HHS Public Access

Author manuscript

J Biol Chem. Author manuscript; available in PMC 2015 May 29.

Published in final edited form as:

J Biol Chem. 1989 September 25; 264(27): 16249–16255.

Expression Cloning and Regulation of Steroid 5α-Reductase, an Enzyme Essential for Male Sexual Differentiation*

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Abstract

The conversion of testosterone into the more potent androgen, dihydrotestosterone, catalyzed by the enzyme steroid 5α-reductase, is required for the differentiation of male external genitalia. Here, we report the isolation of cDNA clones encoding the rat steroid 5α-reductase using expression cloning in *Xenopus* oocytes. DNA sequence analysis demonstrates that the liver and ventral prostate forms of steroid 5α-reductase are identical hydrophobic proteins of 29 kDa. The amount of steroid 5α-reductase mRNA in liver increased in response to castration, but remained unchanged in the prostate. Testosterone administration to castrates induced expression of mRNA in the prostate but had no effect on liver. The data suggest that the steroid 5α-reductase gene is differentially regulated by testosterone in androgen-responsive *versus* non-responsive tissues.

A unique aspect of male sexual development is the requirement that the testis-derived hormone, testosterone,[1] must be converted into dihydrotestosterone in target tissues that differentiate to form the male external genitalia (1). This conversion is catalyzed by a microsomal enzyme, steroid 5α-reductase, in the anlage of the external genitalia (2). The absence of steroid 5α-reductase activity underlies a rare form of male pseudohermaphroditism, originally termed pseudovaginal perineoscrotal hypospadias, in which the male external genitalia differentiate as female structures (3, 4). In addition to its location in androgen-responsive tissues, high levels of steroid 5α-reductase activity are found in female rat liver but not in male rat liver (5). Whether this liver enzyme is the same as that in male target tissues is both controversial and unknown. Moreover, the factors that regulate the expression of this enzyme and the reason for the required conversion of testosterone into dihydrotestosterone for normal male differentiation are poorly understood (2).

*This research was supported in part by National Institutes of Health Grant HL 20948, the Perot Family Foundation, and Robert A. Welch Foundation Grant I-0971.

‡Recipient of National Institutes of Health Research Career Development Award HL01287.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05035.

[1]The abbreviations and trivial names used are: testosterone, 17β-hydroxy-4-androsten-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 4-MA, 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one; androstenedione, androst-4-ene-3,17-dione; androstanedione, 5α-androstane-3,17-dione; progesterone, 4-pregnene-3,20-dione; 5α-dihydroprogesterone, 5α-pregnane-3,20-dione; kb, kilobase(s).

The action of steroid 5α-reductase is a late event in male sexual development, a complex process that requires the correct developmental interpretation of both genetic and hormonal signals (1, 6). The process is thought to begin after the establishment of chromosomal sex at fertilization with the expression of a master regulatory protein termed the testis determining factor. The gene for this protein has recently been cloned and shown to encode a protein with a structural motif (the zinc finger) commonly found in transcription factors (7, 8). This finding is consistent with a role for this protein in the induction of a differentiation program leading to development of the testis (7). The testis in turn produces two hormones, testosterone, and a protein called the Müllerian inhibiting substance (9). The latter hormone causes regression of the Müllerian ducts, which are the anlage of female internal genitalia (1, 9). Testosterone promotes development of the male internal genitalia (epididymides, vasa deferentia, and seminal vesicles), and after conversion to dihydrotestosterone by steroid 5α-reductase, the differentiation of the external male structures (penis, scrotum, and prostate) (1).

The actions of both testosterone and dihydrotestosterone in male development are mediated through a single protein, the androgen receptor, a recently cloned member of the steroid hormone receptor family (10–13). Genetic defects in the androgen receptor prevent the differentiation of both internal and external male structures (14). Although dihydrotestosterone has been shown to bind to the androgen receptor with higher affinity than testosterone (15), it is presently not known why the action of the receptor in promoting the differentiation of the external male genitalia requires synthesis of the higher affinity ligand. This requirement must be attributable to the presence of other regulatory factors in the development of the external genitalia (2).

Although the role of steroid 5α-reductase in male sexual differentiation has been elucidated, molecular insights into the gene and protein have not yet been possible due to the lack of genetic and immunochemical probes. The enzyme has been partially purified from the rat and shown to be an integral membrane protein of the endoplasmic reticulum or nuclear membrane (16). Much controversy exists in the literature as to the number of steroid 5α-reductase isozymes present in the liver and prostate of the rat and their cofactor requirements (17, 18). Both the liver and prostate enzymes are inhibited in a competitive fashion by the steroid analogue 17β-*N*,*N*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA), suggesting that these proteins must at least share sequence homology in their substrate binding domains (19). Consistent with this prediction are the findings that the liver and prostate enzymes catalyze the reduction of similar steroid substrates, including testosterone, androstenedione, and progesterone (5, 20).

In this paper, we describe the cloning and sequence of cDNAs encoding the rat liver and prostate steroid 5α-reductase enzymes. To circumvent the lack of probes for these cDNAs, we modified a *Xenopus* oocyte expression cloning strategy originally developed for the isolation of lymphokines (21), neurotransmitter receptors (22–24), and membrane transporters (25). The amino acid sequences deduced from the cDNAs reveal that the liver and prostate forms of steroid 5α-reductase are identical. However, RNA blotting experiments suggest that the expression of steroid 5α-reductase in these two tissues is differentially regulated by testosterone.

EXPERIMENTAL PROCEDURES

Steroid 5α-Reductase Enzyme Assay

Stage 5 and 6 oocytes were surgically removed from female *Xenopus laevis* (NASCO, Fort Atkinson, WI) and collagenase-treated as described by Julius *et al.* (24). Oocytes were injected with 50–100 nl of RNA (1 μg/μl) as described by Peacock *et. al.* (26). After injection the oocytes were incubated at 19 °C for 24 h in modified Barth's saline solution (26) containing 1 mg/ml bovine serum albumin to allow expression of the injected RNA. Five to ten viable oocytes were then transferred to 1 ml of modified Barth's saline solution containing 5 μM ^{14}C-labeled steroid (50 mCi/mmol, Du Pont-New England Nuclear), and incubated at 37 °C for 2–24 h. This temperature-jump protocol is based on the observation that expression of mRNA in *Xenopus* is maximal at 19 °C, whereas rat steroid 5α-reductase expressed in *Xenopus* has a temperature optima of 37 °C (unpublished results). After the 37 °C incubation, the oocytes were homogenized in the incubation medium and steroids were extracted with 10 ml of dichloromethane. The solvent was evaporated under air and the residue was dissolved in 0.1 ml of chloroform/methanol (2:1, v/v) and subjected to thin-layer chromatography using Silica Gel 60 thin-layer chromatography plates (E. Merck, 5748-7, Darmstadt, West Germany). The mobile phase was chloroform/ethyl acetate (3:1, v/v). The chromatoplates were autoradiographed for 18 h at −70 °C and the radioactive zones were cut out and subjected to liquid scintillation counting in Complete Counting Cocktail (Research Products International). The identities of the products were determined by comparison to the R_F values of known standards.

cDNA Cloning

Total RNA from female rat liver was extracted by a guanidinium isothiocyanate/CsCl procedure (27). Poly(A^+)-enriched RNA was isolated and size-fractionated by density gradient centrifugation on 10–25% (w/v) sucrose gradients containing methylmercury hydroxide (28). After centrifugation at 4 °C for 15 h at 76,800 × *g*, aliquots of RNA from each gradient fraction were assayed for steroid 5α-reductase mRNA by injection into *Xenopus* oocytes. Positive fractions from the sucrose gradients were combined and the RNA was concentrated by ethanol precipitation. First strand cDNA was synthesized using mRNA pretreated with 2.5 mM methylmercury hydroxide and AGCGGCCGC$(T)_{20}$ as a primer. Second strand synthesis, *Eco*RI methylation, flushing of ends with bacteriophage T4 DNA polymerase, and addition of phosphorylated *Eco*RI linkers were performed according to standard procedures (27). The resulting cDNA was digested with *Not*I and *Eco*RI and size-fractionated on a 1% (w/v) agarose gel. Complementary DNAs greater than 1.3 kb were inserted into the EcoRI and *Not*I sites of pBluescript (Stratagene, La Jolla, CA). Recombinant plasmids were propagated in *Escherichia coli* DH5αF′IQ (GIBCO). A rat ventral prostate cDNA library was constructed as described above except that random hexanucleotides we e used as primers and total poly(A^+) RNA was used as template. Si: e-fractionated cDNAs derived from prostate mRNA were inserted into the EcoRI site of λZapII (Stratagene). Recombinant bacteriophage were propagated in *E. coli* XLl-Blue. Bluescript plasmids were subsequently rescued from λZap recombinants by superinfection with helper Fl bacteriophage.

In the initial screening of the female rat liver cDNA library, plasmid minipreps were prepared from 20 pools containing 150–200 cDNA clones/pool. Plasmid DNA was linearized with *Not*I and RNA was transcribed *in vitro* using bacteriophage T7 RNA polymerase (Pharmacia LKB Biotechnology Inc.) as described by Julius *et al.* (24). *Xenopus* oocyte injection was carried out as described above. Plasmid DNA from one positive pool was retransformed and 960 colonies were randomly picked into individual 0.3-ml cultures maintained in 96-well microtiter plates. Plasmid DNAs were subsequently prepared from pools of 100-μ1 aliquots from each well and assayed by microinjection. Sibling selection from the microtiter plate was carried out by matrix analysis as described in the text.

Nucleic Acid Sequencing and Primer Extension

Overlapping fragments from both DNA strands were subcloned into bacteriophage M13 vectors and sequenced by automated methods (29) using an Applied Biosystems model 370A DNA sequencer. For primer extension analysis, an antisense oligonucleotide complimentary to nucleotides 70–109 of Fig. 4*A* was annealed at 68 °C to rat liver poly(A^+) RNA and extended with reverse transcriptase as described by Südhof *et al.* (30). Direct RNA sequencing of the steroid 5α-reductase mRNA was carried out as described by Geliebter *et al.* (31).

In Vitro Translation of RNA

Approximately 100 ng of RNA was translated *in vitro* using [^{35}S] methionine (1100 Ci/mmol) and a rabbit reticulocyte lysate (Promega, Madison, WI) in the presence or absence of dog pancreas microsomes (32). After incubation for 1 h at 30 °C, the reactions were terminated by adding cycloheximide to a final concentration of 0.2 m_M or RNase A to 2 mg ml^{-1}. Experiments with products translated *in vitro* in the presence of 50 μg/ml trypsin (GIBCO) were performed with or without 2% (w/v) Triton X-100 (Boehringer Mannheim) for 30 min at 22 °C. The protease reactions were terminated by adding soybean trypsin inhibitor (Cappel, Malvern, PA) to a final concentration of 1 mg ml^{-1}.

Physiology Experiments

Experiments were designed to allow comparison of mRNA levels in liver and prostate of normal rats, of 7-day castrated animals, of 10-day castrated animals, and of normal or 10-day castrated animals given testosterone on days 7–9 of the experiment. Sexually mature Sprague-Dawley male rats were castrated by standard surgical procedures on day 0. On day 7, experimental groups were subcutaneously injected for 3 consecutive days with 2 mg of testosterone acetate or testosterone propionate dissolved in 0.2 ml of sesame oil (5). Control animals were injected with sesame oil alone. On day 10 of the experiment, RNA was prepared from the livers and prostates of up to 15 animals in each experimental group, and analyzed by blotting as described in the legend to Fig. 8.

RESULTS AND DISCUSSION

Expression Cloning of the Rat Liver Steroid 5α-Reductase cDNA

Fig. 1 outlines the strategy used to obtain a full length cDNA for the rat liver steroid 5α-reductase. As a source of mRNA, we used female rat liver, which for physiologically

unknown reasons expresses high levels of steroid 5α-reductase enzyme activity (16). Microinjection into *Xenopus* oocytes indicated that this mRNA could direct the synthesis of an enzyme that catalyzed the conversion of steroids into their 5α-reduced forms (see below). Sucrose gradient fractionation of rat liver mRNA indicated that this activity was encoded by an mRNA of about 2.5 kb (Fig. 1). Similar results have recently been obtained by Farkash *et al.* (33). The mRNA in this fraction was converted into cDNA, size-fractionated, and cloned into an RNA expression vector. To avoid problems with anti-sense inhibition, the cDNA library was constructed in an oriented manner (Fig. 1). Twenty pools, each containing 150–200 cDNA clones, were then used to synthesize mRNA that was in turn injected into oocytes to allow determination of steroid 5α-reductase activity by thin-layer chromatography analysis. From one active pool, a near full length cDNA encoding this enzyme was subsequently isolated by dilution cloning (Fig. 1).

Fig. 2 illustrates the results of thin-layer chromatography assays from the dilution cloning. In all experiments, assay of steroid 5α-reductase activity in injected oocytes was carried out using a temperature-jump protocol as detailed under “Experimental Procedures.” Microinjection of water into *Xenopus* oocytes revealed an endogenous activity capable of converting the testosterone substrate into androstenedione, and little or no ability to convert these steroids into their 5α-reduced forms (*lane 1*). In contrast, when female rat liver mRNA was injected, the oocytes expressed an activity that generated both dihydrotestosterone and 5α-androstanedione, as well as at least two other steroid metabolites (*lane 2*). These latter unidentified steroids were derived from the 5α-reduced metabolites generated by the injected mRNA (see below). *Lane 3* shows the results obtained when RNA was synthesized from one of the initial 20 cDNA plasmid pools that contained 150–200 independent clones. The spectrum of steroid metabolites observed was identical to that seen upon injection of liver mRNA, indicating that this pool must contain at least one steroid 5α-reductase cDNA. The cDNAs from this pool were retransformed into *E. coli* and individual colonies were picked into microtiter plates. *Lane 4* shows the results obtained after microinjection of RNA prepared from plasmids isolated from a 96-well plate that contained a steroid 5α-reductase cDNA from this transformation. Subsequent analysis of mRNA from pools of plasmids corresponding to the rows and columns of this microtiter plate identified a row (*lane 5*) and column (*lane 6*) containing a steroid 5α-reductase plasmid. The intersection of this row and column on the microtiter plate localized the positive cDNA (*lane 7*).

Substrate Specificity of Cloned Liver Steroid 5α-Reductase

RNA synthesized from the steroid 5α-reductase cDNA plasmid identified in Fig. 2 was microinjected into oocyte for a 24-h period. The oocytes were then incubated with different radiolabeled steroids for an additional 24 h and the products formed were analyzed by thin-layer chromatography. Fig. 3, *lane 1*, shows the typical pattern of 5α-reduced metabolites formed from testosterone. *Lane 2* indicates that co-incubation of the injected eggs with equimolar amounts of testosterone and the competitive steroid 5α-reductase inhibitor 4-MA resulted in a substantial decrease in the formation of these products. As a control for nonspecific inhibition, the conversion of testosterone into androstenedione catalyzed by an endogenous *Xenopus* enzyme (presumably a 17β-hydroxysteroid dehydrogenase) (34), was not inhibited by 4-MA in this experiment (*lane 2*). The data in *lanes 3* and 7 indicate that

both androstenedione and progesterone were substrates for the cloned enzyme. As with testosterone, 4-MA efficiently blocked the reduction of these steroids (*lanes 4* and *8*, respectively). When radiolabeled dihydrotestosterone was used as a substrate (*lane 5*), the inhibitor had no effect on the conversion of this compound into other 5α-reduced metabolites by endogenous *Xenopus* enzymes (*lane 6*).

Sequence of Liver Steroid 5α-Reductase

Fig. 4*A* shows the nucleotide sequence of the liver steroid 5α-reductase cDNA and the deduced amino acid sequence of the protein. The cDNA insert in the expressing clone was 2,465 base pairs in length and included a long 3′-untranslated region of 1,691 base pairs and an extended translation reading frame of 765 base pairs. A potential polyadenylation signal is present at position 2,446, upstream of a tract of A residues, suggesting that the 3′ end of this cDNA is authentic. In the predicted amino acid sequence, there are three methionine residues in the first 19 amino acids. The context of the first ATG is identical in six out of nine nucleotides with the ideal Kozak consensus sequence (35), suggesting that this codon may specify the amino-terminal methionine of steroid 5α-reduc-tase. With this assumption, the open reading frame would encode a hydrophobic protein of 255 amino acids with a predicted M_r of 29,343. Over 50% of the amino acids in the protein sequence have hydrophobic side chains. Consistent with this amino acid composition, a hydropathy plot (Fig. 4*B*) suggests a protein with many hydrophobic regions. A comparison of the sequence shown in Fig. 4*A* to others in the National Biomedical Research Foundation protein data bank and the GenBank DNA sequence collection did not reveal any sequences that were homologous to steroid 5α-reductase.

Characterization of Steroid 5α-Reductase Protein and mRNA

Several reports in the literature have identified a rat liver protein of M_r 50,000 that either has steroid 5α-reductase activity or can be cross-linked to a photoactivatable derivative of 4-MA (36, 37). To ensure that the sequence shown in Fig. 4*A* represented the complete coding region of steroid 5α-reductase, we carried out three kinds of experiments. First, as shown in Fig. 5, *in vitro* translation in a rabbit reticulocyte lysate of RNA generated from the steriod 5α-reductase cDNA yielded a protein product with an apparent M_r of 26,000 (*lane 3*). When the translation reactions were carried out in the presence of dog pancreas microsomes, a protein product of identical size was observed (*lane 4*), suggesting the absence of a cleavable signal sequence in this protein. That the steroid 5α-reductase translated *in vitro* was incorporated into microsomes was demonstrated by protease protection experiments. If the vesicular structure of the microsomes was maintained, the translated product was largely resistant to digestion by trypsin (*lane 5*). However, if the microsomes were disrupted with the detergent Triton X-100 prior to protease treatment, then the steroid 5α-reductase protein was susceptible to digestion (*lane 6*).

We next determined the approximate location of the car-boxyl terminus of the protein by analyzing the expression of RNA derived from a series of 3′-truncated derivatives of the cDNA. The steroid 5α-reductase cDNA plasmid was linearized by cleavage with four restriction enzymes that left intact or removed progressively larger portions of the predicted 3′-untranslated region and/or carboxyl terminus of the protein. RNA was transcribed *in vitro*

from these templates, microinjected into oocytes, and the oocytes were assayed for steroid 5α-reductase activity using testosterone as a substrate. As summarized in Fig. 6*A*, expression of the intact steroid 5α-reductase RNA resulted in the reduction of 67% of the testosterone substrate. Removal of 1474 nucleotides from the 3′-untranslated region of the mRNA did not substantially affect expression of enzyme activity (*Bam*HI-cleaved template, Fig. 6*A*). However, removal of 1830 nucleotides from the 3′ end, which removes 47 amino acid residues from the predicted carboxyl terminus of the protein, eliminated steroid 5α-reductase activity (*Pvu*II-cleaved template, Fig. 6*A*). Similar results were obtained with a truncated RNA that removed 57 residues from the carboxyl terminus of the protein (*Sac*I-cleaved template, Fig. 6*A*). In experiments not shown, all of these mRNAs yielded a protein of the appropriate size after *in vitro* translation in a reticulocyte lysate.

The amino-terminal region of steroid 5α-reductase was examined by carrying out primer extension experiments on liver mRNA. An oligonucleotide primer 40 bases in length and complementary to nucleotides 70–109 of Fig. 4*A* was radiolabeled, annealed to mRNA from female and male rat liver, and extended with reverse transcriptase. As shown in Fig. 6*B*, a single product of 125 nucleotides was detected when RNA from female or male liver was used as a template. These results are consistent with a single 5′ end for the steroid 5α-reductase mRNA in this tissue and suggests that the cDNA sequence shown in Fig. 4*A* represents a near full length clone. In additional experiments not shown, the steroid 5α-reductase mRNA in female rat liver was sequenced directly using the above primer. The results indicated that the mRNA extends only 17 nucleotides upstream of the 5′ end of the cDNA sequence shown in Fig. 4*A*. There were no inframe translation stop codons in this 5′ sequence.

The Liver and Ventral Prostate Forms of Steroid 5α-Reductase Are Identical

To determine if the steroid 5α-reductase activities in the liver and prostate were due to the expression of a single mRNA, we screened a randomly primed cDNA library derived from ventral prostate mRNA with the insert derived from the liver cDNA clone. A single prostate cDNA was isolated after screening approximately 150,000 independent clones. DNA sequence analysis of the 5′ and 3′ ends of this clone indicated that it began at nucleotide 1 and terminated at nucleotide 1955 of the liver cDNA sequence shown in Fig. 4*A*. The sequences were identical between the two clones in these regions. The complete coding region of the prostate-derived cDNA was further subjected to DNA sequence analysis and comparison to that of the liver cDNA again revealed no differences. These results suggested that the enzyme activities in these two tissues were the consequence of expression of the same mRNA. It remained possible, however, that the liver form of steroid 5α-reductase was also expressed in the prostate and that we had in fact isolated a second cDNA encoding the liver enzyme.

We next carried out a series of blot hybridization experiments (Fig. 7). RNA was isolated from female and male rat liver and prostate, electrophoresed on an agarose gel, transferred to a nylon membrane, and probed with the full length liver cDNA insert. An identically sized mRNA of approximately 2.4 kb was detected in all three tissues (Fig. 7*A*). The relative abundance of this mRNA (female liver > male liver > prostate) closely paralleled steroid 5α-

reductase enzyme activity in these three tissues (16). In agreement with the identically sized mRNAs detected in these two tissues, primer extension analysis of prostate mRNA indicated that the 5′ end of the steroid 5α-reductase mRNA in this tissue was identical to that in the liver (data not shown).

Hybridization of the steroid 5α-reductase cDNA to rat genomic DNA provided additional evidence that the liver and prostate forms of this enzyme are encoded by the same gene. As shown in Fig. 7*B*, a small number of hybridizing bands were detected after digestion of genomic DNA with four different restriction enzymes. In some lanes (*e.g. Bam*HI-digested DNA) there were differences in the intensities of the hybridization signals. We believe this result is due to the presence of a larger or smaller number of exons on a given DNA fragment rather than to the existence of multiple steroid 5α-reductase genes. This conclusion is substantiated by the results obtained with the enzyme *Eco*RI that cleaves the cDNA roughly in half (nucleotide position 1334, Fig. 4*A*), and thus would be expected to, and does, generate at least two hybridizing bands of equal intensity on the autoradiogram (Fig. 7*B*).

Regulation of Steroid 5α-Reductase Expression in the Ventral Prostate

The expression of steroid 5α-reductase enzyme activity in the liver and prostate has been shown to be under hormonal control (5, 38, 39). To examine the possibility that this regulation might be exerted at the transcriptional level, and to provide further physiological evidence that our cDNA encoded steroid 5α-reductase, we determined mRNA levels in the liver and prostate after administration of androgens to castrated animals (Fig. 8). In the liver of untreated animals, the steroid 5α-reductase cDNA probe hybridized to an mRNA of 2.4 kb (*lane 1*). Subcutaneous administration of testosterone to normal animals did not affect the expression of this mRNA (*lane 5*). In contrast, the levels of steroid 5α-reductase mRNA in the liver increased approximately 5-fold in the 7-day (*lane 2*) and 10-day (*lane 4*) castrated animals. In this experiment, testosterone did not decrease the enhanced expression of the steroid 5α-reductase mRNA in the castrated animals (*lane 3*), suggesting that this hormone may work in concert with other regulatory influences to control expression of steroid 5α-reductase in the liver.

In the ventral prostate of normal animals, a much lower level of an identically sized mRNA was detected (Fig. 8, *lane 6*). Pharmacological levels of testosterone did not increase this level of expression (*lane 10*), nor did castration for a 7-day or 10-day period (*lanes 7* and *9*, respectively). However, a dramatic 9-fold increase in steroid 5α-reductase mRNA levels was detected in castrated animals given testosterone for a 3-day period (*lane 8*). Subsequent hybridization of this filter with actin and cyclophilin cDNA probes indicated that near identical amounts of RNA were electrophoresed in each lane (data not shown). The results obtained with the prostate RNA provide strong evidence that the cDNA we have isolated does in fact encode steroid 5α-reductase.

Conclusions

The data presented here provide evidence that a single mRNA encodes the enzyme steroid 5α-reductase in both the liver and prostate of the rat. DNA sequence analysis indicates that this mRNA encodes a hydrophobic protein with a M_r of 29,000. This size was confirmed by

in vitro translation, mRNA truncation experiments, and primer extension analysis of liver mRNA. Blot hybridization analyses of RNA and genomic DNA support the existence of a single mRNA and gene for steroid 5α-reductase. Experiments in a castrated animal model suggest that the expression of steroid 5α-reductase may be under transcriptional control in both the liver and ventral prostate. Additional experiments are currently in progress to determine the mechanism of the apparent differential regulation by testosterone in these two tissues.

The contributions of steroid 5α-reductase and its product dihydrotestosterone to male sexual development are clearly illustrated by the clinical syndrome of pseudovaginal perineoscrotal hypospadias (14). In addition to this developmental role, dihydrotestosterone in the mature organism is involved in the normal maintenance of many different cellular and organ processes. In fact, the suggestion has been made that it is dihydrotestosterone and not testosterone that is the more important androgen in this regard (2). As such, abnormal expression of steroid 5α-reductase and subsequent dihydrotestosterone synthesis may contribute to a large number of human diseases and endocrine abnormalities. Localized overproduction of dihydrotestosterone in the prostate is postulated to be a factor in benign prostate hypertrophy, a condition that affects a majority of elderly men (40). Similarly, dihydrotestosterone has been implicated in the formation of acne and in the manifestation of male pattern baldness (41). Finally, a role for this hormone in the development and or susceptibility to cancer of the prostate, the second most prevalent form of cancer in the United States, has been hypothesized (42). The precise contribution of steroid 5α-reductase to these disease states has so far remained uncertain due to the absence of biochemical and genetic tools. The results presented here clarify many controversies that have existed in the literature for 20 years concerning this enzyme and they may provide these necessary tools.

Acknowledgments

We thank Gloria Brunschede, Daphne Davis, and Daphne Norsworthy for excellent technical assistance, David Julius for helpful advice, J. Ian Mason for steroids and advice, Jim Metherall for microsomes, Richard Gibson for veterinary assistance, and Mike Brown, Joe Goldstein, and Jean Wilson for advice and critical reading of the manuscript.

REFERENCES

1. Wilson JD. Annu. Rev. Physiol. 1978; 40:279–306. [PubMed: 345951]
2. Wilson JD. Handb. Physiol. 1975; 5:491–508.
3. Walsh PC, Madden JD, Harrod MJ, Goldstein JL, MacDonald PC, Wilson JD. N. Engl. J. Med. 1974; 291:944–949. [PubMed: 4413434]
4. Imperato-McGinley J, Guerrero L, Gautier T, Peterson RE. Science. 1974; 186:1213–1217. [PubMed: 4432067]
5. Moore RJ, Wilson JD. Endocrinology. 1973; 93:581–592. [PubMed: 4146481]
6. Jost A. Harvey Lect. 1960; 55:201–226. [PubMed: 13790784]
7. Page DC, Mosher R, Simpson EM, Fisher EMC, Mardon G, Pollack J, McGillivray B, de la Chapelle A, Brown LG. Cell. 1987; 51:1091–1104. [PubMed: 3690661]
8. Evans RM, Hollenberg SM. Cell. 1988; 52:1–3. [PubMed: 3125980]
9. Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, Fisher RA, Bertonis JM, Torres G, Wallner BP, Ramachandran KL, Ragin RC, Manganaro TF, MacLaughlin DT, Donahoe PK. Cell. 1986; 45:685–698. [PubMed: 3754790]

10. Chang C, Kokontis J, Liao S. Proc. Natl. Acad. Sci. U. S. A. 1988; 85:7211–7215. [PubMed: 3174628]
11. Lubahn DB, Joseph DR, Sar M, Tan J, Higgs HN, Larson RE, French FS, Wilson EM. Mol. Endocrinol. 1988; 2:1265–1275. [PubMed: 3216866]
12. Tilley WD, Marcelli M, Wilson JD, McPhaul MJ. Proc. Natl. Acad. Sci. U. S. A. 1989; 86:327–331. [PubMed: 2911578]
13. Evans RM. Science. 1988; 240:889–895. [PubMed: 3283939]
14. Griffin, JE.; Wilson, JD. The Metabolic Basis of Inherited Disease. Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. New York: McGraw-Hill; 1989. p. 1919-1944.
15. Wilbert DM, Griffin JE, Wilson JD. J. Clin. Endocrinol. Metab. 1983; 56:113–120. [PubMed: 6183286]
16. Moore RJ, Wilson JD. J. Biol. Chem. 1972; 247:958–967. [PubMed: 4400474]
17. McGuire JS Jr, Tomkins GM. J. Biol. Chem. 1960; 235:1634–1635.
18. Ichihara K, Tanaka C. Biochem. Int. 1987; 15:1005–1011. [PubMed: 3124852]
19. Liang T, Heiss CE, Ostrove S, Rasmusson GH, Cheung A. Endocrinology. 1983; 112:1460–1468. [PubMed: 6832056]
20. McGuire JS Jr, Hollis VW Jr, Tomkins GM. J. Biol. Chem. 1960; 235:3112–3117.
21. Noma Y, Sideras P, Naito T, Bergstedt-Lindquist S, Azuma C, Severinson E, Tanabe T, Kinashi T, Matsuda F, Yaoita Y, Honjo T. Nature. 1986; 319:640–646. [PubMed: 3005865]
22. Masu Y, Nakayama K, Tamaki H, Harada Y, Kuno M, Nakanishi S. Nature. 1987; 329:836–838. [PubMed: 2823146]
23. Lübbert H, Hoffman BJ, Snutch TP, Van Dyke T, Levine AJ, Hartig PR, Lester HA, Davidson N. Proc. Natl. Acad. Sci. U. S. A. 1987; 84:4332–4336. [PubMed: 3473504]
24. Julius D, MacDermott AB, Axel R, Jessell TM. Science. 1988; 241:558–564. [PubMed: 3399891]
25. Hediger MA, Coady MJ, Ikeda TS, Wright EM. Nature. 1987; 330:379–331. [PubMed: 2446136]
26. Peacock SL, Bates MP, Russell DW, Brown MS, Goldstein JL. J. Biol. Chem. 1988; 263:7838–7845. [PubMed: 3372507]
27. Maniatis, T.; Fritsch, EF.; Sambrook, J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1982. p. 1-545.
28. Schweinfest CW, Kwiatkowski RW, Dottin RP. Proc. Natl. Acad. Sci. U. S. A. 1982; 79:4997–5000. [PubMed: 6956910]
29. Smith LM, Sanders JZ, Kaiser RJ, Hughes P, Dodd C, Connell CR, Heiner C, Kent SBH, Hood LE. Nature. 1986; 321:674–679. [PubMed: 3713851]
30. Südhof TC, Russell DW, Brown MS, Goldstein JL. Cell. 1987; 48:1061–1069. [PubMed: 3030558]
31. Geliebter J, Zeff RA, Melvold RW, Nathenson SG. Proc. Natl. Acad. Sci. U. S. A. 1986; 83:3371–3375. [PubMed: 3458188]
32. Walter P, Ibrahimi I, Blobel G. J. Cell Biol. 1981; 91:545–550. [PubMed: 7309795]
33. Farkash Y, Soreq H, Orly J. Proc. Natl. Acad. Sci. U. S. A. 1988; 85:5824–5828. [PubMed: 2457902]
34. Miller WL. Endocr. Rev. 1988; 9:295–318. [PubMed: 3061784]
35. Kozak M. Cell. 1986; 44:283–292. [PubMed: 3943125]
36. Liang T, Cheung AH, Reynolds GF, Rasmusson GH. J. Biol. Chem. 1985; 260:4890–4895. [PubMed: 3988737]
37. Cheng K-C. FASEB J. 1988; 2:355. (abstr.).
38. McGuire JS Jr, Tomkins GM. J. Biol. Chem. 1959; 234:791–794. [PubMed: 13654264]
39. Gustafsson JÅ, Mode A, Norstedt G, Skett P. Annu. Rev. Physiol. 1983; 45:51–60. [PubMed: 6133501]
40. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y. Endocr. Rev. 1987; 8:338–362. [PubMed: 3308446]
41. Mooradian AD, Morley JE, Korenman SG. Endocr. Rev. 1987; 8:1–28. [PubMed: 3549275]

42. Lippman, ME. William's Textbook of Endocrinology. 7th Ed.. Wilson, JD.; Foster, DW., editors. Philadelphia: W. B. Saunders Company; 1981. p. 1309-1326.
43. Kyte J, Doolittle RF. J. Mol. Biol. 1982; 157:105–132. [PubMed: 7108955]
44. Lehrman MA, Russell DW, Goldstein JL, Brown MS. J. Biol. Chem. 1987; 262:3354–3361. [PubMed: 3818645]
45. Lehrman MA, Schneider WJ, Südhof TC, Brown MS, Goldstein JL, Russell DW. Science. 1985; 227:140–146. [PubMed: 3155573]
46. Church GM, Gilbert W. Proc. Natl. Acad. Sci. U. S. A. 1984; 81:1991–1995. [PubMed: 6326095]

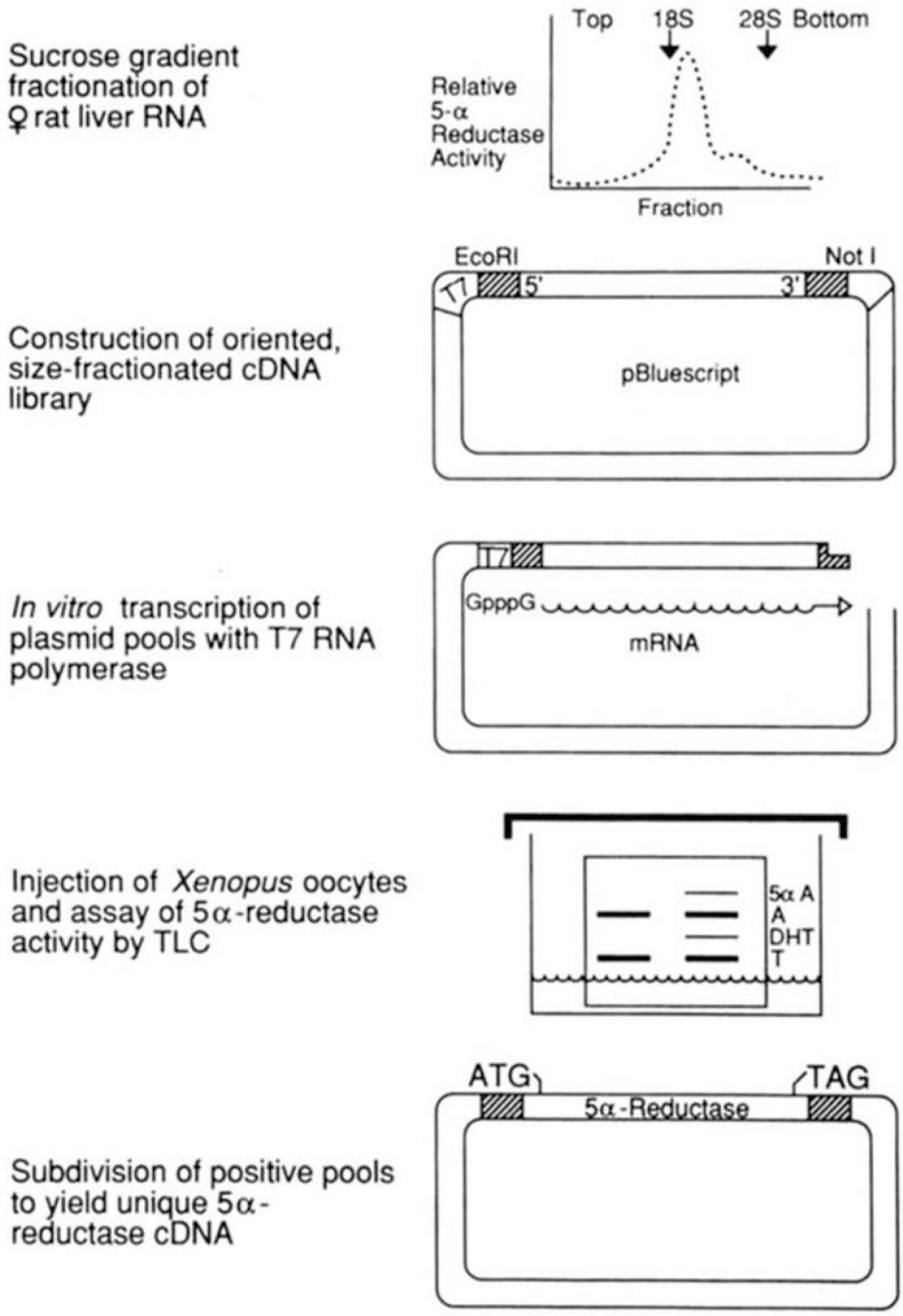

Fig. 1. Expression cloning of steroid 5α-reductase

Female rat liver RNA was size fractionated on 10–25% sucrose gradients and aliquots of RNA were assayed for steroid 5α-reductase activity in *Xenopus* oocytes. Peak activity fractions were used to construct an oriented cDNA library in a plasmid RNA expression vector. *E. coli* transformants from this library were pooled in groups of 150–200 clones and assayed for enzyme expression. A thin layer chromatography assay was employed in which the substrate testosterone (*T*) could be separated from androstenedione (*A*) and the 5α-

reduced forms of these two steroids (*DHT* and 5α*A*, respectively). Sibling selection of a positive pool of clones was carried out as described in the text.

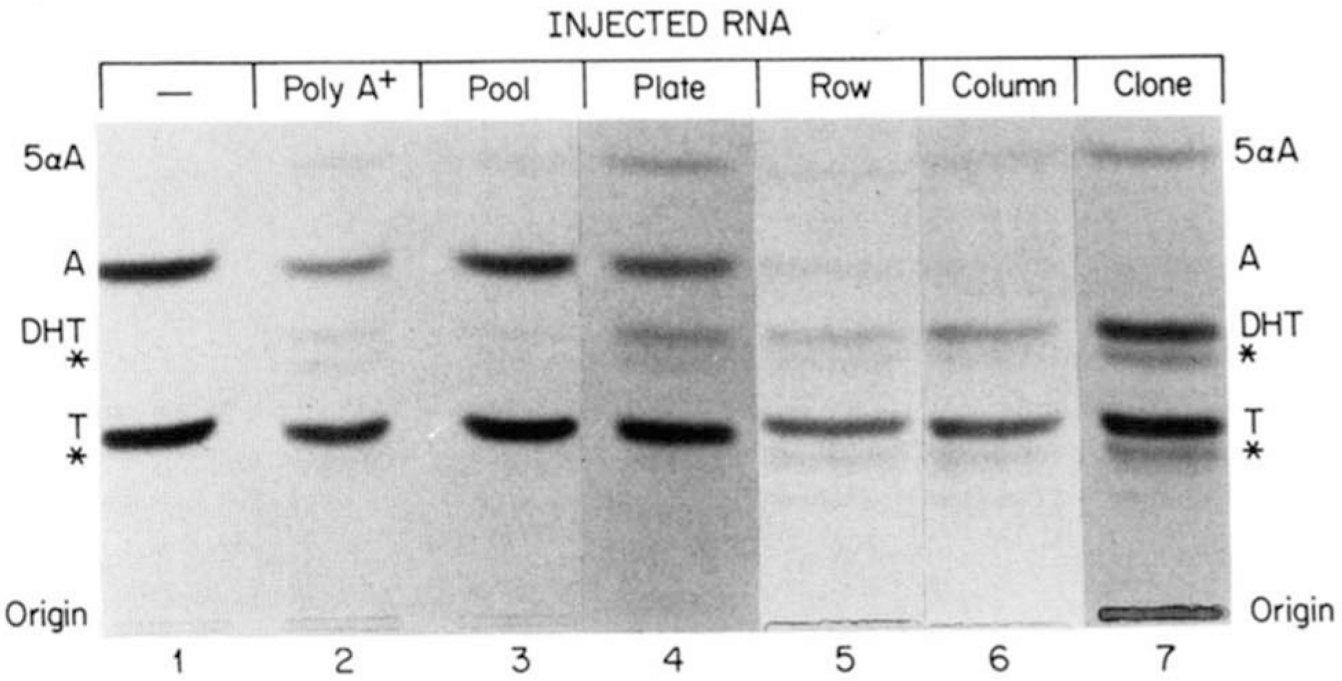

Fig. 2. Dilution cloning of a liver steroid 5α-reductase cDNA

Xenopus oocytes were injected with RNA from the indicated source and assayed for steroid 5α-reductase activity by thin-layer chromatography using [^{14}C]testosterone as a substrate as described under "Experimental Procedures." *Lane 1*, H_2O-injected; *lane 2*, RNA from female rat liver; *lane 3*, RNA synthesized *in vitro* from a pool of 150–200 cDNA clones; *lane 4*, RNA synthesized from cDNAs inoculated in a 96-well microtiter plate; *lane 5*, RNA synthesized from a pool of 12 clones corresponding to a row from the microtiter plate; *lane 6*, RNA synthesized from eight clones corresponding to a column from this plate; and *lane 7*, RNA derived from a cDNA clone corresponding to the intersection of the row and column. Chromatograms from the various experiments were exposed to Kodak XAR-5 film for 16 h. In the chromatographic system employed, hydrophobic steroids migrate further than hydrophilic steroids. The positions of authentic steroid standards are shown on the left of the autoradiograms. *T*, testosterone, *A*, androstenedione, *DHT*, 5α-dihydrotestosterone, *5αA*, 5α-androstanedione. An endogenous *Xenopus* enzyme in the oocyte converts testosterone into androstenedione. Steroids marked with a *asterisk* are uncharacterized metabolites derived from the 5α-reduce compounds by endogenous *Xenopus* enzymes (see Fig. 3). The amount of 5α-reduced metabolites in a given experiment varied depending on the batch of oocytes injected and is thus not calculated here.

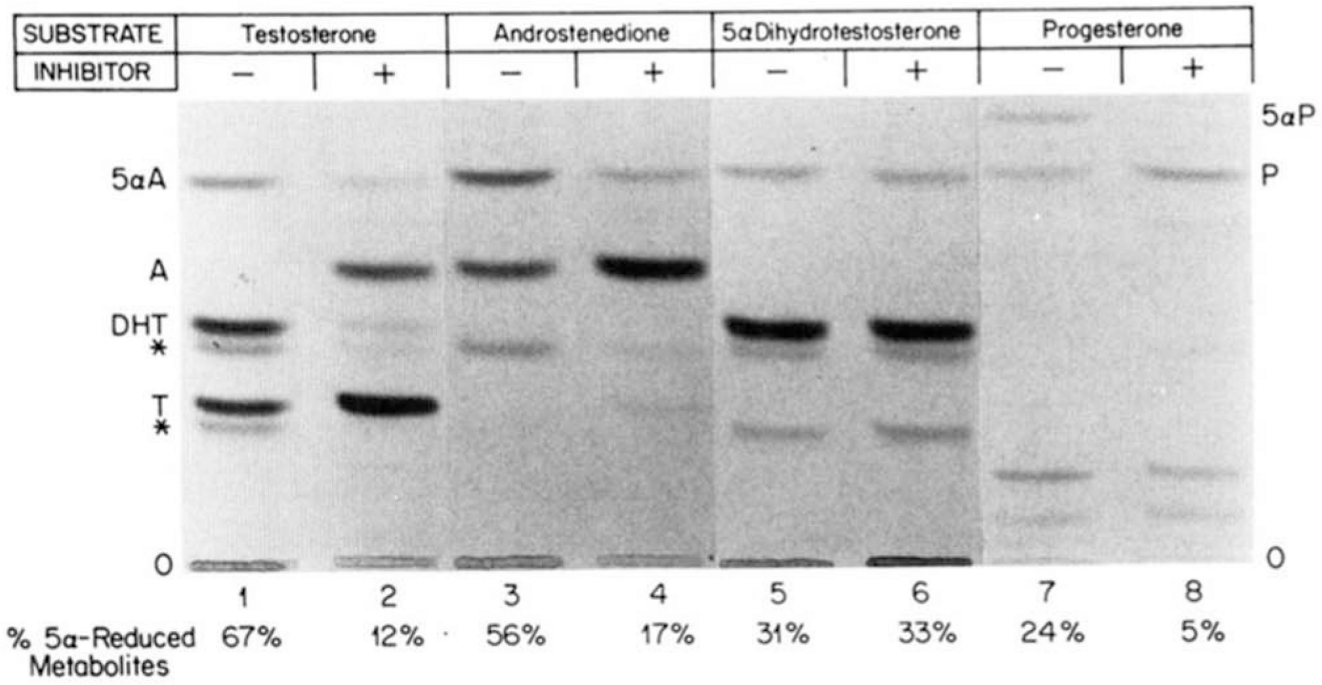

Fig. 3. Substrate specificity of the cloned steroid 5α-reductase

Xenopus oocytes obtained from a single animal were injected with *in vitro* synthesized RNA derived from the steroid 5α-reductase cDNA clone and then assayed for enzyme activity using the indicated ^{14}C-labeled steroid substrates (5 μM) in the absence (−) or presence (+) of the competitive inhibitor 4-MA (5 μm). The various steroids and metabolites are identified on the left and right of the autoradiograms: *P*, progesterone; 5α*P*, 5α-dihydroprogesterone; others are as indicated in the legend to Fig. 2. The amount of 5α-reduced metabcolites for each substrate is indicated at the bottom of the figure and was determined by liquid scintillation counting after cutting out appropriate zones from the chromatograms. In *lanes 5* and *6*, all radioactive derivatives of dihydrotestosterone were counted. In experiments not shown, the pattern of metabolites obtained when dihydrotestosterone was employed as a substrate was identical in both H_2O-injected and steroid 5α-reductase RNA-injected oocytes.

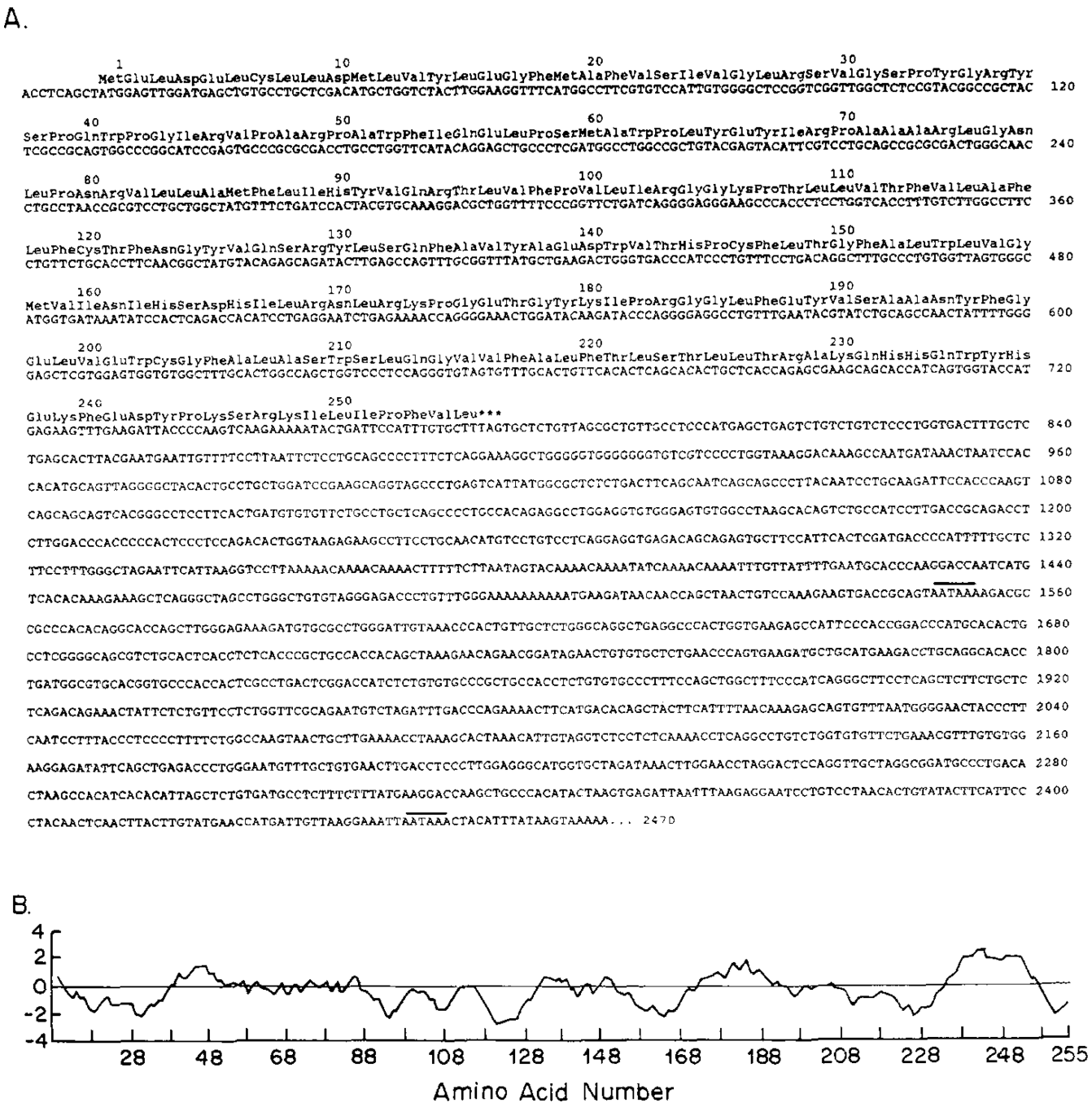

Fig. 4. Nucleotide sequence of the cDNA corresponding to the rat steroid 5α-reductase mRNA, predicted amino acid sequence, and hydropathy profile of the protein

A, nucleotides are numbered on the *right-hand side*. The amino acids are numbered above the sequence with position 1 arbitrarily assigned to the first methionine codon in the nucleotide sequence. Two polyadenylation signals are *overlined. B*, the sequence of the steroid 5α-reductase protein was subjected to a hydropathy analysis using the algorithm of Kyte and Doolittle (43). Sequences above the central dividing line are hydrophilic, and those below the line are hydrophobic.

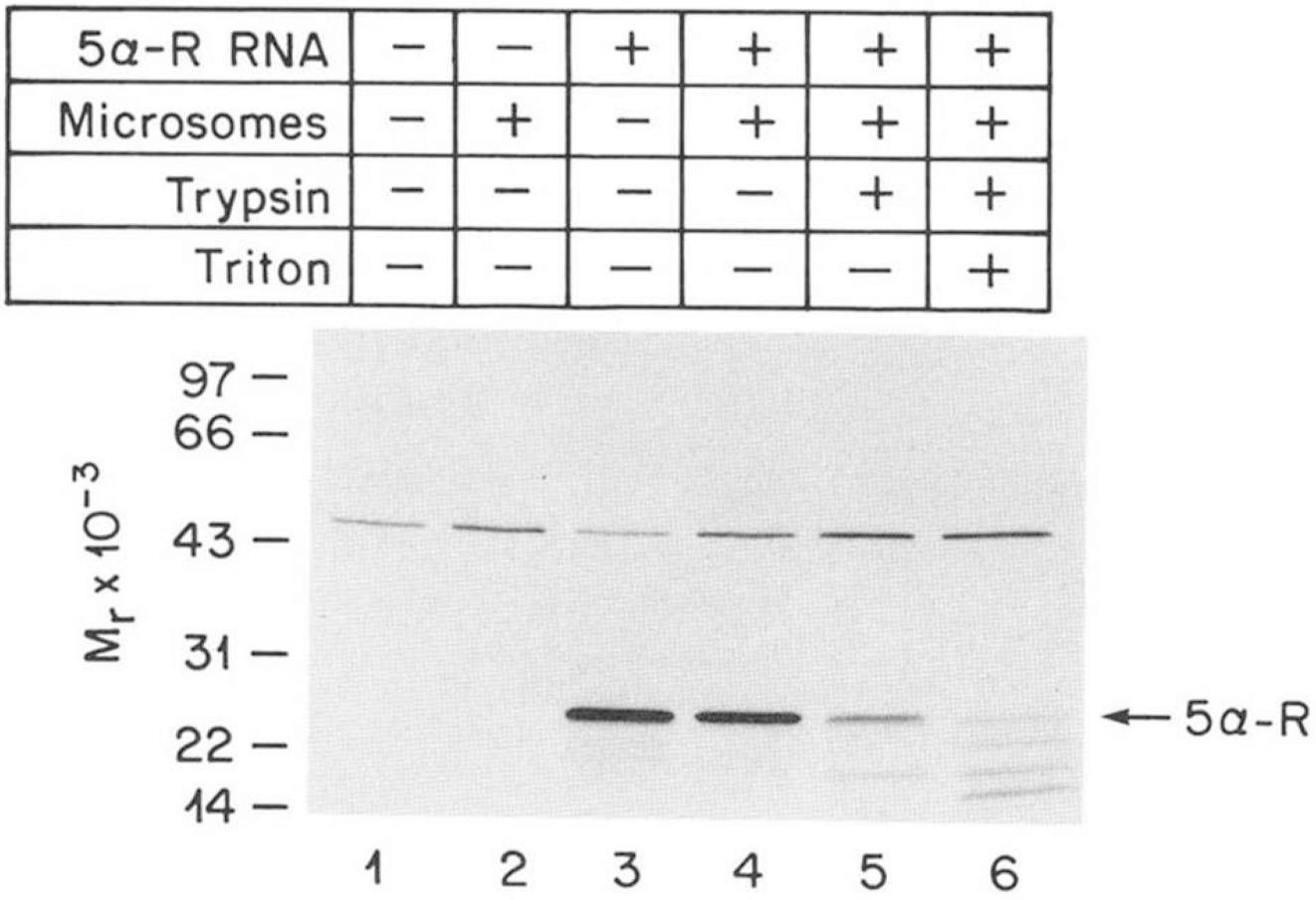

Fig. 5. *In vitro* translation analysis of steroid 5α-reductase RNA

In vitro synthesized steroid 5α-reductase RNA was translated in a reticulocyte lysate as described under “Experimental Procedures.” Additions to individual tubes are indicated above the autoradiogram. Approximately 8% of each translation reaction was analyzed by electrophoresis on 7–15% gradient polyacrylamide-sodium dodecyl sulfate gels. Size standards are indicated on the *left*. The band at M_r 45,000 represents an endogenous methionine binding protein in the reticulocyte lysate. The band corresponding to steroid 5α-reductase is indicated on the *right* of the autoradiogram.

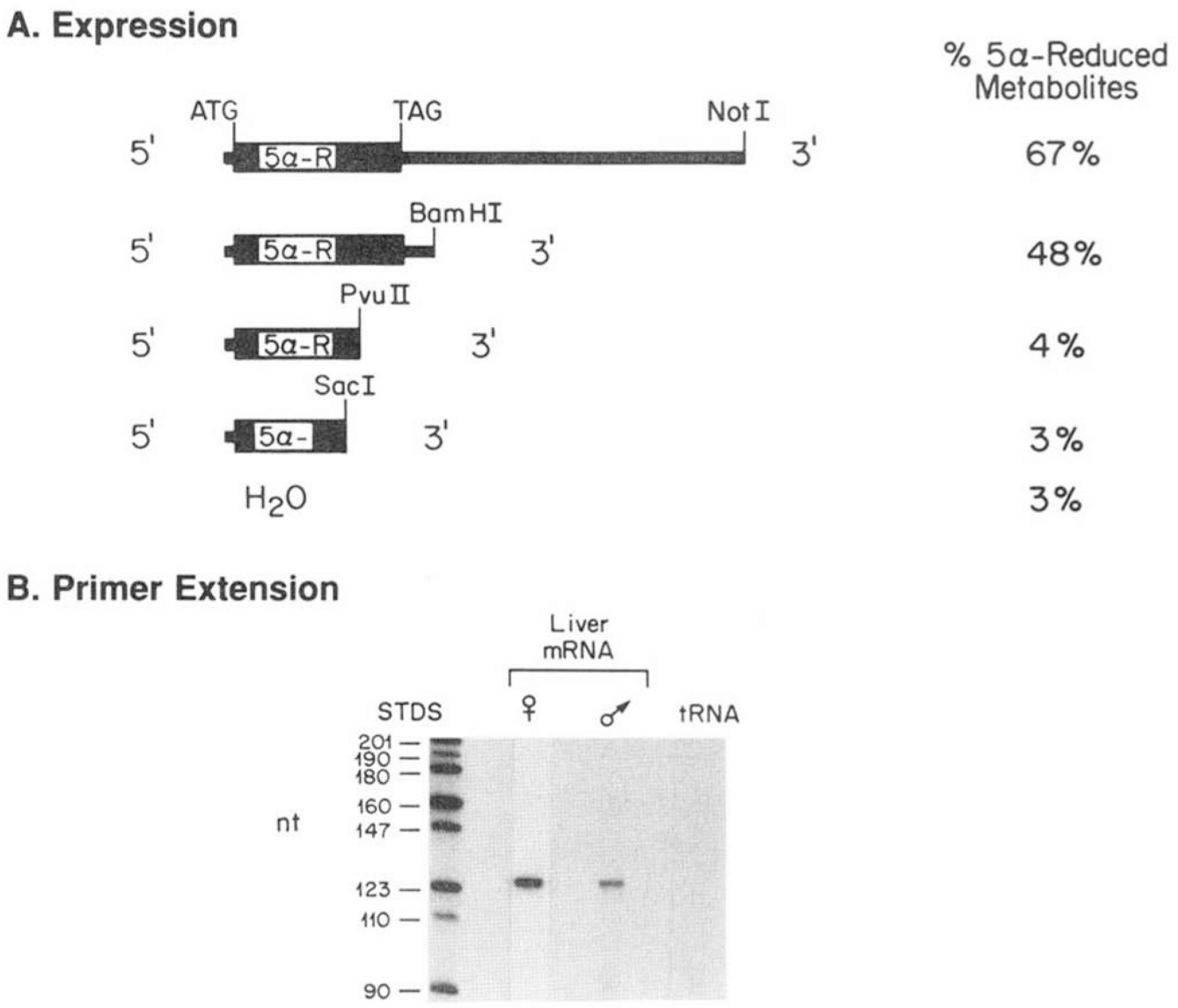

Fig. 6. Characterization of the 5′ and 3′ ends of the steroid 5α-reductase cDNA and mRNA

A, expression of 3′-truncated RNAs in *Xenopus* oocytes. The steroid 5α-reductase cDNA plasmid was linearized with the indicated restriction enzyme and the resulting template was used to synthesize RNA *in vitro*. Oocytes were injected with the RNA and assayed for activity using testosterone as a substrate. The amount of 5α-reduced steroid metabolites was determined as described in the legend to Fig. 3. The values shown are the average of two or three separate experiments for each RNA. *B*, primer extension analysis of the 5′ end of liver steroid 5α-reductase mRNA. Ten μg of poly(A^+) mRNA from the indicated source was subjected to primer extension analysis as described under "Experimental Procedures." Size standards (*STDS*) are indicated on the *left* of the autoradiogram. Exposure times at −70 °C with an intensifying screen were 13 h for *lanes 1*, *3*, and *4*, and 1 h for *lane 2*. *nt*, nucleotides.

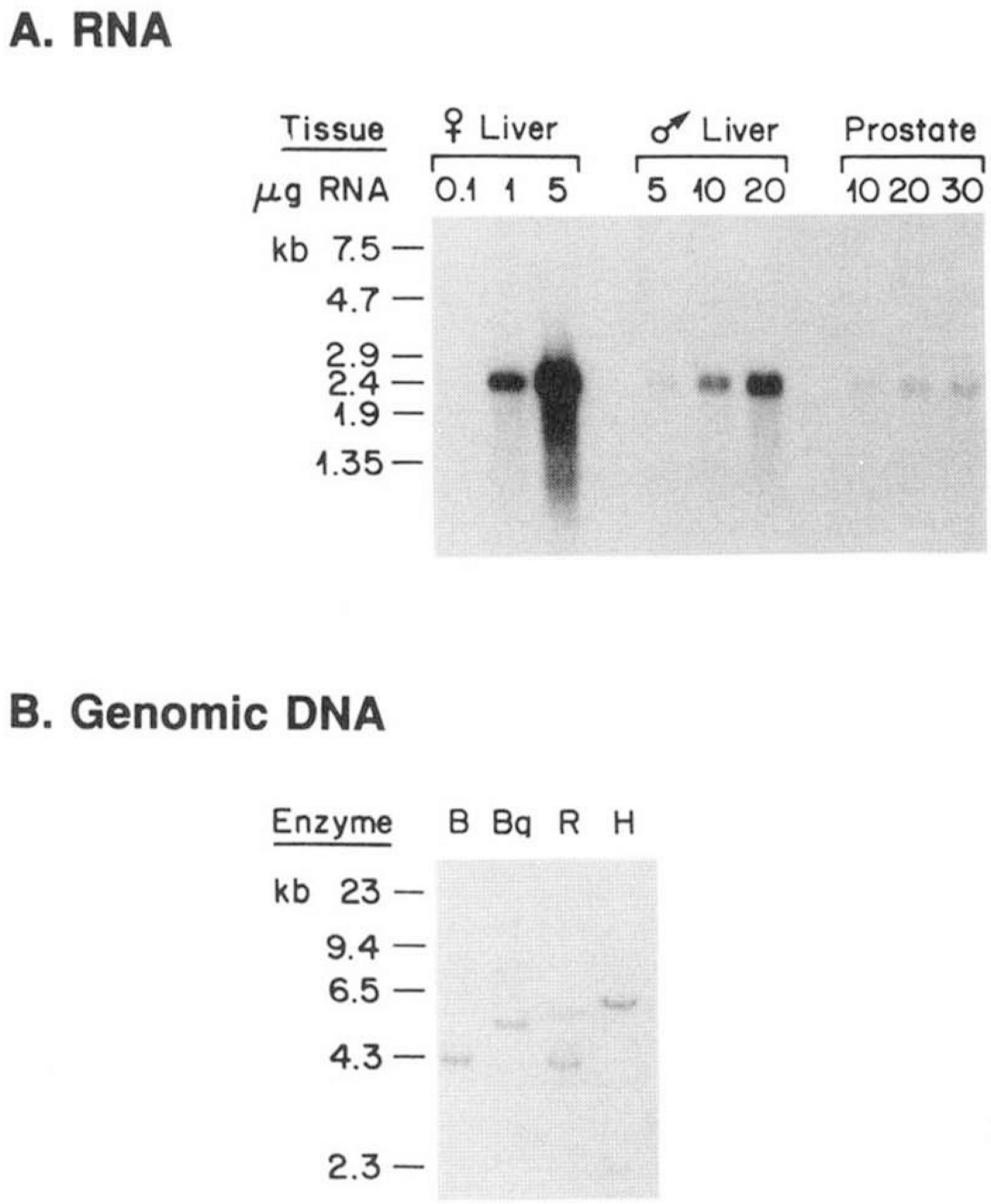

Fig. 7. Blot hybridization of rat RNA and DNA with steroid 5α-reductase cDNA probes

A, poly(A^+) RNA was isolated from female liver, and male liver and ventral prostate, and amounts as indicated were subjected to electrophoresis and blotting as described previously (44). The probe used corresponded to the complete cDNA insert shown in Fig. 4*A*. The positions to which standards migrated in adjacent lanes on the gel are indicated on the *left* of the autoradiogram. The filter was exposed to x-ray film for 16 h at −70 °C with an intensifying screen. *B*, genomic DNA was isolated from liver using an Applied Biosystems model 340A DNA extractor. Ten-μg aliquots were digested with the indicated restriction

enzymes (*B*, *Bam*HI; *Bg*, *Bgl*II; *R*, *Eco*RI; and *H*, *Hind*III) and subjected to Southern blotting as described (45). DNA size standards are indicated on the *left* of the autoradiogram. The entire steroid 5α-reductase cDNA insert was used as a probe, and the washed filter was exposed to film for 5 days.

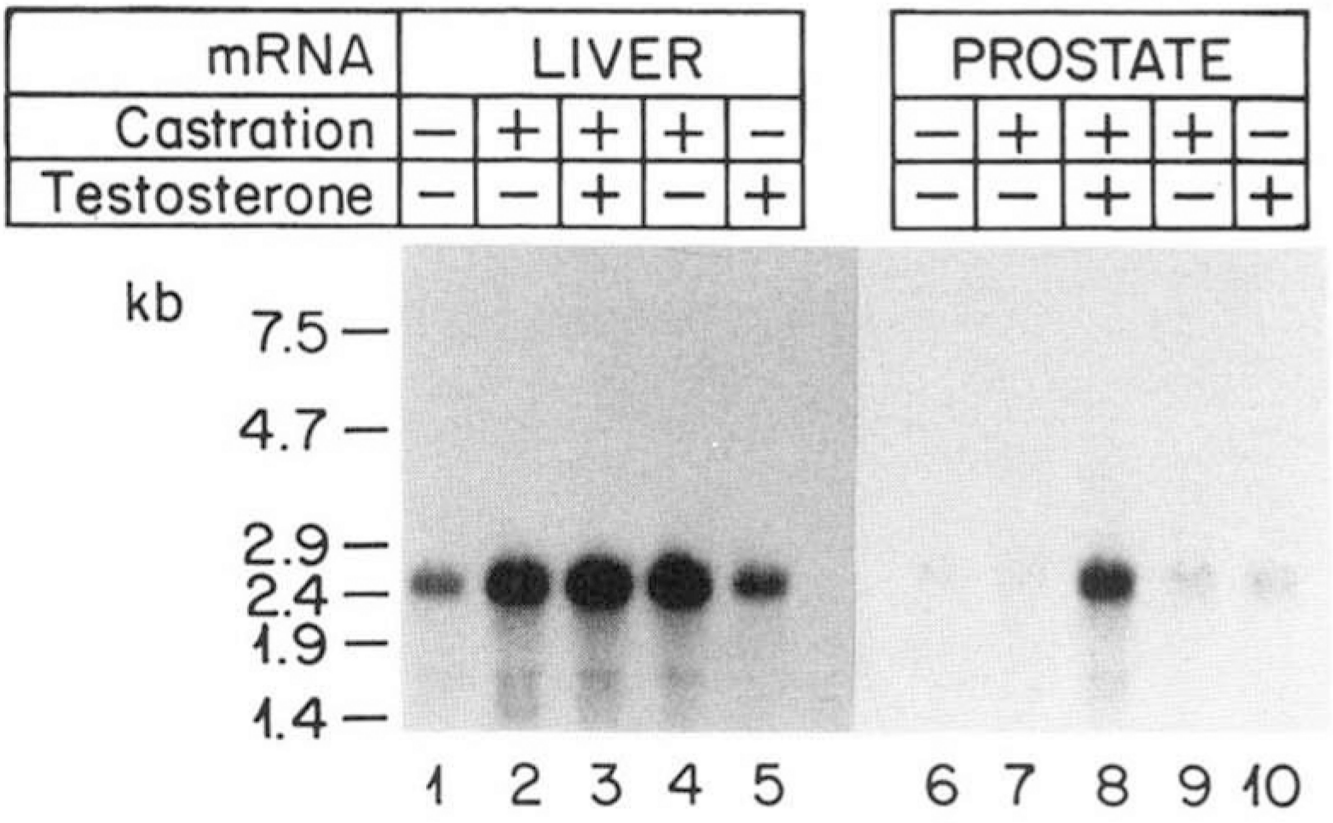

Fig. 8. Expression of steroid 5α-reductase mRNA in rat ventral prostate and liver

Orchiectomy, testosterone administration, RNA isolation, and blot hybridization were carried out as described under "Experimental Procedures." Thirty μg of total RNA were electrophoresed in each lane. *Lane 1*, liver RNA from normal male rat; *lane 2*, liver RNA from 7-day castrates; *lane 3*, liver RNA from 10-day castrates given testosterone acetate on days 7–9; *lane 4*, liver RNA from 10-day castrates; *lane 5*, liver RNA from normal animals given testosterone acetate on days 7–9; *lanes 6–10*, prostate RNAs from the same animals. A combination of three single-stranded DNA probes generated from bacteriophage M13 clones (46) were used as a probe. RNA size standards are indicated on the *left* of the autoradiogram. Exposure times were 16 h for *lanes 1–5*, and 3 days for *lanes 6–10*. The weakly hybridizing mRNA of about 1.7 kb in the liver samples may represent the use of a polyadenylation signal at nucleotide position 1549 of Fig. 4*A*.