A frame-shift mutation in the androgen receptor gene causes complete androgen insensitivity in the testicular-feminized mouse

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ABSTRACT

The testicular feminized (Tfm) mouse lacks completely androgen responsiveness; and therefore, is unique for studying the role of androgenic steroids in different biological processes. In order to understand the molecular basis of this mutation, 2.8 kilobases of cDNA encoding the Tfm mouse androgen receptor (AR) were amplified with a polymerase chain reaction (PCR) technique. No large deletion in the coding region of the Tfm mouse AR was detected. However, sequence analysis revealed a single base deletion in the coding region of the Tfm AR mRNA. This mutation, which is located in the amino-terminus domain of the receptor, is predicted to cause a frame-shift in translation resulting in a premature termination of AR synthesis at amino acid 412. *In vitro* translation studies of the recombinant wild type and Tfm AR's demonstrated that the Tfm AR cDNA failed to produce a full-length receptor. Furthermore, the Tfm AR was demonstrated to lack transcriptional activation capability by cotransfection experiments using the Tfm AR with a reporter plasmid of mouse mammary tumor virus long terminal repeat linked to the chloramphenicol acetyltransferase gene. These studies provide evidence of the molecular defect which causes androgen insensitivity in the Tfm mouse.

INTRODUCTION

Although many endocrine disorders are the result of abnormal hormonal secretions, some diseases are caused by the inability of target tissues to respond to circulating hormones. Androgen insensitivity syndrome (AIS) in man (1) is an X-linked genetic disease resulting from defects in the androgen receptor (AR) gene. Mutations in the AR gene can result in a wide spectrum of abnormalities in male sexual development, ranging from a complete female phenotype to a male phenotype exhibiting infertility (2). Examples of androgen insensitivity in other mammalian species, referred to as testicular feminization (Tfm), have been noted in the mouse (3), rat (4), and cow (5). In the mouse, Tfm is an X-linked genetic disorder in which genetic males carrying the Tfm gene (Tfm/Y) have testes and normal testosterone production but female phenotypic characteristics (3,6). These mice have been shown previously to be resistant to both endogenous and exogenous testosterone. Initial studies suggested that the resistance to androgens in Tfm mice is due to both quantitative and qualitative abnormalities of the AR (6–9). Both the Tfm mouse and the Tfm rat have served as useful models for androgen insensitivity. However, the Tfm rat has been shown to respond to pharmacological doses of androgens and therefore is not completely resistant to androgens (10,11). On the other hand, the Tfm mouse is completely resistant to androgens, and therefore is considered to be the most appropriate animal to examine various androgen-dependent phenomena. For this reason, the Tfm mouse has been used widely as a negative control in monitoring androgenic responses in a variety of biological processes ranging from stromal-epithelial cell interaction (12), and hepatocarcinogenesis (13), to testicular descent (14). Despite extensive studies on the AR in the Tfm mouse during the past two decades, the molecular basis of this mutation remains unknown. The fact that the Tfm mouse is a commonly used animal model resembling the human syndrome of complete AIS prompted us to study the molecular basis of this mutation.

MATERIALS AND METHODS

Animals

Wild type male (C57BL/6J-A^{W-J}-Ta+/Y) and Tfm (C57BL/6J-A^{W-J}-Ta Tfm/Y) mice were obtained from Jackson Laboratories, Bar Harbor, ME. Tfm mice were also kindly provided by Dr. J. D. Wilson, The University of Texas Southwestern Medical Center, Dallas, TX.

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Polymerase Chain Reaction (PCR)

First strand cDNA was synthesized in a 50 μl reaction mixture consisting of 5 μg of poly(A^+) RNA or 100 μg of total RNA isolated from wild type male or Tfm mouse kidneys, 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 2 mM each of dATP, dGTP, dCTP, and dTTP, 2.5 μg of oligo $(dT)_{12-18}$, 50 units of human placental ribonuclease inhibitor and 100 units of avian myeloblastosis virus (AMV) reverse transcriptase. The reaction mixture was incubated at 42°C for 1 h followed by incubation at 65°C for 10 min. The reaction mixture was diluted to 150 μl with H_2O. PCR was carried out in a 25 μl reaction mixture with 1 μl of the above first strand cDNA sample, 50 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 μM each of dATP, dCTP, dGTP, and dTTP, 25 pmol of each primer and 0.5 unit of Taq polymerase (Perkin-Elmer Cetus). Thirty five cycles of PCR (consisting of denaturation at 94°C for 1 min; annealing at 55°C for 2 min; and elongation at 72°C for 3 min) were performed with a Perkin-Elmer Cetus automated thermal cycler. Oligonucleotide primers for PCR amplification were synthesized with an Applied Biosystems 380A DNA synthesizer. The sequences of primers used for PCR were the following: mAR (1−15) U (5'GAATTCGGTGGAAGCT3') and mAR (943−959) T7 D (5'TAATACGACTCACTAT AGGGAG-AGTAACCTCCCTTGAAAG3') for the amplification of fragment mARpa; mAR (787−802) U (5'AACATCTGAGTCC-AGG3') and mAR (1577−1592) T7 D (5'TAATACGACTC-ACTATAGGGAGAC ATGTCCCCATAAGGT3') for the amplification of mARpb; and mAR (1409−1424) U (5'TATGGCTACACTCGGC3') and mAR (2761−2777) T7 D (5'TAATACGACTCACTATAGGGAGAA AAGGGAACAA-GGT3') for the amplification of mARpc (Figure 1). The numbers represent the positions of the mAR cDNA sequences from which each primer is derived (15). T7 signifies the T_7 promoter sequence. D or U indicates whether the primer is downstream or upstream for PCR amplification.

Sequence Analysis

PCR amplified products were sequenced directly by RNA amplification with transcript sequencing (RAWTS) (16). Briefly, the PCR amplified products were separated on a 1.5% agarose gel, the band of interest was sliced out, and DNA was purified with a Gene Clean kit (Bio 101). The purified DNA was used to transcribe RNA with a Gemini Transcription System (Promega Corporation) in a 100 μl reaction mixture. The appropriate reverse transcriptase primers were end-labeled with [γ^{32}P]ATP in a reaction mixture containing 10 μl of [γ^{32}P]ATP (10 mCi/ml, >5000 Ci/mmol), 1 μl of 10× labeling buffer (500 mM Tris-HCl, pH 7.4, 100 mM $MgCl_2$, 50 mM DTT, and 1 mM spermidine), 1 μl T_4 polynucleotide kinase (10 units) and 1 μl of primer (25 pmol/μl) at 37°C for 30 min. The reaction was inactivated by heating at 65°C for 5 min, and 7 μl of H_2O was added to the reaction mixture. The annealing mixture containing 2 μl of the transcription reaction mixture, 10 μl of annealing buffer (10 mM Tris-HCl, pH 8.3, and 250 mM KCl) and 1 μl of end-labeled primer was heated to 80°C for 3 min and annealed at 45°C for 30 min. The final sequencing reaction mixture containing 1 μl each of one of the four ddNTP stock solutions (1 mM ddATP, 1 mM ddTTP, 1 mM ddGTP, and 0.25 mM ddCTP), 3.3 μl of reverse transcription buffer (24 mM Tris-HCl, pH 8.3, 16 mM $MgCl_2$, 8 mM DTT, 0.8 mM dATP, 0.4 mM dCTP, 0.8 mM dGTP and 1.2 mM dTTP), 2.0 μl of the annealing mixture and 1 unit of AMV reverse transcriptase was incubated at 55°C for 45 min, and the reaction was terminated with 2.5 μl of stop solution (85% deionized formamide, 25 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). The samples were heated at 85°C for 5 min, and 2 μl of the samples were loaded onto a 6% polyacrylamide/8 M urea sequencing gel. After electrophoresis the gel was dried and exposed to Kodak XAR-5 film for 12−48 h at room temperature. Regions of DNA, which were difficult to sequence by this technique, were subcloned into pBluescript II SK(+) (Stratagene), and three to four independent clones were sequenced with Sequenase (United States Biochemical) according to the manufacturer's protocol.

Construction of Full-Length Wild Type and Tfm mAR cDNA Expression Vectors

Full-length wild type and Tfm AR cDNA constructs (Fig. 3a, constructs A and B, respectively) were made from the PCR products and the mAR genomic clone (15). To clone the wild type AR in pBluescript II SK(+), the amino-terminus EcoRI-Hind III fragment was constructed first by ligating the EcoRI-BsmI fragment from the mAR genomic clone mARg (15) with the BsmI-HindIII fragment from a PCR product amplified from mAR cDNA. The EcoRI-HindIII fragment was ligated further with the C-terminus HindIII-PstI fragment to generate the full-length mAR in pBluescript II SK(+). The Tfm AR was constructed in the same manner except that the BsmI-HindIII fragment was amplified from the Tfm mouse cDNA. All clones were sequenced after construction to ensure that no mutation was generated by PCR amplification. The full-length wild type and Tfm AR were transferred to the BamHI site of the eukaryotic expression vector pcDNAI/Neo (In Vitrogen), which contains a human cytomegalovirus (CMV) promoter and allows the expression of foreign products in eukaryotic cells. The full-length wild type and Tfm AR cDNA constructs in pcDNAI/Neo were named AR-CMV and tAR-CMV, respectively.

In Vitro Expression of Wild Type and Tfm mAR

The pBluescript II SK(+) vector containing wild type and Tfm mAR inserts were linearized by KpnI restriction digestion. Capped RNA was synthesized using the T_3-RNA polymerase according to a method described previously (17) with minor modifications. RNA templates were synthesized with 0.5 mM each of ATP, UTP, and CTP, 50 μM GTP, and 0.5 mM RNA cap analog (m7G(5')ppp(5')G, Boehringer Mannheim Biochemicals). The synthesized RNAs were purified by phenol extraction and ethanol precipitation. *In vitro* translation was carried out with 2−3 μg of RNA, 35 μl of rabbit reticulocyte lysate (Promega), 4 μl of [^{35}S]methionine (Amersham, 1 μCi/μl at 1000 Ci/mmol) in a final volume of 50 μl according to the manufacturer's protocol. After allowing the reaction to proceed at 30°C for 60 min, 20 μl of the lysates were incubated with 10 μl of human autoimmune antibodies to AR (18) overnight at 4°C. Antibody-antigen complexes were precipitated with protein A Sepharose (Pharmacia) and analyzed on 10% SDS polyacrylamide gels.

Cell Culture and Transfection

The cell line QT6 (19), which is a chemically induced quail fibroblast tumor cell line, was obtained from Dr. N. Maihle, Mayo Clinic. This cell line lacks detectable androgen receptors and is amenable to transfection with the calcium phosphate protocol. The cells were maintained in Dulbecco's Modified

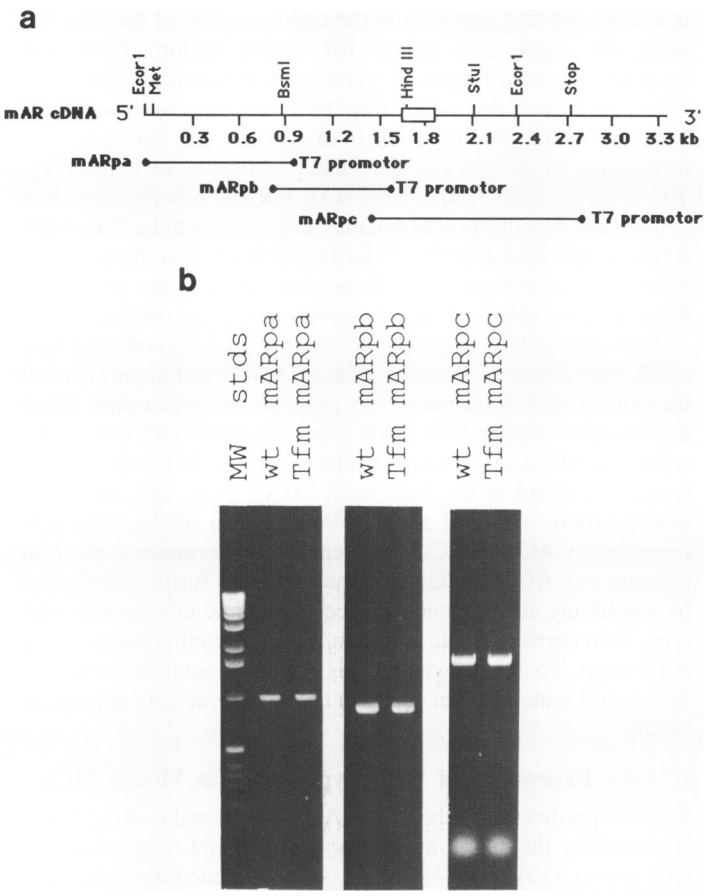

Fig. 1. PCR amplification of wild type and Tfm mAR cDNAs. **(a)** Map of mAR cDNA. mARpa, mARpb and mARpc depict three PCR fragments amplified with primers derived from the mAR cDNA sequence reported previously (15). The open rectangle depicts the putative DNA-binding domain of the mAR. **(b)** Amplification of mAR transcripts from wild type and Tfm mice. Amplified products of wild type (wt) and Tfm AR mRNA were separated on a 1% agarose gel and stained with ethidium bromide for visualization under UV light.

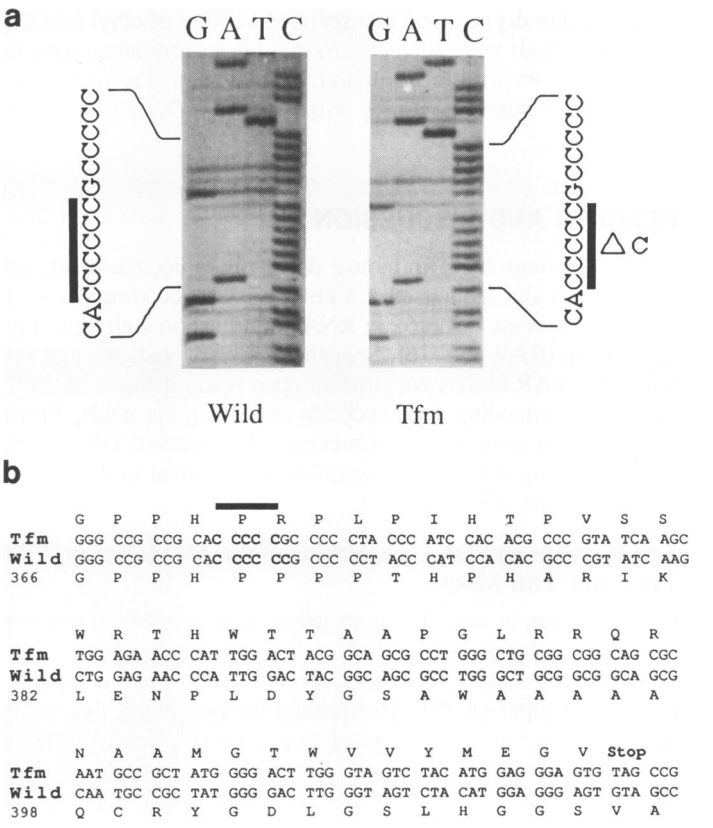

Fig. 2. A frame-shift mutation in the Tfm mAR cDNA. **(a)** Sequence of the mAR cDNA from wild type and Tfm mice in the region of the mutation. PCR products were subcloned into pBluescript II SK(+) and sequenced with Sequenase. The partial DNA sequence of the mAR cDNA from wild type and Tfm mice are shown. A single base (ΔC, vertical bars) is deleted from the Tfm mAR cDNA when compared to the wild type mouse AR sequence (vertical bar). **(b)** The DNA and the predicted amino acid sequences from wild type and Tfm mouse ARs in the region of the mutation. The horizontal bar indicates the site of the mutation. A stop codon is observed about 120 nucleotides downstream of the mutation in the Tfm mAR cDNA.

Eagle's medium (DMEM) containing 6% glucose (Sigma), 5% fetal calf serum (BRL-Gibco), 1% chicken serum (BRL-Gibco), 50 units/ml of penicillin-G (Sigma) and 50 μg/ml of streptomycin sulfate (Sigma) in a cell incubator at 37°C and 5% CO_2. Endogenous hormones in serum were removed with dextran-coated charcoal as described previously (20).

QT6 cells were transfected with DNA by a calcium phosphate protocol (21). Briefly, cells at 35−45% confluency were changed into fresh medium 5 to 6 h prior to transfection. A 2×-HBPS buffer was prepared as a mixture of 5 ml of HBS (10 mM HEPES, pH 7.1, and 90 mM NaCl) and 100 μl of sodium phosphate buffer (70 mM NaH_2PO_4 and 70 mM Na_2HPO_4) in a 15 ml sterile tube. Individual DNA samples containing 5 μg of MMTV-CAT, 5 μg of β-galactosidase reporter plasmid pCH110 (Pharmacia) and 0.1 μg of mAR full-length expression vectors (wild type or Tfm) were mixed with 10 μg of carrier DNA (pGEM7Z+, Promega) and 30 μl of 2 M $CaCl_2$ in a final volume of 245 μl. The DNA solution was added to 255 μl of 2X-HBPS with gentle mixing. After a 30 min incubation at room temperature, the precipitate was layered onto cells with the freshly changed media. After 16 h of incubation, the medium was replaced with DMEM either with or without 10 nM of dihydrotestosterone (DHT). Cells were grown for 48 h prior to preparing cytosols.

Confluent monolayers of cultured cells were washed three times in 10 ml of PBS buffer. The cells were detached with a cell lifter from the flasks after a 5 min incubation in 1 ml of TEN buffer (40 mM Tris, pH 8.0, 1 mM EDTA and 150 mM NaCl) at room temperature. The cells were centrifuged briefly, and the pellets from each flask were resuspended in 150 μl of 0.25 M Tris HCl, pH 8.0, and subjected to three freeze and thaw cycles. The disrupted cells were pelleted in a microcentrifuge for 10 min, and the supernate was assayed for chloramphenicol acetyltransferase (CAT) activity. β-galactosidase activity, measured according to a method described previously (22), was used as an internal control for transfection efficiency. The transfection experiment was repeated three times.

Chloramphenicol Acetyltransferase (CAT) Assay

CAT activity was assayed as described previously (23) with slight modifications. Duplicate aliquots, representing about 50 μg of total protein, were adjusted for equivalent amounts of β-galactosidase activity and adjusted to a final volume of 105 μl with 250 mM Tris-HCl, pH 7.8. This mixture was added to 10 μl of 10 mM Acetyl-CoA (Sigma) prepared fresh in 250 mM Tris-HCl, pH7.8, 10 μl of [^{14}C] chloramphenicol (4 μCi at 57 mCi/mM, Amersham) and incubated for 30 min at 37°C. Samples were extracted in 1 ml of ethyl acetate (Pierce),

evaporated to dryness and resuspended in 30 μl of ethyl acetate. Aliquots (10 μl) were subjected to thin layer chromatography in a mixture of 95% chloroform and 5% methanol. Products were visualized by autoradiography with XAR film (Kodak) for 18 h at −70°C.

RESULTS AND DISCUSSION

In order to study the Tfm mouse AR at the molecular level, we have cloned and sequenced 2.8 kb of cDNA encoding the wild type Balb/c mouse AR (15) by RNA amplification with transcript sequencing (RAWTS) (16). Sequence analysis predicted that the wild type mAR cDNA contains an open reading frame of 2697 nucleotides encoding a polypeptide of 899 amino acids. From the predicted amino acid sequences, the putative DNA- and steroid-binding domains of the mAR are identical to that of the human and rat ARs (24−28).

Amplification and DNA Sequencing of the AR cDNAs in Wild Type and Tfm Mouse

Recent studies in our laboratory using sucrose gradient and gel filtration techniques together with antibodies against AR demonstrated that the Tfm receptor is smaller when compared to the wild type AR (9). To examine the possibility that there is a large deletion mutation in the coding region of the Tfm AR gene, the entire Tfm mouse AR coding region cDNA was amplified in three fragments using a PCR technique (Fig. 1a). When these three fragments from the Tfm mouse were compared with that of the wild type AR in an agarose gel, no significant differences in the sizes of the PCR products were found (Fig. 1b). A recent study (29) of Tfm mAR mRNA with Northern blot and RNase-A protection techniques suggested that the Tfm mAR RNA is expressed at a 10−20 fold lower level than the wild type mAR but is the same size. These data rule out the possibility that the Tfm mAR contains a large deletion in the coding region.

The entire cDNA sequence coding for the Tfm and wild type mAR were determined with both the RAWTS technique (15) and the cloning of PCR fragments into plasmids for sequencing. When the sequence of the Tfm mAR was compared with that of the wild type AR, a single base deletion was revealed in the amino-terminal domain of the Tfm mAR cDNA (Fig. 2a). At amino acid position 369−371 the cDNA sequence of the wild type receptor is CACCCCCCG, whereas the same region in the Tfm receptor is CACCCCCG. This mutation was further confirmed by amplifying and sequencing directly genomic DNAs from wild type, Tfm carrier female and Tfm/Y mice simultaneously (data not shown). To our knowledge, this is the first naturally occurring frame-shift mutation ever found in the steroid receptor supergene family.

In Vitro Expression of Wild Type and Tfm Mouse AR's

It can be predicted that the Tfm mAR translational reading frame is shifted by the single nucleotide deletion and that translation will stop at a cryptic TAG stop codon generated at amino acid 412 (Fig. 2b). Thus, the protein product should be terminated at about 45% of the length of the wild type AR. To test this hypothesis, the full-length wild type and Tfm AR cDNAs (Fig. 3a, A and B, respectively) were cloned into pBluescript II SK(+) vector, transcribed *in vitro*, translated in a rabbit reticulocyte lysate system, and immunoprecipitated with antibodies to AR. The wild type AR construct expressed a large protein of about 100,000 daltons (Fig.3b, lane A), which is the expected molecular weight calculated from the deduced amino acid sequence. In contrast, the Tfm mAR construct failed to produce the 100,000 dalton band. Instead, the Tfm construct produced three smaller

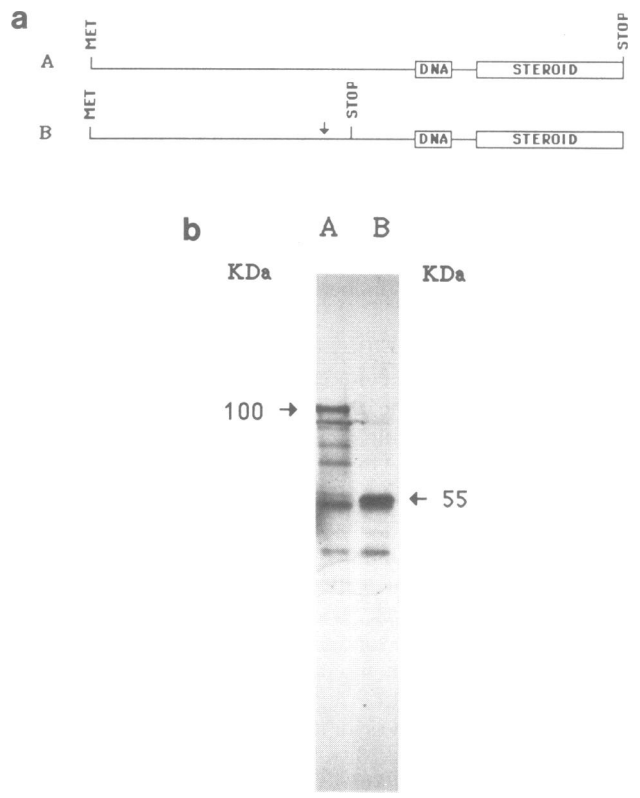

Fig. 3. Expression of the wild type and Tfm mARs *in vitro*. (**a**) Construction of wild type and mutant AR cDNAs. Construct **A** depicts the full-length wild type AR. The first methionine, DNA- and steroid-binding domains are indicated. Construct **B** depicts the full-length Tfm receptor. The arrow indicates the site of mutation. The stop codon indicates the predicted stop site generated due to the frame-shift mutation. (**b**) Expression of the mAR constructs *in vitro*. Constructs A and B were linearized and transcribed into RNAs *in vitro*. The RNAs were used to direct translation in a rabbit reticulocyte lysate system. Lanes A and B represent protein products from constructs **A** and **B**.

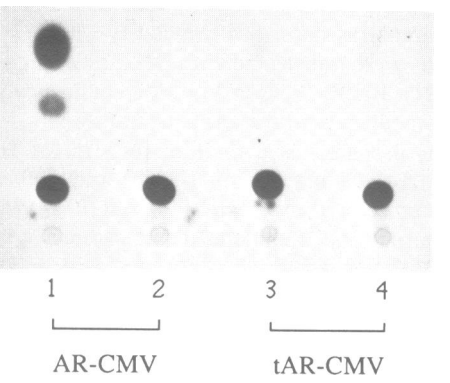

Fig. 4. Effect of the Tfm mutation on activation of MMTV-CAT transcription. QT-6 cells were transfected with CMV expression vectors containing either full-length wild type AR (AR-CMV, lane 1 and 2) or full-length Tfm AR (tAR-CMV, lane 3 and 4). Cells were incubated either with (lane 1 and 3) or without (lane 2 and 4) 10 nM DHT. The AR-CMV and tAR-CMV cDNAs were constructed as described in 'Materials and Methods.'

bands of about 55,000, 52,000 and 35,000 daltons (Fig.3b, lane B). These protein bands were not observed in the translation product from a transcript which lacked the AR cDNA insert (data not shown). Moreover, a control construct, with deletion of both the DNA- and steroid-binding domains, exhibited the 55,000 dalton band only (data not shown). Therefore, the 55,000 dalton band, which was not present in the wild type AR, may represent the prematurely terminated protein in the Tfm mouse. These data suggest that the translation of mAR in the Tfm mouse is indeed disrupted by the frame-shift mutation.

If the amino-terminus portion of the Tfm mAR is the only product produced in the Tfm mouse, it is in contrast to the previous observation (9) that the AR in the Tfm mouse exhibits low levels of high affinity binding to both androgen and DNA. One explanation of this discrepancy would be that more than one AR gene exists in the mouse genome. However, Southern blot analysis suggested that the AR is a single copy gene in the mouse genome (data not shown).

Another possible explanation is that translation of the Tfm AR mRNA is initiated from an internal AUG translational signal. Although most eukaryotic mRNAs are monocistronic, an increasing number of exceptions to the 'first AUG rule' have been noted (30–32). One mechanism for reaching an internal AUG codon exists when the up-stream AUG is followed by an in-frame terminator codon (33,34) as in the case of the Tfm mouse. In such cases, ribosomes can reinitiate translation at the next AUG codon downstream. A model for termination-reinitiation has been suggested (33) in which the ribosome remains associated with the mRNA following translation termination and can reinitiate translation if it encounters a nearby AUG. Interestingly, there are three in-frame AUG sites preceding the DNA-binding domain after the premature stop codon in the Tfm mAR mRNA. It can be predicted that the Tfm mAR RNA would direct the synthesis of a smaller-sized protein containing both DNA- and steroid-binding domains if one of these AUG translational initiation sites is used. Studies to test this hypothesis are being carried out currently in our laboratory.

Functional Analysis of the Wild Type and Tfm Mouse AR's

The ability of wild type AR and Tfm AR to transactivate an androgen responsive gene was tested by cotransfection of the wild type (AR-CMV) or Tfm (tAR-CMV) receptors together with the MMTV-CAT reporter gene. The MMTV-LTR has been shown previously to contain response elements to androgen, glucocorticoid, and progesterone (35). When MMTV-CAT was co-transfected with the wild type AR, CAT enzyme activity was induced with 10 nM of DHT (Fig 4, lane 1); whereas no CAT activity was detected in the absence of DHT (Fig 4, lane 2). In contrast, when MMTV-CAT was co-transfected with the Tfm AR, no CAT activity was observed either with (Fig 4, lane 3) or without DHT stimulation (Fig 4, lane 4). These data demonstrate that the frame-shift mutation in the Tfm mAR would impair the AR transactivation activity resulting in insensitivity of Tfm mice to androgens.

Our data demonstrate that a frame-shift mutation is present in the coding region of the Tfm mAR gene. This mutation would disrupt the reading frame of the normal AR translation as shown by in vitro translation experiments. The disruption of translation appears to be responsible for the absence of androgen responsiveness in these mice, since Tfm mAR constructs fail to transactivate an MMTV-CAT reporter gene. Moreover, the generation of an in-frame stop codon is most likely responsible for the reduced AR mRNA levels observed in Tfm mice (29,36). Reduction in mRNA levels due to a premature termination codon within an mRNA has been observed in many genes, including the human β-globin (37), the mouse muscular dystrophy (38), the human insulin (39), and the human AR (40) genes.

Recent studies of AIS patients suggest that a variety of mutations in the AR gene can cause androgen insensitivity in humans. Mutations identified thus far include a large deletion (41) and a point mutation (42) in the steroid binding domain, aberrant splicing (43), amber and ochre mutations (40,44,45). A single nucleotide change in the steroid binding region has been found to be responsible for androgen insensitivity in the Tfm rat (46). The Tfm mouse remains the most widely used animal model to examine the role of androgenic steroids in different biological processes. Therefore, an understanding of this mutation should facilitate a more detailed explanation of the mechanism of androgen action in target cells.

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ABBREVIATIONS

AIS: androgen insensitivity syndrome; Tfm: testicular feminization or testicular feminized; AMV: avian myeloblastosis virus; DHT: dihydrotestosterone; mAR: mouse androgen receptor; MMTV-CAT: mouse mammary tumor virus promoter linked to the chloramphenicol acetyltransferase gene; MMTV-LTR: mouse mammary tumor virus long terminal repeat; PCR: polymerase chain reaction; SDS: sodium dodecyl sulfate; RAWTS: RNA amplification with transcript sequencing; AR-CMV: wild type androgen receptor-cytomegalovirus; tAR-CMV: testicular feminization androgen receptor-cytomegalovirus.

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