Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 731–735, February 1984
Biochemistry

Androgen induction of ornithine decarboxylase mRNA in mouse kidney as studied by complementary DNA

(androgen action/recombinant DNA/dot blots/blot hybridization/testicular feminization)

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Communicated by Roy Hertz, October 14, 1983

ABSTRACT **To investigate the mechanisms by which androgens regulate ornithine decarboxylase (OrnDCase; L-ornithine carboxy-lyase, EC 4.1.1.17) in mouse kidney, a cDNA clone encoding OrnDCase mRNA was prepared. Purification of OrnDCase mRNA from kidneys of androgen-treated mice was accomplished by immunoadsorption of renal polysomes to a protein A-Sepharose column and enrichment for poly(A)-containing RNA by oligo(dT)-cellulose. Double-stranded cDNA synthesized from this mRNA was inserted into the *Pst* I site of plasmid pBR322 by using oligo(dG·dC)-tailing and was propagated in *Escherichia coli*. Plasmids containing cDNA sequences coding for OrnDCase were identified by differential colony hybridization, by radioimmunological detection of OrnDCase-like antigens in bacterial cultures, and by cell-free translation of hybrid-selected mRNA followed by immunoprecipitation with monospecific OrnDCase antiserum. A restriction endonuclease fragment of the selected plasmid DNA (pODC54) was labeled by nick-translation and used to study changes in OrnDCase mRNA concentration. After a single dose of testosterone, renal OrnDCase mRNA concentration increased as soon as 6 hr and peaked 24 hr after steroid injection, as measured by RNA blot hybridization. Continuous androgen treatment for 4 days resulted in a 10- to 20-fold increase in OrnDCase mRNA concentration in normal animals, but no induction of this mRNA was detected in mice that have an inherent defect of the androgen receptor (testicular feminization). These results indicate that androgens regulate OrnDCase synthesis in mouse kidney, at least in part, by increasing OrnDCase mRNA accumulation.**

Ornithine decarboxylase (OrnDCase; L-ornithine carboxy-lyase, EC 4.1.1.17) catalyzes the conversion of ornithine to putrescine and is the first and apparently rate-limiting enzyme in polyamine biosynthesis. A striking feature of this enzyme is that it has extremely rapid induction kinetics and a high turnover rate (for review, see refs. 1–4). Several studies (1–6) have suggested that regulation of OrnDCase activity occurs through modulation of the amount of the enzyme protein, although other factors such as inhibitors (7–10) or activators (11) of OrnDCase and post-translational enzyme modifications (12, 13) also may be involved. A major reason for difficulties in studying the regulation of this enzyme is that it represents only 0.01–0.05% of the total cellular protein, even when maximally induced. Nonetheless, purification of the enzyme to apparent homogeneity (5, 14–17) and development of radioimmunoassays (5, 6) have permitted studies on OrnDCase turnover.

We reasoned that further understanding of the factors that regulate OrnDCase would be facilitated if a probe were available that would allow direct measurement of OrnDCase mRNA sequences. Kidneys of testosterone-treated mice were chosen as a source for isolation of OrnDCase mRNA because androgen administration increases enzyme protein in this organ to a higher concentration than is known in most tissues (2, 5, 6). In the present study, we describe the cloning of DNA complementary to mouse renal OrnDCase mRNA and report how androgens increase the concentration of this mRNA.

MATERIALS AND METHODS

Animals. Mature male and female NCS mice [randomly bred strain Rku:NCS(s) SPF] were from the Rockefeller University (New York). Androgen-insensitive (Tfm/Y) mice, which lack functional androgen receptors (18), were kindly supplied by C. D. Toran-Allerand (Columbia University, New York).

Purification of OrnDCase mRNA. Kidneys from 150 female mice treated for 1 wk with testosterone-releasing implants were homogenized (4 ml/g of tissue) with a glass/teflon homogenizer in 20 mM Tris·HCl/10 mM $Mg(OAc)_2$/75 mM KCl/7 mM mercaptoethanol/0.25 M sucrose containing heparin (5 mg/ml) and cycloheximide (5 μg/ml) (pH 7.6 at 20°C). Membrane-bound polysomes were released by adding 0.1 vol of 10% (wt/vol) deoxycholate/10% (vol/vol) Triton X-100. Polysomes were sedimented at 100,000 × *g* for 16 hr in a Beckman SW 28 rotor through discontinuous gradients of 24% and 60% (wt/vol) sucrose. The pellets were suspended in polysome buffer [25 mM Tris·HCl/150 mM NaCl/5 mM $MgCl_2$/0.1% Nonidet P-40 containing cycloheximide (1 μg/ml), RNasin (100 units/ml; Biotec, Madison, WI), and heparin (5 mg/ml) (pH 7.6 at 20°C)] to yield a polysomal RNA concentration of 1 mg/ml. To minimize subsequent nonspecific trapping, the polysomal suspension was first passed through a protein A-Sepharose column (10 × 65 mm). The eluate was then reacted for 2 hr at 0°C with an IgG fraction (1 mg of IgG per 7 mg of polysomal RNA) purified from a monospecific OrnDCase antiserum (5) as described by Kraus and Rosenberg (19). Antibody-bound polyribosomes were then adsorbed to protein A-Sepharose, and RNA subsequently was eluted with 20 mM EDTA as described by Shapiro and Young (20). The released RNA was made 0.5 M in KCl and 0.1% in $NaDodSO_4$ and further purified by two cycles of oligo(dT)-cellulose chromatography (21) to yield 2–4 μg of poly(A)-RNA.

Preparation of cDNA from the Purified mRNA and Cloning of Double-Stranded cDNA. cDNA was prepared from 2 μg of the purified poly(A)-RNA as described by Stein *et al.* (22). Preparation of double-stranded cDNA, cleavage with S1 nuclease, and annealing at the *Pst* I site of the plasmid pBR322 with poly(dC)·poly(dG) homopolymeric extensions were carried out by standard techniques (22). Transformation of *Escherichia coli* strain LE 392 with the chimeric plasmids was accomplished as described by Dagert and Ehrlich (23).

Colony Hybridization. Bacterial clones containing chimeric plasmids were first selected by differential colony hybrid-

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Abbreviations: OrnDCase, ornithine decarboxylase; kb, kilobase(s).
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ization (24) with two different [^{32}P]cDNA probes: one (+ probe) prepared by reverse transcription of the mRNA used for the cloning and another (− probe) complementary to the poly(A)-RNA-enriched fraction from the RNA that failed to bind to the protein A-Sepharose.

Screening of Bacterial Colonies for OrnDCase Immunoreactivity. Selected bacterial colonies were grown overnight at 37°C in 5 ml of L broth. After pelleting the bacteria at 3000 × *g* for 15 min, aliquots of the supernatants were subjected to radioimmunoassay for OrnDCase as described (5).

Hybridization Selection. Plasmid DNA was prepared from 100-ml cultures after amplification with chloramphenicol (200 μg/ml) by the method of Ish-Horowicz and Burke (25). Poly(A)-containing polysomal RNA from kidneys of androgen-treated mice was used for hybridization selection according to Parnes *et al.* (26).

Analytical Plasmid DNA Extraction. Plasmid DNA for restriction enzyme digestion was prepared as described by Ish-Horowicz and Burke (25). The digests were analyzed on 6% polyacrylamide gels (27) and stained with ethidium bromide (1 μg/ml).

RNA Blot Hybridization Analyses. Total RNA from mouse kidney was isolated by the lithium chloride/urea method (28) and enriched for poly(A)-RNA by oligo(dT)-cellulose (21). Dot blot and gel blot hybridization analyses of the mRNA were conducted essentially as described by Thomas (29). The [^{32}P]cDNA probe for these hybridizations was prepared as follows: 250 μg of plasmid DNA from a clone (designated pODC54) containing a sequence complementary to OrnDCase mRNA was digested with the restriction endonucleases *Pst* I and *Hin*dIII, and a 330-base-pair internal *Pst* I/*Hin*dIII fragment was isolated by preparative 6% polyacrylamide gel electrophoresis and labeled by nick-translation (30) to a specific activity of about 10^8 cpm/μg.

Other Techniques. Cell-free translation of mRNA preparations was carried out with L-[^{35}S]methionine and a rabbit reticulocyte lysate kit (Bethesda Research Laboratories) according to the supplier's instructions. Immunoprecipitation of the translation products was performed essentially as described by Kraus and Rosenberg (19). The total translation products and immunoprecipitated peptides were fractionated by electrophoresis on a 10% polyacrylamide gel containing 0.1% NaDodSO$_4$ (31). After the electrophoresis, the gels were treated with EN^{3}HANCE (New England Nuclear). Fluorography of the dried gels and nitrocellulose filters was carried out by exposing them to Kodak XAR film for 1 hr to 7 days at −70°C.

RESULTS

Purification of OrnDCase mRNA. At least 300-fold purification of OrnDCase mRNA was achieved by a modification of the protein A-Sepharose immunoadsorbent chromatography described by Shapiro and Young (20). The additional steps introduced to the procedure, including passing of polysomes through protein A-Sepharose column prior to antibody addition, use of ribonuclease inhibitor RNasin, and inclusion of a high concentration of heparin in the buffers, increased the purity of OrnDCase mRNA. Similar results were recently reported by Boyer *et al.* (32) using formalin-fixed *Staphylococcus aureus* cells in an immunological mRNA purification. The final purity of the mRNA in different preparations ranged from 5% to 15%, as judged by the immunoprecipitable counts in the cell-free translation system. The purity of the mRNA preparation used for cDNA synthesis and cloning was about 5%.

The cell-free translation products of the purified mRNA samples immunoprecipitated with a monospecific antiserum against OrnDCase contained three peptides with estimated molecular weights of about 54,000, 37,500, and 33,000 (Fig. 1, lanes 4 and 6); the smaller components appeared to be

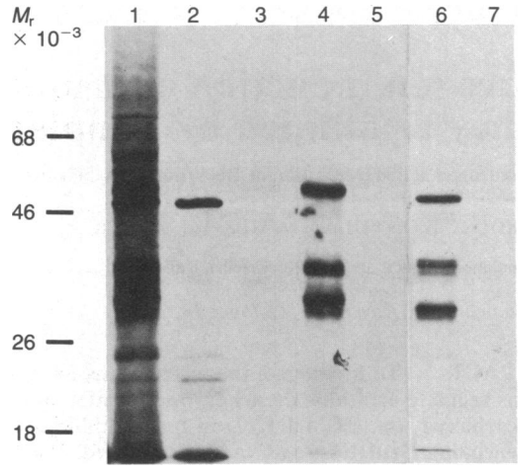

FIG. 1. Electrophoresis of the *in vitro* translation products of the affinity-purified mRNA on 10% polyacrylamide gels containing 0.1% NaDodSO$_4$. Lanes: 1, total translation products of 0.25 μg of the purified mRNA; 2, no exogenous mRNA; 3, purified OrnDCase covalently labeled with ^{3}H-labeled α-difluoromethylornithine; 4, translation products (same as lane 1) after immunoprecipitation with monospecific anti-OrnDCase antibody; 5, translation products of 0.2 μg of rabbit reticulocyte mRNA after immunoprecipitation with anti-OrnDCase antibody; 6, the same as lane 4; 7, translation products (same as lane 1) after immunoprecipitation with anti-OrnDCase antiserum in the presence of 10 μg of purified OrnDCase. The mobility of ^{14}C-labeled reference proteins (bovine serum albumin, ovalbumin, α-chymotrypsinogen, β-lactoglobulin) are shown on the left.

doublets. The largest component comigrated with the homogeneous OrnDCase standard (Fig. 1, lane 3) covalently labeled with ^{3}H-labeled α-difluoromethylornithine (5). All these bands were also clearly visible in the total translation products (Fig. 1, lane 1). The specificity of the immunoprecipitation reaction was indicated by the inability of the OrnDCase antiserum to precipitate cell-free translation products of rabbit reticulocyte mRNA (Fig. 1, lane 5) and the ability of excess homogeneous OrnDCase protein to prevent immunoprecipitation of all three peptides (Fig. 1, lane 7).

Screening of the Bacterial Colonies for cDNA to OrnDCase mRNA. Transformation of *E. coli* with the chimeric plasmids yielded 1615 tetracycline-resistant ampicillin-sensitive colonies, which were subjected to differential colony hybridization (Fig. 2). Each of the 377 colonies that gave a clear differential hybridization signal was further analyzed by an additional colony hybridization with a [^{32}P]cDNA probe complementary to a mRNA preparation of the highest purity (about 15%, Fig. 2 *Lower Left*). On the basis of this screening, 110 hybridization-positive colonies and 16 hybridization-negative colonies (as controls) were subjected to radioimmunoassay for OrnDCase-like antigens. Cultures of 26 of the former and none of the latter contained material that reacted with OrnDCase antiserum (Table 1).

Plasmid DNA was isolated from four colonies positive in OrnDCase radioimmunoassay and used for hybridization selection of mRNA from renal polysomal RNA. In all four cases, the hybridization-selected mRNA directed the synthesis of peptides which, after immunoprecipitation, had electrophoretic mobility identical with that of the cell-free translation products of the purified OrnDCase mRNA preparation (results for two clones shown in Fig. 3). Also in this case, the immunoprecipitation of the labeled peptides was abolished by excess of purified OrnDCase (data not shown).

Plasmid DNA from one of the clones (designated pODC54) positive in hybridization selection contained a 730-base-pair insert, as judged by analysis with selected restriction enzymes. An internal *Pst* I/*Hin*dIII fragment of 330 base pairs

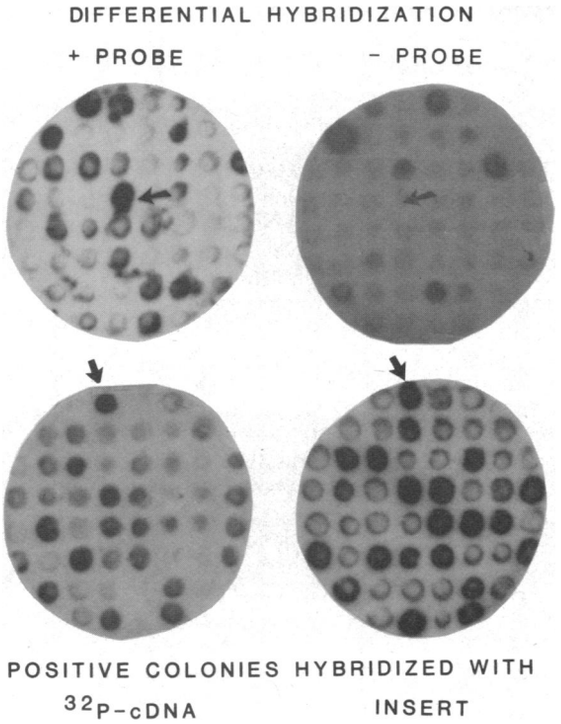

FIG. 2. Screening of bacterial colonies for DNA sequences complementary to OrnDCase mRNA. (*Upper*) Initial differential screening of the tetracycline-resistant ampicillin-sensitive clones with [^{32}P]cDNAs prepared from the mRNA used in cloning (+ probe) and from the poly(A)-RNA preparation not bound to the protein A-Sepharose column (− probe). The example shown is 1 of 32 pairs of filters screened. (*Lower Left*) Screening of the colonies positive in the differential hybridization with [^{32}P]cDNA prepared from the most purified OrnDCase mRNA sample (about 15% pure). (*Lower Right*) Screening with the nick-translated internal *Pst* I/*Hin*dIII fragment of the plasmid pODC54. The positive clone pODC54 on the filters is marked by the arrow.

from pODC54 was used as a hybridization probe for another colony hybridization experiment (Fig. 2 *Lower Right*): of the 110 colonies selected by hybridization to the [^{32}P]cDNA from the mRNA fraction of highest purity, 79 were positive upon insert hybridization, whereas no colony negative in the original assay was strongly positive in this assay. Of the 26 colonies showing OrnDCase immunoreactivity, 23 were positive in the pODC54-insert hybridization.

Table 1. Immunological screening for OrnDCase-like antigens in bacterial culture media

Group*	Colonies, no.	Experiment I,† cpm	Experiment II,† cpm
Controls	9	7305 ± 589	6641 ± 316
Colonies negative in colony hybridization			
RIA$^-$ colonies	16	7171 ± 414	6642 ± 351
RIA$^+$ colonies	0		
Colonies positive in colony hybridization			
RIA$^-$ colonies	84	7246 ± 378	6649 ± 242
RIA$^+$ colonies	26	5216 ± 632‡	4990 ± 1219§

*Controls were bacterial colonies transformed with the plasmid pBR322 only. RIA$^-$ and RIA$^+$ designate colonies that were negative and positive in OrnDCase radioimmunoassay, respectively. The limit of positivity was defined as the mean minus 2 SD for the control colonies. In the radioimmunoassay for OrnDCase, 50% displacement of the tracer was achieved by 1 ng of the enzyme protein (5).

†Colonies (135 in total) were equally divided into two consecutive series of OrnDCase radioimmunoassay. Each value is the mean ± SD.

‡Range: 3642–6042.

§Range: 3006–5992.

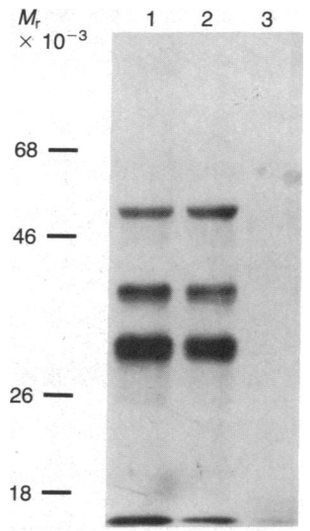

FIG. 3. Analysis of the immunoprecipitated cell-free translation products of hybrid-selected RNA preparations by NaDodSO$_4$/polyacrylamide gel electrophoresis. Lanes: 1 and 2, immunoprecipitated translation products of the mRNAs selected by plasmids pODC54 and pODC152, respectively; 3, immunoprecipitated translation products of the material eluted from a blank nitrocellulose filter. The molecular weight markers are the same as in Fig. 1.

Androgen Induction of OrnDCase mRNA in Mouse Kidney. The OrnDCase mRNA sequences were barely detectable in kidneys of untreated female mice, started to increase between 6 to 12 hr, and attained the maximum level 24 hr after a single dose of testosterone (Fig. 4). Prolonged testosterone treatment for 4 days caused a 10- to 20-fold accumulation of OrnDCase mRNA (Fig. 5). The basal level of OrnDCase mRNA sequences was very low in kidneys of androgen-insensitive (Tfm/Y) animals and showed no significant increase in response to prolonged testosterone administration. Regardless of the animal and treatment used, the blot hybridizations always revealed two hybridizable RNA species: a major band of 2.15 kilobases (kb) in length and a minor one of 2.7 kb (Figs. 4 and 5). Testosterone treatment resulted in roughly proportional increases in these two mRNA species.

DISCUSSION

Testosterone administration markedly increases the activity of OrnDCase and other proteins in mouse kidney (5, 6, 14, 15, 33–35) and produces cellular hypertrophy rather than hyperplasia (35). The induction requires functional androgen receptors and is seen as early as a few hours after hormone administration (5, 34). Treatment of mice with pharmacological doses of androgen for five days results in a 400- to 600-fold increase in renal OrnDCase (5, 6, 34) with a parallel rise in catalytic activity and enzyme protein concentration (5, 6). In addition to increased *de novo* synthesis of OrnDCase, androgen treatment has been shown to prolong the biological half-life of the enzyme by 4- to 10-fold (5, 36). The present study shows that one of the mechanisms by which androgens regulate OrnDCase synthesis is to increase OrnDCase mRNA concentration.

Several lines of evidence show that we have synthesized and cloned a DNA sequence complementary to OrnDCase mRNA. First, the percentage of positive clones in colony hybridization was in good agreement with the estimated purity of the OrnDCase mRNA used for cloning. Second,

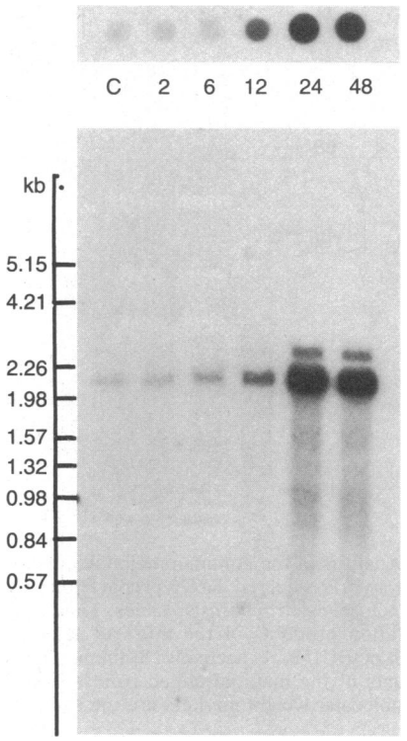

FIG. 4. Dot blot (*Upper*) and gel blot hybridization (*Lower*) analyses of poly(A)-RNA from kidneys of androgen-treated female mice. For the dot blots, RNA was bound to the nitrocellulose filter (5 μg of RNA per sample); for the gel blot hybridizations, RNA (8 μg of RNA per sample) was first denatured with glyoxal, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose. Hybridization of the filter-bound RNA was carried out with the *Pst* I/*Hin*dIII insert of pODC54 labeled by nick-translation. End-labeled *Eco*RI/*Hin*dIII fragments of bacteriophage λ served as references for molecular sizes. Lanes: C, renal poly(A)-RNA from nontreated mice; 2, 6, 12, 24, and 48, renal poly(A)-RNAs from mice killed 2, 6, 12, 24, and 48 hr after treatment with 10 mg of testosterone. There were six animals in each group.

mRNA selected by hybridization to plasmid pODC54 directed cell-free synthesis of peptides which, after immunoprecipitation with OrnDCase antiserum, migrated on $NaDodSO_4$/polyacrylamide gels identically with the translation products of the purified OrnDCase mRNA. Furthermore, excess of nonlabeled OrnDCase protein abolished immunoprecipitation of the labeled translation products. It should be emphasized that the enzyme protein used in the competition studies was at least 95% pure (5) and that the antiserum was monospecific, as judged by crossed immunoelectrophoresis (5) or electrophoretic blotting of renal cytosol (unpublished observations). Third, the kinetics of induction of the mRNA that hybridized to pODC54 was in accordance with that reported for the OrnDCase protein (5, 6, 34). Finally, the molecular size of the hybridizable mRNA was compatible with the predicted size for a message coding for OrnDCase (see below).

The mechanisms by which the three immunoreactive peptides (Figs. 1 and 3) were formed in the cell-free translation of OrnDCase mRNA remain unknown. Size heterogeneity of peptide products has been observed with a number of mRNAs translated *in vitro* and has been attributed to inappropriate initiation of translation by internal initiation codons, premature termination of the translation, or degradation of the translation products by proteases present in the cell-free system (37–43). Whatever the mechanism of their formation is, all the three major bands seen on polyacrylamide gel electrophoresis seemed to represent OrnDCase or its fragments because purified OrnDCase protein abolished immunoprecipitation of these peptides.

The present study demonstrated the feasibility of using a modification of an immunoadsorption technique (20) for the purification of low-abundance mRNA species. It also introduced a convenient immunological method for screening recombinant bacterial colonies. Direct measurement of OrnDCase-like antigens (fusion proteins between β-lactamase and OrnDCase) from the culture media of bacterial colonies yielded no false-positive results as compared to the differential colony hybridization (Table 1) and was in agreement with the insert hybridization in 23 of 26 cases. Moreover, the four colonies selected from the 26 positives in radioimmunoassay recognized the same mRNA species in hybridization selection. Random insertion to the *Pst* I site should result in 1/6th of the clones being expressed as an immunoreactive β-lactamase fusion protein. In view of this, the number of colonies expressing OrnDCase-like immunoreactivity (26 of 110 positive colonies) was somewhat higher than expected.

Two mRNA species with molecular sizes of 2.15 and 2.7 kb, hybridizable to the plasmid pODC54, were induced by testosterone treatment. The molecular size of the major mRNA component (2.15 kb) is compatible with that of an intact OrnDCase mRNA, if one assumes that the molecular weight of OrnDCase subunit is 54,000 (5, 15–17) and that the mature mRNA contains about 500 nucleotides in the nontranslated regions. The size heterogeneity observed in this study for OrnDCase mRNA is not an uncommon phenomenon for eukaryotic mRNAs (42, 44, 45) and may arise from different degrees of post-transcriptional processing of the primary transcript, for example, because of the presence of multiple polyadenylylation sites (46).

In summary, we have cloned a DNA complementary to OrnDCase mRNA and have shown that testosterone administration greatly increases OrnDCase mRNA concentration

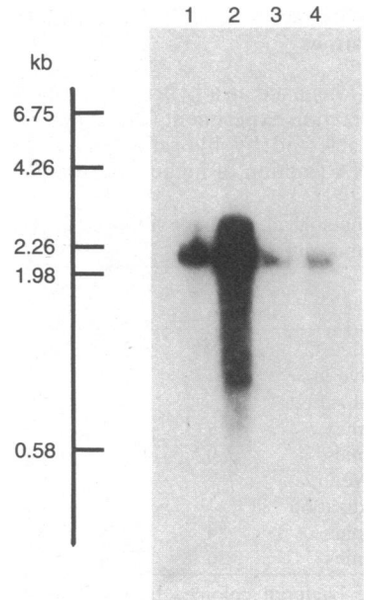

FIG. 5. Blot hybridization analysis of poly(A)-RNA from kidneys of androgen-treated castrated male mice and of androgen-insensitive (Tfm/Y) mice. The experimental protocol was as in the legend to Fig. 4 except that end-labeled *Hin*dIII fragments of bacteriophage λ were used as molecular size markers. Lanes: 1, untreated castrated male mice; 2, castrated male mice treated for 4 days with testosterone-releasing implants; 3, untreated Tfm/Y mice; 4, Tfm/Y mice treated for 4 days with testosterone-releasing implants. There were eight animals in each group.

in mouse kidney. This induction is apparently mediated by an androgen receptor-dependent mechanism because no androgenic induction of OrnDCase mRNA took place in kidneys of Tfm/Y mice. The cDNA should now permit a detailed study on the structure and regulation of the OrnDCase gene.

The skillful technical assistance of Ms. Cecilia Liu and Mr. Paul Van Duyne is gratefully acknowledged. We also wish to thank Dr. James F. Catterall for many helpful suggestions and Ms. Jean Schweis for invaluable secretarial help. This study was supported by National Institutes of Health Grants HD-13541 and 3 FO5 TW3192-0151.

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