain of the cDNA (designated hAR for human androgen receptor) (Fig. 1C) most closely resembles the amino acid sequences of the human progesterone, mineralocorticoid, and glucocorticoid receptors. Cysteine residues thought to be involved in "finger" formation are conserved along with other residues. Amino acid residues 1 through 31 of hAR are encoded by the exon in genomic clone X05AR. The 3' intron-exon boundary within the glycine codon (amino acid residue 31) (Fig. 1C) is in an identical position to that reported for the human mineralocorticoid receptor gene (6) and exon 2 in the chicken progesterone receptor gene (15). Histidine 32 or 33 (Fig. 1C, underlined), which may be involved in "second finger" formation, is absent, as it is in the human retinoic acid receptor (10), avian vitamin D receptor, human hap protein, and human estrogen receptor-related proteins (16).

Transient expression in monkey kidney cells (COS M6) demonstrated that the human foreskin fibroblast cDNA encodes the steroid-binding domain of hAR. A DNA fragment (ARHFL1H-X) (Fig. 1B) extending 5' to 3' from the Hind III site within the putative DNA-binding domain through the stop codon (TGA) was cloned into pCMV (Fig. 2A). Expression was facilitated by

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adding to the 5' end a consensus translation initiation sequence (17) containing the methionine codon (ATG) in reading frame. Transfection of the recombinant construct produced a protein with high affinity for [³H]dihydrotestosterone, saturable at

physiological levels of hormone (Fig. 2B). The binding constant [$K_d = 2.7 (\pm 1.4) \times 10^{-10} M$] (Fig. 2C) was nearly identical to that of native AR (18). The level of expressed protein, 1.3 pmol per milligram of protein, was 20 to 60 times greater than that

in male reproductive tissues (19). Mock transfections without plasmid or transfections with plasmid DNA lacking the AR insert yielded no specific binding of dihydrotestosterone. Steroid specificity was identical to that of native AR (18), with highest affinity for dihydrotestosterone and testosterone, intermediate affinity for progesterone and estradiol, and low affinity for cortisol (Fig. 2D). That this truncated receptor (molecular weight 41,000 estimated from the cDNA clone ARHFL1H-X) displayed characteristic AR-binding properties was not unexpected since proteolytic fragments of AR with molecular weight of 30,000 retain the androgen-binding site (18).

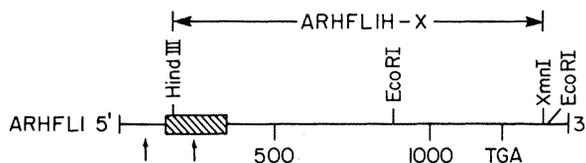
The X chromosomal origin of AR was established by dosage dependence relative to the number of copies of the human X chromosome and by hybridization with DNA from human-rodent cell hybrids in which the X chromosome was the only human chromosome present (19). A dosage-dependent increase in hybridization intensity of 6.5- and 2.3-kb Eco RI DNA fragments was observed on Southern blot analysis of DNA isolated from blood leukocytes of increasing X chromosome copy number (Fig. 3A). Essentially identical results were obtained when either cDNA (Fig. 3A) or genomic DNA was used as radiolabeled probe. A known X-linked probe (coagulation factor IX) showed similar dosage dependence, whereas the signal intensity of an autosomal probe (glucocorticoid receptor cDNA) was independent of X chromosome dosage. In hybrid cells containing the human X chromosome or in human leukocytes, a 5.5-kb Hind III fragment hybridized with a fibroblast AR cDNA fragment (Fig. 3C, lanes 1 and 4) and was undetected in hybrid cells lacking the human X chromosome (Fig. 3C, lane 3). These results indicate that the hAR gene is located on the X chromosome.

Sublocalization of hAR was achieved with known fragments of the X chromosome in human-rodent hybrids. A 900-bp Hind III DNA probe from the hAR genomic isolate (X05AR) hybridized to a 0.9-kb Hind III DNA fragment from hybrid cells that contained human Xcen-qter or Xp21-qter fragments (Fig. 3B, lanes 3 and 4), but not to DNA fragments from hybrid cells containing Xq21-qter or Xq13-qter (Fig. 3B, lanes 2 and 5). Neither did a 500-bp cDNA probe hybridize with DNA from hybrid cells containing a human Xq22-qter chromosome fragment (Fig. 3C, lane 2). Thus, the AR gene is located between the X centromere and Xq13. This chromosome map position is similar to that determined by expression of AR from X chromosome fragments in hybrid cells and is consistent with X linkage of the androgen insensitivity syndrome (1,

A.

Oligo A Complement	5'-ACG	TGT	GAG	GGC	TGT	AAG	GTC	TTC	TTC	AAA	AG-3'	(100%)
	**	***	*	*	**	***	***	***	***	***	**	
hAR (X)	ACA	TGT	GGA	AGC	TGC	AAG	GTC	TTC	TTC	AAA	AG	(84%)
hPR (11)	ACC	TGT	GGG	AGC	TGT	AAG	GTC	TTC	TTT	AAG	AG	(88%)
hMR (4)	ACC	TGT	GGC	AGC	TGC	AAA	GTT	TTC	TTC	AAA	AG	(81%)
hGR (5)	ACT	TGT	GGA	AGC	TGT	AAA	GTT	TTC	TTC	AAA	AG	(81%)
hER (6)	TCC	TGT	GAG	GGC	TGT	AAG	GCC	TTC	TTC	AAG	AG	(91%)
hT3R (3, 17)	ACG	TGT	GAA	GGC	TGC	AAG	GGT	TTC	TTT	AGA	AG	(78%)
hRAR (17)	GCC	TGT	GAG	GGC	TGC	AAG	GGC	TTC	TTC	GGC	CG	(78%)

B.



C.

	DNA-Binding Domain																																				
	+	+	10						+	20+			30																								
hAR	C	L	I	C	G	D	E	A	S	G	C	H	Y	G	A	L	T	C	G	S	C	K	V	F	F	K	R	A	A	E	G	(100%)					
hPR (aa 567)	C	L	I	C	G	D	E	A	S	G	C	H	Y	G	V	L	T	C	G	S	C	K	V	F	F	K	R	A	A	E	G	(94%)					
hMR (aa 603)	C	L	V	C	G	D	E	A	S	G	C	H	Y	G	V	V	T	C	G	S	C	K	V	F	F	K	R	A	V	E	G	(87%)					
hGR (aa 421)	C	L	V	C	S	D	E	A	S	G	C	H	Y	G	V	L	T	C	G	S	C	K	V	F	F	K	R	A	V	E	G	(87%)					
hER (aa 185)	C	A	V	C	N	D	Y	A	S	G	Y	H	Y	G	V	W	S	C	E	G	C	K	A	F	F	K	R	S	I	Q	G	(55%)					
cVDR	C	G	V	C	G	D	R	A	T	G	F	H	F	N	A	M	T	C	E	G	C	K	G	F	F	R	R	S	M	K	R	(48%)					
hT3R (aa 102)	C	V	V	C	G	D	K	A	T	G	Y	H	Y	R	C	I	T	C	E	G	C	K	G	F	F	R	R	T	I	Q	K	(48%)					
vERBA (aa 37)	C	V	V	C	G	D	K	A	T	G	Y	H	Y	R	C	I	T	C	E	G	C	K	S	F	F	R	R	T	I	Q	K	(48%)					
hRAR (aa 58)	C	F	V	C	Q	D	K	S	S	G	Y	H	Y	G	V	S	A	C	E	G	C	K	G	F	F	R	R	S	I	Q	K	(45%)					
hAR	K	Q	K	Y	L	C	A	S	R	N	D	C	T	I	D	K	F	R	R	K	N	C	P	S	C	R	L	R	K	C	Y	E	A	G	M	(100%)	
hPR	Q	H	N	Y	L	C	A	G	R	N	D	C	I	V	D	K	I	R	R	K	N	C	P	A	C	R	L	R	K	C	Q	A	G	M	(71%)		
hMR	Q	H	N	Y	L	C	A	G	R	N	D	C	I	I	D	K	I	R	R	K	N	C	P	A	C	R	L	Q	K	C	L	Q	A	G	M	(71%)	
hGR	Q	H	N	Y	L	C	A	G	R	N	D	C	I	I	D	K	I	R	R	K	N	C	P	A	C	R	Y	R	K	C	L	Q	A	G	M	(71%)	
hER	H	N	D	Y	M	C	P	A	T	N	Q	C	T	I	D	K	N	R	R	K	S	C	Q	A	C	R	L	R	K	C	Y	E	V	G	M	(63%)	
cVDR	K	A	M	F	T	C	P	F	N	G	D	C	K	I	T	K	D	N	R	R	H	C	Q	A	C	R	L	K	R	C	V	D	I	G	M	(40%)	
hT3R	N	L	H	P	S	S	C	K	Y	E	G	K	C	V	I	D	K	V	T	R	N	Q	C	Q	E	C	R	F	K	K	C	I	Y	V	G	M	(40%)
vERBA	N	L	H	P	T	T	S	C	T	Y	D	G	C	V	I	D	K	I	T	R	N	Q	C	Q	L	C	R	F	K	K	C	I	S	V	G	M	(37%)
hRAR	N	M	V	Y	T	C	H	R	D	K	N	C	I	N	K	V	T	R	N	R	C	Q	Y	C	R	L	Q	K	C	F	E	V	G	M	(43%)		

Fig. 1. Comparison of DNA-binding domains of the human androgen receptor (hAR) with members of the nuclear receptor family. (A) Comparison of oligo A nucleotide sequence with sequences of hAR and other nuclear receptors: hPR, human progesterone receptor (7); hMR, human mineralocorticoid receptor (6); hGR, human glucocorticoid receptor (5); hER, human estrogen receptor (8); hT3R, human thyroid hormone receptor (9); and hRAR, human retinoic acid receptor (10). Chromosomal locations are shown in parentheses at the left. Nucleotide identity between oligo A and hAR is indicated with an asterisk. The percent homology with oligo A is in parentheses at the right of each sequence. (B) Structure of fibroblast clone ARHFL1. Nucleotide residues are numbered from the 5' terminus. Restriction endonuclease sites were determined by mapping or were deduced from DNA sequence. The TGA translation termination codon, determined by comparison with hPR (7), hMR (6), and hGR (5), follows a long open reading frame containing sequences homologous to those of other steroid receptors. Arrows indicate exon boundaries in genomic clone X05AR. The hatched area is the putative DNA binding domain. (C) Comparison of amino acid sequence of the AR DNA-binding domain with sequences of the nuclear receptor family. AR amino acid sequence was deduced from nucleotide sequence of clone ARHFL1 and is numbered beginning with the first conserved cysteine residue (+). Amino acid (aa) numbers in parentheses at the left indicate the residue number of the first conserved cysteine from the references indicated above. Percent homology with hAR is indicated in parentheses on the right. The region of the DNA-binding domain from which the oligo A sequence was derived is underlined in hAR. Coding DNA of residues 1 to 31 is contained within genomic clone X05AR. Abbreviations in addition to those described above are cVDR, chicken vitamin D receptor, and vERBA, *erb A* protein from avian erythroblastosis virus (16). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequence of hAR will be available from GenBank under accession number J03180.

2). Thus the androgen insensitivity syndrome likely results from a defect in the AR gene. Definitive linkage between AR and human androgen insensitivity requires identification of a mutant AR gene from an

affected individual.

Expression of messenger RNA (mRNA) AR as determined by Northern blot analysis of polyadenylated [poly(A)] RNA paralleled the known tissue distribution of AR andro-

gen-binding activity. A predominant 9.6-kb mRNA was evident in poly(A) RNA of cultured human foreskin fibroblasts and rat testis, epididymis, kidney, and prostate (Fig. 4). The spleen lacks AR androgen-binding activity (20) and the 9.6-kb mRNA (Fig. 4A, lane 5). AR mRNA was also undetectable in liver and kidney of androgen-insensitive Tfm mice (Fig. 4B, lanes 2 and 4). Normal male littermates displayed the 9.6-kb mRNA (Fig. 4B, lanes 1 and 3). RNA integrity in each lane was verified by rehybridization with a rat glucocorticoid receptor cDNA probe (21). On the basis of additional experiments, the hybridizing band at about 7 kb (Fig. 4A, lanes 1 and 8) may represent an AR mRNA that results from alternative processing. However, the hybridizing bands at about 4.4 and 2.0 kb (Fig. 4, A and B) are likely due to cross-hybridization with contaminating ribosomal RNA.

Design of the oligonucleotide probe, oligo A, used in isolating hAR DNA was prompted by the supposition, now confirmed, that AR belongs to a family of

Fig. 2. Steroid-binding properties of expressed AR cDNA. (A) Structure of pCMVAR in the expression vector pCMV (22) containing the human cytomegalovirus (CMV) promoter of the immediate early gene, poly(A) addition-transcription terminator region of the human growth hormone gene [hGH poly(A), SV40 origin of replication (SV40 ori), and a poly-linker region for insertion of cDNAs. The plasmid pTEBR contains the ampicillin resistance gene (Amp). Construction of pCMVAR and transfection of COS M6 cells are described (23). Restriction endonuclease sites in parentheses are lost during cloning. (B) Saturation analysis of [³H]dihydrotestosterone binding in extracts of pCMVAR-transfected COS M6 cells. Portions of cytosol (0.1 ml; 0.3 mg of protein per milliliter) were incubated overnight at 4°C with increasing concentrations of ³H-labeled hormone and analyzed by charcoal adsorption (18). Nonspecific binding increased from 18% to 37% of total bound radioactivity. (C) Scatchard plot analysis of [³H]dihydrotestosterone binding. Error estimation was based on linear regression analysis ($r = 0.966$). (D) Competition of unlabeled steroids for binding of 5 nM [³H]dihydrotestosterone in transfected COS M6 cell extracts. Unlabeled steroids were added at 10- and 100-fold excess of labeled hormone. Specific binding was determined as previously described (18). Unlabeled competitor steroids included dihydrotestosterone (○), testosterone (●), estradiol (△), progesterone (▲), and cortisol (□).

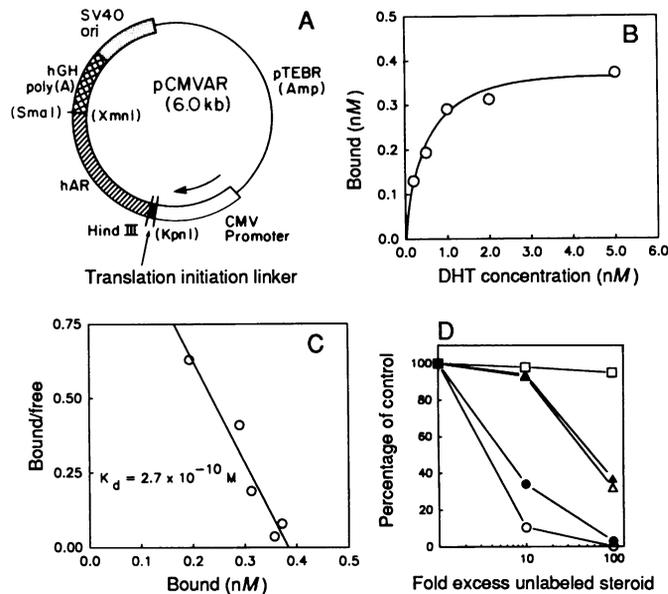


Fig. 3. X Chromosome localization of the hAR gene on Southern blots of DNA from human cells containing multiple X chromosomes and mouse or hamster cells with X:autosome translocation chromosomes. DNA (5 µg) was digested with Eco RI (A) or Hind III (B and C) and analyzed by Southern hybridization with hAR DNA as probe (24). The ³²P-labeled DNA hybridization probes included (A) the 750-bp Hind III-Eco RI fragment of cDNA clone ARHFL1, (B) the 900-bp Hind III fragment from genomic clone X05AR (containing three-fourths of the first finger DNA-binding domain and 830 bp of 3' intron DNA), or (C) the 500-bp Eco RI fragment of cDNA clone ARHFL1. DNA size markers were ³²P-labeled Hind III digest of lambda DNA and Hae III digest of phiX174 DNA. (A) (Lane 1) 46,XY normal male leukocytes; (lane 2) 46,XX normal female leukocytes; (lane 3) 48,XXXX (GM1416B) lymphoblasts; and (lane 4) 49,XXXXX (GM6061A) lymphoblasts. Lanes 3 and 4 contain DNA from human cell lines with different numbers of human X chromosomes (25). Rehybridization of lanes 1 to 4 with an autosomal hGR probe verified the equal quantity of DNA in each lane. (B) (Lane 1) 46,XX normal female leukocytes; (lane 2) Xq21-qter in mouse (A50-1A); (lane 3) Xcen-qter in mouse (A48-1F); (lane 4) Xp21-qter in mouse (A2-4); and (lane 5) Xq13-qter in hamster (W4-1A). Lanes 2 to 5 contain DNA from human X chromosome fragments each in a heterologous cell line (20). Rehybridization of the blot with a Xq27.1-27.2 human blood coagulating factor IX probe verified the presence of human X fragment DNA in each lane. (C) (Lane 1) Human X chromosome in hamster (C1-2D); (lane 2) Xq22-qter in hamster (W53-5Bc15); (lane 3) hamster DNA; and (lane 4) 46,XY normal male leukocytes. The 2.3-kb hybridizing fragment [lanes 1 to 3 in (C)] represents hamster X chromosome DNA endogenous to hybrid cells. Hybridization to rodent genomic DNA was not observed with the 900-bp genomic probe from X05AR [lanes 2 to 5 in (B)] as it was with the 500-bp cDNA fragment containing a portion of the putative steroid-binding domain [lanes 1 to 3 in (C)].

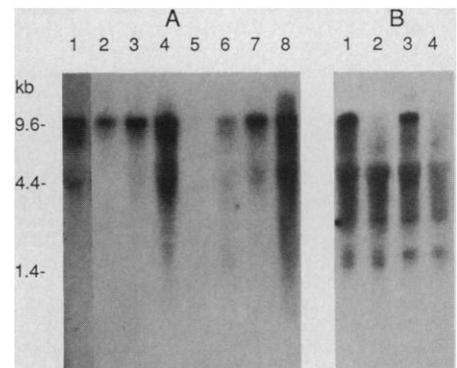
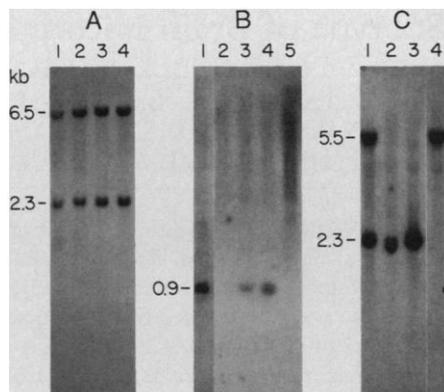


Fig. 4. Northern blot analysis of AR mRNA in human, rat, and mouse tissues. Poly(A) RNA was treated with glyoxal, fractionated on agarose gels, and analyzed by blot hybridization (26), except that GeneScreen Plus (New England Nuclear) was used as the transfer membrane and 0.5% sodium dodecyl sulfate was included in the hybridization solutions. (A) Poly(A) RNA (20 µg) of human and rat tissue was analyzed by blot hybridization with the ³²P-labeled 750-bp Eco RI-Hind III fragment of ARHFL1 as probe. (Lane 1) Human foreskin fibroblast; (lane 2) Sprague-Dawley adult rat testis; (lane 3) 26-day-old rat testis; (lane 4) rat epididymis; (lane 5) male rat spleen; (lane 6) male rat kidney; (lane 7) rat ventral prostate; and (lane 8) human liver (10 µg). (B) Kidney and liver poly(A) RNA (15 µg) from Tfm male mice and normal littermates hybridized with a 2000-bp rat AR cDNA probe (27). (Lane 1) Normal male kidney; (lane 2) Tfm male kidney; (lane 3) normal male liver; (lane 4) Tfm male liver. Tfm mice (N3F15) and their normal male littermates were from Jackson Laboratory. Size markers in adjacent lanes were denatured ³²P-labeled Hind III digest of lambda DNA and Hae III digest of phiX174 DNA. Care of the animals was in accordance with institutional guidelines.

nuclear receptors containing a conserved cysteine-rich DNA-binding domain. This family may be larger than anticipated, including yet to be discovered genes with the capability of hybridizing to consensus oligonucleotide probes based on known DNA-binding domain sequences. Genomic DNA libraries prepared from flow-sorted human chromosomes are now available, making it possible to screen specific chromosome DNA for additional nuclear receptor genes.

Cloning of the AR cDNA opens new approaches to studies on the biological actions of male sex hormones. The molecular mechanisms of androgen-induced male sexual development and reproductive function can be investigated in normal and diseased conditions. Identification of mutations causing androgen insensitivity should provide insight into structure-function relationships of the AR and, by analogy, of other members of the nuclear receptor family.

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- A bacteriophage lambda Charon 35, chromosome-sorted, X genomic library (partial Sau 3A, XLAOXNL01) was obtained from L. L. Deaven at Los Alamos National Laboratory in Los Alamos, NM. Recombinant phages (300,000) were screened with ³²P-labeled oligo A (Fig. 1A) (1.2 × 10⁷ cpm/pmol) as probe. Oligonucleotides were ³²P-labeled with T4 polynucleotide kinase and [γ-³²P]ATP. Hybridization [G. M. Wahl, M. Stern, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3683 (1979)] in 30% formamide, was for 16 hours at 30°C. Stringent washing was with 0.2 × SSC (1 × SSC is 0.15M NaCl and 0.015M sodium citrate) with 0.5% sodium dodecyl sulfate at 37°C for 2 minutes. The positive clones were characterized by partial DNA sequencing [J. Messing, *Methods Enzymol.* **101**, 20 (1983); F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
- Oligonucleotide B (38 residues) was made from the coding sequence 5' to the DNA-binding region of genomic clone X05AR.
- Poly(A) RNA was prepared [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972); J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)] from human epididymis and cultured human foreskin fibroblasts and used to prepare cDNA by the method of U. Gubler and B. J. Hoffman [*Gene* **25**, 263 (1983)]. After blunt-ending of the cDNAs with T4 DNA polymerase, Eco RI methylation and the addition of Eco RI linkers, the cDNAs were cloned in bacteriophage λgt11 [R. A. Young and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1194 (1983)]. The recombinant phages were screened for AR cDNA with oligo B as described for oligo A above (12).
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- The vector pCMV was obtained from D. W. Russell, Department of Molecular Genetics, University of Texas Health Science Center at Dallas.
- The 1200-bp Hind III-Xmn I fragment ARHFL1H-X (Fig. 1B) was cloned into pCMV (Hind III-Sma I cleaved). After insertion of the AR DNA, a 26-bp synthetic fragment containing a consensus translation initiation site, CCACCATGG, was placed in reading frame into the Kpn I-Hind III restriction sites of pCMV DNA. Subconfluent COS M6 cells (10⁶ cells per 100-mm dish) were transfected with 3 μg of pCMVAR DNA per dish by the DEAE-dextran method [R. J. Deans, K. A. Denis, A. Taylor, R. Wall, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1292 (1984)] and assayed for steroid binding as previously described (18, 20).
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Functional Expression of a New Pharmacological Subtype of Brain Nicotinic Acetylcholine Receptor

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A new type of agonist-binding subunit of rat neuronal nicotinic acetylcholine receptors (nAChRs) was identified. Rat genomic DNA and complementary DNA encoding this subunit (alpha2) were cloned and analyzed. Complementary DNA expression studies in *Xenopus* oocytes revealed that the injection of messenger RNAs (mRNAs) for alpha2 and beta2 (a neuronal nAChR subunit) led to the generation of a functional nAChR. In contrast to the other known neuronal nAChRs, the receptor produced by the injection of alpha2 and beta2 mRNAs was resistant to the α-neurotoxin Bgt3.1. In situ hybridization histochemistry showed that alpha2 mRNA was expressed in a small number of regions, in contrast to the wide distribution of the other known agonist-binding subunits (alpha3 and alpha4) mRNAs. These results demonstrate that the alpha2 subunit differs from other known agonist-binding α-subunits of nAChRs in its distribution in the brain and in its pharmacology.

THERE IS A FAMILY OF GENES THAT encodes functional subunits of rat neuronal nicotinic acetylcholine receptors (nAChRs) (1-3). The genes identified thus far have been designated alpha3, alpha4, and beta2. The first two genes encode agonist-binding subunits (1, 2) which, when combined with the beta2 gene product, form a functional neuronal nAChR in *Xenopus* oocytes (2, 3). In addition, our previous study (4) indicated the existence of another gene, alpha2, which encodes a pro-