Isolation and Characterization of a DNA Sequence Complementary to an Androgen-inducible Messenger RNA from Mouse Kidney*

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Clones containing sequences corresponding to testosterone-inducible RNAs of mouse kidney have been identified within a cDNA clone bank prepared from size-fractionated poly(A)-containing kidney RNA. A novel screening method was employed to specifically detect such sequences. One of these, pMK908, containing a 1.2 kilobase pair insert, was studied in detail. RNA blotting experiments show that the inserted DNA and functions at detailed levels. An important application of this technology has been in the purification of sequences corresponding to hormone-responsive genes, resulting in the generation of biochemical probes useful in studying the molecular details of a particular hormonal effect. In this paper, we report the construction and isolation of a recombinant plasmid containing a DNA sequence complementary to an androgen-inducible mRNA from mouse kidney. We have used this plasmid to define the corresponding mRNA and polypeptide, and to study the nature of the androgen-mediated induction. The potential importance of this study in understanding hormonal response mechanism is discussed.

MATERIALS AND METHODS

Animals—Mice of strains C57BL/6J, C57BL/6-Tfm, and C57BL/6-lit/lit were obtained from the Production Department of the Jackson Laboratory. Hypophysectomized C57BL/6 mice were obtained from the Charles River Mouse Farms. All mice were used at 2 months of age.

Mice were treated with testosterone by subcutaneous application of 30-µg pellets (11) for 1-2 weeks unless otherwise stated. Castrated males were used 14 days after surgery; we thank J. Knopf of Roswell Park Memorial Institute for performing the castrations.

Isolation and Fractionation of mRNA—RNA was isolated from mouse tissues by the guanidine-HCl method (13) as described by Labarca and Paigen (14). To enrich for poly(A)-containing sequences, total RNA, dissolved in 0.5 M NaCl, 0.5% sodium dodecyl sulfate was passed through an oligo (dT)-cellulose (Collaborative Research) column. The bound RNA was eluted with 10 mM Tris-Cl, pH 7.5, 1 mM sodium EDTA to yield RNA that was ~50% rRNA and 50% heterogeneous-sized RNA.

Poly(A)-enriched RNA was size-fractionated in 5-20% sucrose gradients containing 6 M urea, 0.5% SDS, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.5). Centrifugation was at 39,000 rpm for 22 h in a SW-41 rotor. Gradient fractions corresponding to RNA with an S0.2 of ~200 were diluted with water and precipitated twice with 2 volumes of ethanol.

Preparation of Double-stranded DNA Complementary to Kidney Poly(A)* RNA—Double-stranded DNA complementary to kidney mRNA was prepared using AMV reverse transcriptase as polymerizing enzyme (15). First strand synthesis was performed by reverse transcription of sucrose gradient-enriched poly(A)+ containing RNA. The reaction mixture contained, in a total volume of 0.8 ml, the following: 50 mM Tris-Cl, pH 8.3, 6 mM MgCl2, 1 mM β-mercaptoethanol, 0.24 mM each of dATP, dCTP, and TTP, 100 µCi [4,5-3H]TTP (60 Ci/mmol), (Amersham), 10 µg/ml of oligo (dT)n-Is (Collaborative Research), 50 µg/ml of RNA, and 300 units/ml of AMV reverse transcriptase (supplied by Dr. J. W. Beard). After incubation at 45 °C for 30 min, the mixture was brought to 0.2% SDS and 0.3 M NaOH and incubated for an additional 1 h at 45 °C. It was neutralized with glacial acetic acid and chromatographed on a column (1.5 × 25 cm) of SP-Sephadex C-50 (Pharmacia) in 10 mM Na acetate, pH 5.0, containing 0.3 M NaCl and 0.2% SDS. Fractions containing the cDNA were combined, 50 µg of carrier yeast RNA were added, and the solution was precipitated twice with ethanol.

The reaction mixture for second strand synthesis contained, in a total volume of 0.5 ml, the following: 50 mM Tris-Cl, pH 8.3, 10 mM and functions at detailed levels. An important application of this technology has been in the purification of sequences corresponding to hormone-responsive genes, resulting in the generation of biochemical probes useful in studying the molecular details of a particular hormonal effect. In this paper, we report the construction and isolation of a recombinant plasmid containing a DNA sequence complementary to an androgen-inducible mRNA from mouse kidney. We have used this plasmid to define the corresponding mRNA and polypeptide, and to study the nature of the androgen-mediated induction. The potential importance of this study in understanding hormonal response mechanism is discussed.

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1 The abbreviation used is: SDS, sodium dodecyl sulfate.

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MgCl₂, 1 mM β-mercaptoethanol, 0.2% vol each of dATP, dCTP, dGTP, and [α-32P]dTTTP (Amersham) (600 Ci/mmol), 10 μg/ml of cDNA, and 300 units/ml of reverse transcriptase. The mixture was incubated at 45 °C for 2 h, then terminated by addition of 10 μl of 10% SDS. The double-stranded DNA was isolated as described above for isolation of cDNA.

The double-stranded DNA was digested with S1 nuclease to remove single-stranded tails. In a total volume of 140 μl, 300 units of S1 nuclease (Sigma) were incubated in 30 mM Na acetate, pH 4.5, 0.2 mM NaCl, 0.2 mM NaEDTA, 0.6 mM ZnSO₄, and 10 μg/ml of double-stranded cDNA for 45 min at 37 °C. The mixture was extracted with an equal volume of phenol and passed over a column (0.5 x 12 cm) of Sepharose-4C. Fractions containing S1-treated double-stranded DNA were pooled and precipitated twice in ethanol. Starting with 40 μg of pol(A)-containing RNA, we obtained 1.2 μg of S1-treated double-stranded cDNA.

**Homopolymeric Tailing of DNA**—Double-stranded cDNA and Pot I-treated pH232 DNA were used to prime poly-dC and poly-dG addition, respectively, as catalyzed by terminal deoxynucleotidyltransferase (16). Reactions contained, in a total volume of 50 μl, 20 μM [4,5-3H]dGTP or [4,5-3H]dCTP (Amersham), an amount of DNA such that the molar ratio of triphosphate to 3'-OH primer termini was 500:1, and 60 units/ml of terminal deoxynucleotidyltransferase (P. L. Biochemicals, lots 4374-2 or 4374-3). Reactions were performed at 15 °C for 15-20 min, and the kinetics of tailing were monitored by acid precipitation.

The reaction was terminated by addition of excess NaEDTA, made 10 mM with 1 M Na acetate, and autoclaved at 121 °C for 15 min with the equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1), and ether. Tailed DNA was isolated by chromatography on Sephadex G-50 (Pharmacia) in 10 mM Tris, pH 8, 500 mM NaCl, 1 mM NaEDTA, and was recovered by ethanol precipitation after addition of yeast tRNA carrier.

**Construction of Recombinant Plasmids and Transfection into Escherichia coli**—All manipulations were performed in a B3 facility using an EK2 host according to National Institutes of Health guidelines. More recently, conditions have been changed to P1-ERK1. Tailed double-stranded DNA (350 μg) was incubated with 1.8 μg of pH232 DNA in a total volume of 25 ml for 10 min at 70 °C; the mixture was slowly cooled to 50 °C for 1 h, then brought to room temperature. The resulting DNA preparation was used to transfect E. coli XL77 cells essentially as described by Enea et al. (17). Transfection mixtures were plated on agar containing 10 μg/ml of tetracycline. Colonies were tested for ampicillin sensitivity by spotting onto plates containing 50 μg/ml of ampicillin.

**Preparation of Complementary DNA Probes**—Complementary DNA probes were made against the sucrose gradient-enriched poly(A)-containing RNA essentially as described (10). They were labeled in vitro with 32P or with 3H in a reaction mixture containing, in a total volume of 20 μl, the following: 50 mM Tris-Cl, pH 8.6, 6 mM MgCl₂, 1 mM β-mercaptoethanol, 0.25 mM each of dATP, dCTP, and dGTP, 30 μM TTP, 100 μCi of [α-32P]dTTP, or [4,5-3H]dTTTP, 10 μg/ml of oligo-dT, 150 μg/ml of RNA, and 300 units/ml of polynucleotide kinase as described by Woodworth-Gutai (24). Labeled DNA was isolated exactly as described above for isolation of yeast tRNA carrier.

**Colony Hybridization**—To screen for androgen-inducible sequences, a modification of the colony hybridization method (19) was used. Colonies were grown on nitrocellulose filters (S & S type BA 85, 82-mm circles with grid pattern), lysed, and the DNA was denatured and fixed as originally described (18). Prehybridization and hybridization were done in sealed plastic bags containing 5 filters (multiple filters were separated by nylon mesh). After prehybridization, 1 x 10⁶ cpm of [32P]DNA probe transcribed from 20 S RNA isolated from kidneys of untreated female mice were added. Hybridization to the mixed cDNA probes was at 65 °C for 24 h. Filters were washed, bleotted dry, and autoradiographed. After exposure of the film, which detects only the 32P probe, colonies with their bound, hybridized DNA were counted for both 32P and 1H by liquid scintillation. Colonies having a high ratio of 32P to 1H indicated the presence of sequences corresponding to RNA species that were induced by testosterone (see text).

**Isolation of Plasmid DNA**—Plasmid DNA was obtained from bacterial cells by detergent extraction following chloroform/phenol amplification essentially as outlined previously (19).}

**Agorase-Cell Electrophoresis**—Gel electrophoresis was in 0.5-1.5% agarose using 0.09 mM Tris, 0.09 mM boric acid, 2.8 mM Na₂EDTA, and 0.3 mg/ml of iodoacetate as running buffer. Samples, containing 10% sucrose and 0.002% bromophenol blue, were electrophoresed at 50 V for 5 min, and 18 V for 16-20 h. For staining, gels were washed in running buffer, stained in 10 μg/ml of ethidium bromide, and viewed under UV illumination.

**Nick Translation of Plasmid DNA**—Plasmid DNA was labeled in vitro by the nick translation protocol of Maniatis et al. (20). Labeling was generally done in the presence of both [α-3P]dCTP and [α-32P]dATP. Resulting DNA had a specific activity of 1-2 x 10⁶ cpm/μg. Nick-translated probes were made against the sucrose gradient-enriched poly(A)-containing RNA. Aminobenzoxymethyl paper was purchased from Schleicher and Schuell and diazotized prior to use (21).

**Purification of a Probe Specific for the Insert of pMK908**—Single-stranded DNA complementary to the 908 mRNAs was prepared from pMK908 DNA by preparative RNA excess hybridization. Poly(A)-containing RNA (190 μg) from testosterone-induced kidneys was hybridized to 0.2 μg of denatured nick-translated pMK908 DNA in 0.2 ml of 0.1 M 1,4-piperazinediethanesulfonic acid, pH 6.5, 80% formamide, 0.5 M NaCl, at 50 °C for 4 h; the reaction was terminated by addition of 2 ml of 20% Tris-Cl, pH 7.5, containing 0.4 M NaCl, and 0.1% SDS. The solution was applied to an oligo(dT) column, and the retained RNA-DNA hybrids were eluted with water. The eluant was brought to 80% with 80 mM Na acetate, pH 4.5, 50 mM NaCl, 0.6 M ZnSO₄, and 50 μg/ml NaEDTA, and was dialyzed against 1 M NaCl. To remove unhybridized single-stranded DNA, 125 units of S1 nuclease (Sigma) were added; the mixture was incubated for 1 h at 37 °C, chilled on ice, and 5 μg of heat-denatured salmon sperm DNA were added. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Phenol was removed by several successive ether extractions. The RNA strand of the hybrid was degraded by alkaline digestion at 100 °C for 5 min in 0.3 N NaOH. After neutralization, single-stranded DNA was recovered by ethanol precipitation.

**Hybridization Analysis**—Hybridization reactions were performed according to the conditions of Bishop et al. (21) in a total volume of 50 μl with a paraffin oil overlay. At various times, 3-μl aliquots were diluted into 0.5 ml of buffer and assayed for extent of hybridization by resistance to S1 nuclease (22). Zero time control values were subtracted, and the results were expressed as the percentage of total trichloroacetic acid-precipitable radioactivity that was nucleases-resistant. R₀₂₅ values were calculated by computer after least squares fitting of an idealized curve to the raw data. The program was kindly provided by Dr. K. Manly of Roswell Park Memorial Institute.

**Purification of the pMK908 Insert**—A restriction map for pMK908 was constructed by restriction mapping of pMK908 DNA to the single Eco RI site in the pH232 sequence according to protocols provided by the supplier. Restricted DNA (50 μg) was centrifuged through a 5-20% sucrose gradient in 10 mM Tris-Cl, pH 7.8, 50 mM NaCl, and 2 mM Na₂EDTA for 14 h at 25,000 rpm in a SW-41 rotor; DNA was recovered from fractions containing form III (i.e., linear) pMK908 DNA by ethanol precipitation. DNA was dissolved in 0.01 X SSC and made 50 mM Tris, pH 7.5, 50 mM NaCl.

**Phosphate residues were removed from the 5'-terminal by treating 16 μg of the DNA with 0.4 units of bacterial alkaline phosphatase ( Worthington) at 37 °C for 45 min. After addition of 1/10 volume of 0.5 M Na₂EDTA, the incubation mix was successively extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), and dialyzed against 10 mM Tris-Cl, pH 8.1.

**Labeling of the 5'-end with [γ-32P]ATP was accomplished using polynucleotide kinase as described by Woodworth-Gutai (24). Labeled DNA was isolated from the reaction mixture by Sephadex G-50 chromatography in 10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM MgCl₂, and 0.1 M Na₂EDTA. DNA was digested to completion with HindIII according to directions provided by the supplier. The generated 5'-end labeled fragments of lengths 4333 and 29 base pairs, the latter of which is negligible in the analysis described below. Phage DNA fragments obtained by digestion with HindIII and Eco RI were similarly labeled for use as size standards.
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Trace amounts of the 32P-labeled pMK908 were digested with restriction nucleases under previously-determined limiting conditions and the resulting products were fractionated by electrophoresis in 1.5% agarose gels. Gels were dried onto DEAE-paper and the spectrum of partial digestion products containing the common 5' terminus was visualized by autoradiography. The 32P-labeled pMK908 DNA partially digested with Pst I was loaded onto the gel adjacent to A size marker fragments. The positions of some restriction sites in the insert were confirmed by standard restriction enzyme analyses.

Selection and Translation of 908 RNA—mRNA was selected by hybridization to filter-bounded pMK908 DNA following a procedure modified from Ricciardi et al. (25). pMK908 or pG1 (a globin cDNA plasmid obtained from David Housman of Massachusetts Institute of Technology) was linearized by restriction with HindIII, deproteinized by extraction with phenol/chloroform/isooamyl alcohol (25:24:1), precipitated with ethanol, and dissolved in 10 mM Tris-Cl, pH 8.0. Nitrocellulose filters (Schleicher and Schuell, BA 85) were cut into 13-mm circles, washed in water, and autoclaved. Just prior to use, 5 ml of 1 M NH4 acetate were passed through the filters. For each filter, 10–20 pl of DNA in 150 ml of 10 mM Tris-Cl, pH 8, 1 mM Na2EDTA was denatured by addition of 45 ml of 0.4 N NaOH at room temperature for 10 min. To the denatured DNA was added an equal volume of 2 M NH4 acetate and the mixture was passed through a filter. The filter with its bound DNA was washed 3 times with 5 ml X SSC, floated on 4 X SSC for 10 min, blotted, allowed to dry, and finally baked at 80 °C under vacuum for 2 h.

For hybridization, part or all of a filter was cut into small pieces and prehybridized for 2 h at 43 °C in 100 ml of 50% formamide, 0.1 M 1,4-piperazinediethanesulfonic (pH 6.4), 0.6 M NaCl, and 100 mg/ml of poly(A). The prehybridization mixture was discarded and replaced by 40 ml of an identical mixture except that poly(A) was replaced by sufficient poly(A)-containing RNA to approach saturation of the plasmid DNAs inserted sequence. Incubation at 43 °C continued for 2–5 h. The hybridization mixture was then removed and the filters were washed 4 times with 1 ml X SSC, 0.5% SDS at 65 °C followed by 2 washes with 1 ml of 0.5 mM Tris-Cl (pH 7.9), 2 mM Na2EDTA at room temperature. Hybridized RNA was recovered by heating the filter in 100 ml of 100 °C for 1 min, followed by quick freezing. RNA in aqueous solution was removed from the nitrocellulose, precipitated with ethanol, rinsed once with cold 70% ethanol, dried under vacuum to remove traces of ethanol, and dissolved in a small volume of water.

Selected RNA was translated in vitro using a reticulocyte lysate system in the presence of [35S]methionine as supplied in a translation kit (New England Nuclear). Translation products were fractionated by polyacrylamide gel electrophoresis in the presence of SDS (26) and observed by autoradiography.

RESULTS

Construction and Isolation of pMK908—A library of E. coli colonies bearing DNA complementary to mouse kidney poly(A)-containing RNA was constructed by a protocol similar to that outlined previously (27). Briefly, a double-stranded cDNA complement of sucrose gradient-fractionated poly(A)-containing RNA from mouse kidney was elongated at its 3' ends with deoxy-C residues and annealed in vitro to pBR322 DNA which had been linearized with endonuclease Pst I and elongated with deoxy-G residues. The annealing mixture was used to transform the x1776 strain of E. coli to tetracycline resistance, and colonies from the ampicillin-sensitive subpopulation, containing inserted DNA, were picked for further screening. From 350 ng of tailed complementary DNA, we obtained 6780 tetracycline-resistant transformants, 3138 of which were also ampicillin-sensitive.

To screen sequences complementary to androgen-inducible RNAs, we have used a modification of the colony lysate hybridization method (18). Bacterial colonies were grown and lysed onto nitrocellulose filters (18). The filters were hybridized to a mixture of two radioactive cDNA probes: one probe was labeled with [32P]dCTP and was complementary to RNA from the kidneys of testosterone-treated female mice, while the other was labeled with [3H]dCTP and was complementary to RNA from the kidneys of normal female mice. Following the hybridization, the 32P/3H ratio of the cDNA hybridizing to each colony was determined. Theoretically, any clone containing a sequence corresponding to a testosterone-inducible mRNA should show an increased ratio, for the concentration of [32P]cDNA complementary to this mRNA will be greater than the concentration of [3H]cDNA. Results of such an analysis for a representative filter is shown in Table I. Most colonies gave a 32P/3H ratio that was not significantly different from the input. However, 37 of 1089 colonies tested gave ratios significantly higher than the input, indicating the presence of a cDNA insert corresponding to a mRNA that is induced by testosterone.

One such clone, pMK908, was chosen for further study. Plasmid DNA from this clone contains a 1.2 kilobase insert, which can be cleaved away from pBR322 DNA byendonuclease Pst I (data not shown); this is expected since the cloning protocol regenerates Pst I sites at both vector-insert junctions. Locations of other restriction sites were determined using a DNA fragment containing a 5' terminal 32P label at one end (see “Materials and Methods”); partial digestion of this fragment with a variety of nucleases (23) enabled construction of the map depicted in Fig. 1.

| Clone | Hybridized 32P cpm | Hybridized 3H cpm | Ratio 32P cpm/3H cpm |
|-------|-------------------|-------------------|---------------------|
| pMK907 | 85 | 95 | 1.0 |
| 908    | 337 | 13 | 30.0 |
| 909    | 115 | 218 | 0.6 |
| 910    | 455 | 452 | 1.2 |
| 911    | 24 | 20 | 1.4 |
| 912    | 56 | 52 | 0.6 |
| 913    | 90 | 160 | 0.57 |
| 914    | 37 | 56 | 0.8 |
| 915    | ND | 29 | |
| 916    | ND | 25 | |
| 917    | 61 | 76 | 0.9 |
| 918    | 92 | 63 | 1.5 |
| 919    | 75 | 66 | 1.1 |
| 920    | 54 | 75 | 1.5 |
| 921    | 34 | 42 | 0.9 |
| 922    | 71 | 116 | 6.6 |
| 923    | 46 | 74 | 0.7 |
| 924    | 151 | 1296 | 0.12 |
| 925    | 74 | 49 | 1.5 |
| 926    | 303 | 123 | 1.9 |
| 927    | 127 | 62 | 2.4 |
| 928    | 318 | 36 | 10.0 |

a The observed ratio of hybridized 32P to 3H was normalized to the input ratio, which was set at 1.0.

b ND, no cpm detected.

c | 2.2 | 10 | 18 | 16 | 14 | 12 | 10 | 8 | 6 | 4 | 2 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | 16 | 12 | 10 | 8 | 6 | 4 | 2 | 0 | 0 | 0 | 0 | 0 |

FIG. 1. Restriction map of pMK908. The restriction sites for the various enzymes were determined by the method of Smith and Birnstiel (33) as described under "Materials and Methods." The thick line represents insert DNA while the thin line represents pBR322 DNA. The bottom line shows the number of kilobases from the Eco RI site of pBR322.
Analysis of RNA Corresponding to pMK908—In order to identify and study in detail the RNA corresponding to the DNA insert of pMK908, we have used the RNA blotting technique of Alwine et al. (21). Total RNA from normal female kidneys or from testosterone-induced female kidneys was electrophoresed in a 1.5% agarose gel, and blotted onto diazobenzyloxymethyl paper. The blot was incubated with $^{32}$P-labeled pMK908 DNA and hybridizing RNA was detected by autoradiography. As shown in Fig. 2A, RNA from untreated kidney gave a barely detectable signal; however, RNA from testosterone-induced kidney showed a markedly stronger signal (Fig. 2B), indicating a higher concentration of hybridizing RNA in this preparation. Blot analysis of a 10-fold dilution of the induced kidney RNA generated a signal of similar intensity to that of normal kidney RNA (data not shown); thus, testosterone elicits about a 10-fold increase in the concentration of hybridizing RNA. This result validates the screening procedure described in the preceding section and proves that we are indeed dealing with a testosterone-inducible sequence. The same results were obtained using poly(A)-containing RNAs (data not shown) indicating that the hybridizing RNAs are polyadenylated.

An interesting feature of the data shown in Fig. 2 is that there are apparently two distinct RNA species that hybridize to the probe. These two RNAs are present in the same ratio in all kidney RNA preparations, including those from normal males, normal females, testosterone-treated females, and testosterone-treated males. One species, representing 60–80% of the signal, is ~2500 nucleotides long, while the other is ~1500 nucleotides long. It is unlikely that the presence of these two RNAs results from degradation or aggregation, for the two bands have been consistently observed in numerous RNA samples, including those analyzed immediately after preparation, as well as after prolonged storage at ~20 °C. Preliminary experiments in this laboratory show that the ratio of the two RNA species is unchanged when hybridization of the blotted RNAs is performed under more stringent conditions.

This indicates that there is little sequence divergence between them in the region covered by the insert of pMK908.

The kinetics of induction was examined by implanting female mice with testosterone pellets and analyzing kidneys for 908 RNA levels at various times thereafter. As shown in Fig. 3, following a lag of ~2 h, there is a rapid increase in the 908 RNA level which reaches a maximum between 6 and 14 h after implantation of hormone. In other experiments, we have observed that this induced level is maintained with no significant changes for at least 21 days (data not shown).

We have tested various tissues in the mouse for the presence of RNA complementary to the insert of pMK908 DNA. Total RNA from the liver, brain, heart, or submaxillary of female mice both prior to and after androgen treatment was tested for hybridization to $^{32}$P-labeled pMK908 DNA by the RNA blotting protocol. The submaxillary is a known androgen-target organ (28) and was therefore of special interest. We have been unable to detect any RNA complementary to the plasmid insert from these tissues even after prolonged exposure of the x-ray film (data not shown). It appears, therefore, that the 908 RNAs are specific to the kidney, at the level of sensitivity employed.

Liquid hybridization was used to quantitate the levels of RNA complementary to the insert of pMK908 both before and after induction with testosterone. A radioactive probe complementary to the pMK908 DNA insert (see “Materials and Methods”) was hybridized to a large excess of total RNA from kidneys of normal or androgen-treated mice, and the kinetics of hybridization were determined for each. As shown in Fig. 4 and Table II, RNA from normal female kidney hybridizes with a $R_d/2$ of 47.9 while that from testosterone-induced kidney hybridizes with a $R_d/2$ of 6.4. From these values, we can calculate (Table II) that RNA complementary to the pMK908 DNA insert represents ~0.0036% of total RNA from female kidney and ~0.027% of total RNA from testosterone-treated female kidney. Assuming equal distribution of the 908 RNAs throughout kidney cell types, these values correspond to ~300 copies/kidney cell in normal females and 2400 copies/kidney cell in induced females (Table II). Thus, testosterone elicits a 7–8-fold increase in the concentration of 908 RNA in female mice.

Compared to females, males have barely elevated and highly variable concentrations of the 908 RNAs, while levels in castrated males are significantly lower (Fig. 4) (Table II). The concentration of the 908 RNAs in both normal and castrated males can be rapidly induced by testosterone treatment to levels similar to that in hormone-treated females (data not shown). Thus, the 908 RNAs are only barely, if at all, induced in males and can be further induced by androgen treatment. This response is similar to that for kidney β-glucuronidase$^3$ and contrasts sharply with KAP, which appears to be fully induced in normal males (10).

Isolation and Translation of mRNA Corresponding to the pMK908 Insert—Messenger RNA corresponding to the insert in a recombinant plasmid can be purified by hybridization to the appropriate denatured DNA immobilized on a nitrocellulose filter. Hybridized RNA can then be recovered from the filter and translated in a cell-free reticulocyte lysate system (29); the nature of the encoded polypeptides can be examined following acrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate. When RNA from induced female kidneys was hybridized to a filter containing pMK908 DNA, RNA was recovered that encoded a 43,000-dalton polypeptide chain in vitro (Fig. 5A). This polypeptide was not observed when RNA from untreated female kidneys was tested (Fig. 5B).

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$^3$ G. Watson, unpublished results.

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Fig. 2. RNA blot hybridization of kidney RNA. A total of 15 μg of RNA from female kidney (A) or androgen-induced kidney (B) were electrophoresed in a 1.5% agarose gel and blotted onto DBM-paper (21). The paper was incubated with 10–15 × 10$^6$ cpm heat-denatured, nick-translated pMK908 DNA, and hybridizing RNA was observed by autoradiography. 28 S and 18 S ribosomal RNAs served as the 5.0 and 1.9 kilobase markers, respectively.
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**Fig. 3.** Kinetics of 908 RNA induction. Testosterone pellets were implanted into female mice. At various times, two animals were sacrificed, kidney RNA was extracted, and the levels of the 908 RNAs were determined by RNA blot hybridization. A, untreated control; B, 1 h; C, 2 h; D, 4 h; E, 6 h; F, 14 h; G, 24 h.

**Fig. 4.** Comparison of 908 RNA levels in various mice. A labeled single-stranded probe specific for the pMK908 RNAs was incubated with an excess of kidney RNAs from various mice; the percentage of hybridization, as determined by S1-nuclease resistant label, was plotted as a function of the log of the Rot (see "Materials and Methods"). X, testosterone-treated females; O, normal males; D, normal females; A, castrated males; A, no RNA control.

**TABLE II**

Quantitation of 908 RNA levels

The 908 RNA levels were calculated from the Rot analysis in Fig. 4 as described by Hastie *et al.* (52), assuming the complexity of 908 RNA to be 820,000. Values were corrected for the length of the 908-specific cDNA probe (1.15 kilobases). The number of 908 RNA molecules/cell was determined under the assumption that the 908 RNAs are equally distributed throughout kidney cell types.

| RNA source          | Rot/100 | 908 mRNA level (fraction of total) | Copies of 908 RNA/cell |
|---------------------|---------|----------------------------------|------------------------|
| Female              | 47.9    | $3.6 \times 10^{-5}$             | 320                    |
| Female, testosterone-treated | 6.4     | $27 \times 10^{-5}$              | 2380                   |
| Male                | 36.4    | $4.7 \times 10^{-5}$             | 410                    |
| Male, castrated     | 92.4    | $1.8 \times 10^{-5}$             | 160                    |

**Fig. 5.** In vitro translation of purified 908 RNA. Poly(A)-enriched kidney RNA (18 μg) was hybridized to 4 μg of filter-bound plasmid DNA for 5 h as described under "Materials and Methods." Hybridized RNA was recovered and translated in the presence of [35S]methionine. Translation products were fractionated by SDS-gel electrophoresis. A, induced kidney RNA hybridized to filter-bound pMK908 DNA; B, normal female kidney RNA hybridized to filter-bound pMK908 DNA; C, induced RNA hybridized to filter-bound pG1 DNA; and D, normal RNA hybridized to filter-bound pG1 DNA. The 43,000-dalton polypeptide is indicated. E shows endogenous translation products made in the absence of any added RNA.

**Physiological Requirements for Induction**—Several studies have indicated that androgen receptor protein, which is markedly reduced in mice bearing a mutation at the Tfm locus on the X chromosome (30, 31), is required for testosterone-mediated induction of several kidney proteins (10, 32, 33). We have tested the inducibility of 908 RNA in androgen-insensitive Tfm mice (Fig. 6). Although only barely visible in the depicted exposure, kidneys from untreated Tfm/Y animals (Fig. 6C) appear to maintain wild type (Fig. 6A) levels of the 908 RNAs; however, in contrast to normal mice, testosterone

**Fig. 6.** Levels of the 908 RNAs in Tfm/Y mice. Total kidney RNA was extracted from wild type (i.e. strain C57BL/6J) or C57BL/6-Tfm/Y mice, and 908 RNA levels were determined by blot hybridization. A, wild type; B, testosterone-treated wild type; C, Tfm/Y; D, testosterone-treated Tfm/Y.
treatment of Tfm/Y animals had no effect on the 908 RNA levels (Fig. 6, B and D). Thus, the Tfm mutation removed the inducibility of 908 RNA, indicating that induction of this RNA, like other androgen-inducible functions of kidney, requires androgen receptor protein.

In order to test whether the pituitary is involved in the induction of 908 RNA, we have analyzed hypophysectomized mice in which the pituitary had been surgically removed. RNA blotting experiments (Fig. 7, C and D) indicate that hypophysectomized females have 908 RNA levels comparable to normal females and that testosterone treatment of such animals elicits a normal induction. Thus, the pituitary appears to play little or no role in the androgen-mediated induction of the 908 RNAs; this is in contrast to the involvement of the pituitary in other androgen-inducible functions (34-36).

We have used a genetic test to assess the role of one pituitary hormone, growth hormone, in modulating the response of the 908 RNAs to androgen. Growth hormone affects inductions of rat α2-globulin (37), mouse β-glucuronidase (38), and mouse major urinary proteins. Although the data obtained with hypophysectomized mice minimizes any participation of growth hormone in 908 RNA induction, a mutant mouse specifically lacking the hormone was examined to verify this conclusion. C57BL/6-lit/lit mice have reduced levels of circulating growth hormone, resulting in a stunted postnatal growth pattern (39). As shown in Fig. 7, E and F, 908 RNA levels in these mice appear normal and are induced by testosterone to levels comparable to fully induced normal mice. Thus, the reduction of the growth hormone level due to the lit mutation has no significant effect upon the basal level of the 908 RNAs or its sensitivity to testosterone.

**DISCUSSION**

The mouse kidney offers several advantages as a model system for studying the effect of androgens on gene expression. Previous studies have shown that testosterone functions in kidney in the absence of any detectable cellular hyperplasia (1); thus, the effects of androgen on gene expression can be examined independently of secondary changes accompanying cellular proliferation. In addition, a variety of kidney genes respond to androgen (11); extensive biochemical and genetic studies on several of these androgen-inducible gene products enable comparison of similarities and differences that will be important in understanding the general features of testoster-

A recombinant plasmid was selected that contains an insert corresponding to a testosterone-inducible RNA. Clones were selected on the basis of differential hybridization to cDNA from androgen-induced kidney RNA and cDNA from basal kidney RNA. Differential hybridization has been used before to detect clones containing DNA sequences that correspond to regulat-

The mechanism by which the two 908 RNAs are generated is not known. The smaller may be derived from the larger by some posttranscriptional processing event. It is also possible that the two RNAs are members of a family that is derived in whole or part from closely related genomic sequences. The existence of mRNA families is well documented and includes the mammalian immunoglobulins (42-44) and globins (45, 46), frog vitellogenin (47), insect chorion proteins (48), chicken ovalbumin (49, 50), sea urchin embryo proteins (51), and mouse major urinary proteins (52, 53). The heterogeneity within most of these families is generated by sequence divergence among similarly sized mRNAs. However, heterogeneity of length has been observed (42-44, 50, 51) and is probably not a particularly rare phenomenon.

The 908 RNAs are induced 7-8-fold following testosterone treatment of female mice as shown by hybridization analysis (Fig. 4). The induction is relatively rapid, reaching a maximum between 6 and 14 h after hormone administration (Fig. 3). Thus, the 908 RNAs respond more rapidly to androgen than any other kidney gene product identified to date. Other specific androgen-inducible functions in the kidney require several days or longer to become maximally induced. Full indiction of β-glucuronidase mRNA, for example, takes nearly a month (12). It is highly probable that many other inducible
gene products respond as fast as or faster than the 908 RNAs, for studies on the transcriptional capacity of kidney chromatin and the activity of kidney RNA polymerase show that these parameters respond to androgen within 30 min (4).

Isolation of the 908 RNAs followed by their translation in vitro enables detection of a 43,000-dalton polypeptide chain (Fig. 5). Mills and Bardin (54) have identified an androgen-simulated protein, called C1, of $M_0 = 43,000$ in the lysosomal-mitochondrial fraction of whole kidney homogenates. It is possible that the 908 mRNAs encode C1; in addition to the similarity between the molecular weights of C1 and of the 908 mRNA translation product, the kinetics of their inductions is similar. Without further information, however, definitive conclusions are premature.

Although they differ considerably in length, it is conceivable that both 908 RNAs encode the same polypeptide chain. Such a situation has recently been identified within the ovalbumin gene family (50). A gene, $X$, that is linked to and closely related to the ovalbumin gene, encodes two RNAs that differ in the length of their $3'$-proximal regions. It is unlikely that the RNAs encode different polypeptides, for nucleotide sequence analysis of the $3'$-proximal region reveals the presence of termination codons in all three reading frames (50). By analogy, it is possible that the 908 mRNAs also differ in the length of $3'$-proximal regions, yet encode the same polypeptide. We have recently separated the 908 RNAs on a sucrose gradient and are currently analyzing the in vitro translation products of each to determine if one or both encode the 43,000-dalton polypeptide.

Induction of the 908 RNAs, like all other androgen-dependent functions that have been examined, requires the testosterone receptor protein. However, unlike several others, 908 RNA regulation is independent of the pituitary. That the pituitary exerts effects on androgen-mediated gene expression has been clearly established in both the rat ovo-b-globulin (34, 35) and mouse $\beta$-glucuronidase (36) systems; in addition, the pituitary has recently been implicated in maintaining major urinary protein synthesis in the mouse. Growth hormone appears to be a major pituitary product that is specifically functioning in these systems (37, 38). The 908 RNAs appear to be free of such multihormonal effects, as do some other androgen-inducible kidney functions (36). This is of some advantage in that 908 RNA regulation by androgen may be exerted through a less complicated series of hormonal interactions, making it more amenable to analysis.

We have recently isolated a genomic clone corresponding to 908 RNA and are using this clone to examine the structure of the 908 gene and to gather information concerning the relationship between the 908 gene and the two 908 RNAs. In addition, we are taking advantage of the potential for genetic studies in the mouse by screening a variety of laboratory and inbred strains for polymorphism in the expression of the 908 RNAs. These studies should provide details on the role of the genome in governing 908 gene expression.

In addition to pMK908, recombinant plasmids containing inserts complementary to another androgen-sensitive gene product, kidney androgen-regulated protein mRNA, have been isolated. The availability of recombinant plasmids containing KAP and 908 sequences and comparison of the structure and regulation of the corresponding genes will be useful in delineating features that govern hormonal modulation of gene activity. In addition, since these genes are expressed only in the kidney, it will be possible to determine the details of gene structure that signal cell-type specific expression.

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REFERENCES

1. Kochakian, C. D., and Harrison, D. G. (1962) Endocrinology 70, 98-108.
2. Kochakian, C. D., Nishida, M., and Hirota, T. (1969) Am. J. Physiol. 217, 383-391.
3. Dubovsky, J., and Kochakian, C. D. (1973) Endocrinology 92, 917-920.
4. Janne, O., Bullock, L. P., Bardin, C. W., and Jacob, S. T. (1976) Biochim. Biophys. Acta 418, 239-244.
5. Fishman, W. H. (1985) in Methods in Hormone Research (Dorman, R. L., ed), Vol. 4, pp. 273-326, Academic Press, New York.
6. Swank, R. T., Paigen, K., and Ganschow, R. E. (1973) J. Mol. Biol. 81, 225-243.
7. Grubstein, S., Stenius, C., and Christian, L. C. (1970) Clin. Genet. 1, 35-44.
8. Endahl, B. R., and Kochakian, C. D. (1956) Am. J. Physiol. 185, 250-256.
9. Grahn, B., Henningsson, S. S., Kahlaon, G., and Rosengren, E. (1976) Br. J. Pharmacol. 56, 113-120.
10. Toole, J. J., Hastie, N. D. and Held, W. A. (1979) Cell 17, 441-448.
11. Swank, R. T., Paigen, K., Davye, R., Chapman, V., Labarca, C., Watson, G., Ganschow, R., Brandt, E. J., and Novak, E. (1978) Recent Prog. Horm. Res. 34, 401-436.
12. Watson, G., Davye, R. A., Labarca, C., and Paigen, K. (1981) J. Biol. Chem. 256, 3005-3011.
13. Cox, R. A. (1968) Methods Enzymol. 12, 120-129.
14. Labarca, C., and Paigen, K. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 4463-4465.
15. Rougeon, F., and Mach, B. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3418-3422.
16. Roychoudhury, R., Jay, E., and Wu, R. (1976) Nucleic Acids Res. 3, 101-116.
17. Enea, V., Vovis, G. F., and Zinder, N. D. (1975) J. Mol. Biol. 96, 495-509.
18. Grunstein, M., and Hogness, D. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3961-3965.
19. Tanaka, T., and Weisblum, B. (1975) J. Bacteriol. 121, 354-362.
20. Maniatis, T., Jeffrey, A., and Kleid, D. G. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 1184-1188.
21. Alwine, J. C., Kemp, D. J., and Stark, G. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5350-5354.
22. Bishop, J. O., Morton, J. G., Rosbash, M., and Richardson, M. (1974) Nature 241, 204-207.
23. Smith, H. O., and Burstein, M. I. (1976) Nucleic Acids Res. 3, 2987-2998.
24. Woodworth-Gutai, M. (1981) Virology 109, 344-352.
25. Ricciardi, R. F., Miller, J. S., and Roberts, B. E. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4927-4931.
26. Laemmli, U. K. (1970) Nature (Lond.) 227, 680-685.
27. Gordon, J. I., Burns, A. T. H., Christmann, J. L., and Deeley, R. G. (1978) J. Biol. Chem. 253, 8629-8639.
28. Geesse, A., Wilson, C. M., and Erdos, E. G. (1976) Biochem. Pharmacol. 25, 763-765.
29. Peitham, H. P. B., and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247-257.
30. Attardi, G., and Ohno, S. (1974) Cell 2, 205-212.
31. Gehring, U., and Tomkins, G. M. (1974) Cell 3, 59-64.
32. Dofuku, R., Tettenborn, U., and Ohno, S. (1971) Nature (New Biol.) 232, 5-7.
33. Bullock, L. P., and Bardin, C. W. (1974) Endocrinology 94, 746-756.
34. Kumar, M., Roy, A. K., and Axelrod, A. E. (1969) Nature (Lond.) 233, 399-400.
35. Roy, A. K. (1973) J. Endocrinol. 56, 295-301.
36. Swank, R. T., Davye, R., Joyce, L., Reid, P., and Macey, M. R. (1977) Endocrinology 100, 473-480.
37. Roy, A. K., and Dowdenko, D. J. (1977) Biochemistry 16, 3918-3922.

1 P. Howles and K. Gross, unpublished results.
2 N. Piccini, K. Gross, and W. Held, unpublished results.
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38. Swank, R. T. (1978) Mol. Cell. Endocrin. 12, 139–149
39. Eicher, E. M., and Beamer, W. G. (1976) J. Hered. 67, 87–91
40. St. John, T. P., and Davis, R. W. (1979) Cell 16, 443–452
41. Goldberg, D. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5794–5798
42. Alt, F. W., Bothwell, A. L. M., Knapp, M., Siden, E., Mather, E., Koshland, M., and Baltimore, D. (1980) Cell 20, 293–301
43. Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L., and Wall, R. (1980) Cell 20, 303–312
44. Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., and Hood, L. (1980) Cell 20, 313–319
45. Weatherall, D. J., and Clegg, J. B. (1979) Cell 16, 467–479
46. Efstratiadis, A., Posakony, J. W., Maniatis, T., Lawn, R. M., O’Connell, C., Spritz, R. A., DeRiel, J. K., Forget, B. G., Weissman, S. M., Slightom, J. L., Blechly, A. E., Smithies, O., Baralle, F. E., Shoulders, C. C., and Proudfoot, N. J. (1980) Cell 21, 653–658
47. Wahl, W., David, I. B., Wyler, T., Jagg, R. B., Weber, R., and Ryffel, G. U. (1979) Cell 16, 535–549
48. Spradling, A. C., Digan, M. E., Mahowald, A. P., Scott, M., and Craig, E. A. (1980) Cell 19, 905–914
49. Royal, A., Garapin, A., Cami, B., Perrin, F., Mandel, J. L., LeMeur, M., Bregesegre, F., Gannon, F., LePennec, J. P., Chambon, P., and Kourilsky, P. (1979) Nature 279, 125–132
50. Heilig, R., Perrin, F., Gannon, F., Mandel, J. L., and Chambon, P. (1980) Cell 20, 625–637
51. Lee, A. S., Thomas, T. L., Lev, Z., Britten, R. J., and Davidson, E. H. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 3259–3263
52. Hastie, N. D., Held, W. A., and Toole, J. J. (1979) Cell 17, 443–457
53. Szoka, P. R., Gallagher, J. F., and Held, W. A. (1980) J. Biol. Chem. 255, 1367–1373
54. Mills, N. C., and Bardin, C. W. (1980) Endocrinology 106, 1182–1190