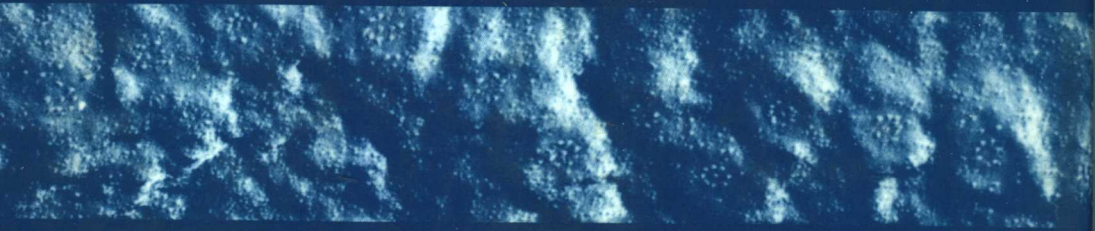
Birgit H. Satir, Series Editor

MODERN CELL BIOLOGY

Volume 6



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Foreword

The goal of this series is to provide researchers in cell and molecular biology with comprehensive articles that review areas of current significance and that raise pertinent questions about future directions. We seek to publish articles that look to the future but provide lasting value.

The articles published here, which cover topics as diverse as structure and regulation of genes and protein modification via sulfation on tyrosine residues, illustrate the breadth of modern cell biology. This volume continues the traditions of the Modern Cell Biology series. Each article has been carefully reviewed by the appropriate editors to ensure accuracy, timeliness, and significance. The authors are important contributors to the fields whose current status they assess for us.

We hope readers find these reviews both useful and exciting.

Birgit H. Satir
October 1987

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Modern Cell Biology 6:1-28

Androgen Action in Mouse Kidney: Structure and Regulation of the β-Glucuronidase and Ornithine Decarboxylase Genes

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I. INTRODUCTION

Steroid hormones act on target organs to influence the synthesis of a specific battery of proteins that constitute the induced phenotype. Steroids impose this phenotype on individual cells through interaction with soluble receptor proteins, which mediate the effects of the hormone in the nucleus. In all cases studied, including the sex steroids and the glucocorticoids, a receptor protein is necessary for the development of the stimulated state. However, presence of the receptor for a given hormone is often not sufficient

to initiate a response upon exposure to that hormone. This indicates the requirement for other tissue-specific factors as modulators of steroid hormone action. Although these factors are currently unspecified, the events they may mediate in the nucleus include the interaction of steroid-receptor complexes with regions of chromatin at or near regulated genes, the extent to which DNA of a target cell is methylated, and the accessibility of genes to transcription by RNA polymerase. The essential link between the androgen receptor and the induced phenotype was established through the identification and study of receptor mutants in the rat and mouse, which were insensitive to many of the effects of androgens [Lyon and Hawkes, 1970; Bardin et al., 1970; Bullock et al., 1971]. Both androgenic effects on reproductive tissues and the growth-promoting (anabolic) effects of these steroids on other tissues were shown to be mediated via androgen receptors [Bardin and Catterall, 1981].

Studies of the molecular mechanism by which androgens regulate gene expression have been carried out using a number of biological markers, such as the abundant gene products in rat prostate [Page and Parker, 1982; Dodd et al., 1983] and rat seminal vesicle [Mansson et al., 1981; Kandala et al., 1983; McDonald et al., 1983]. The mouse kidney has also been utilized as an important experimental system for the study of these aspects of androgen action. Recombinant DNA probes are currently available for several genes that are under androgenic regulation in this tissue [Watson et al., 1984; Berger et al., 1981; Kontula et al., 1984; McConlogue et al., 1984; Catterall and Leary, 1983; Palmer et al., 1983]. In this review, we describe our studies on the control of gene expression by testosterone in murine renal cells. We have developed complementary DNA probes for the ornithine decarboxylase [Kontula et al., 1984] and β-glucuronidase [Catterall and Leary, 1983] genes and have used these to examine their structure and their expression under the various conditions of hormone treatment as well as in different genetic backgrounds.

II. THE MOUSE KIDNEY MODEL SYSTEM

The physiological roles of androgens are to mediate the growth and differentiation of the primary and secondary sex organs of the male. However, many sexually dimorphic responses in nongenital tissues are regulated by androgens by a mechanism (Fig. 1) analogous to androgenic effects in the sex organs [Bardin and Catterall, 1981]. The mouse kidney is a useful model system for the study of androgen action because of several characteristics that simplify experimental approaches to this problem. First, androgens

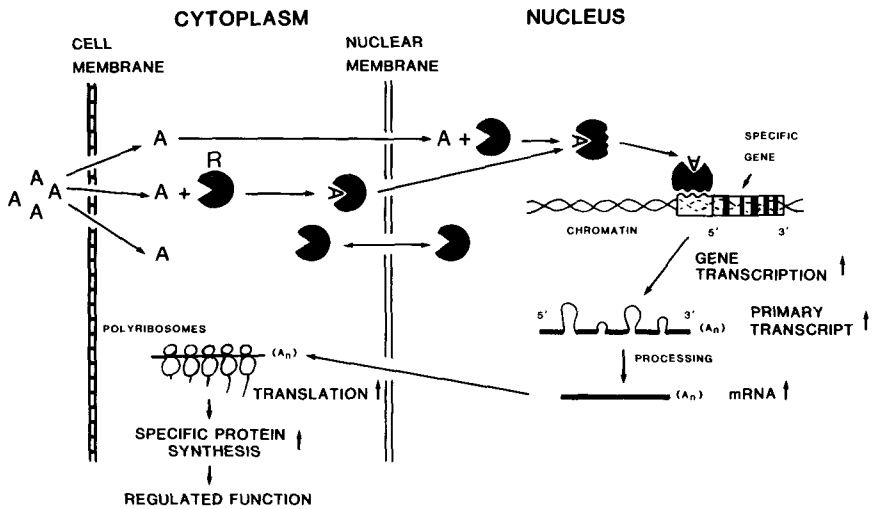

Fig. 1. Schematic representation of the mechanism of androgen action in a target cell. A, androgen; R, androgen receptor.

increase protein synthesis in the mouse kidney without a concomitant stimulation of cell division or DNA replication. Whereas the synthesis of certain proteins is induced as much as 1,000-fold upon treatment with testosterone, renal DNA content has been shown to be unaffected or to increase only by 25% with similar hormone treatment [Henningsson et al., 1978; Kochakian and Harrison, 1962]. Second, testosterone itself is the primary effector of the androgenic response in this tissue. In many other target tissues for androgens, the effects of testosterone require its conversion to the more biopotent metabolite 5α-dihydrotosterone (5α-DHT) [Wilson et al., 1981; Bardin et al., 1973]. Mouse kidney has low 5α-reductase activity, so the effects of testosterone can be measured directly in this tissue.

In addition to these advantages, many enzymes and other proteins have been identified that are under androgenic control in the mouse kidney (Table I). Two of these enzymes, β-glucuronidase (β-GLUC) and ornithine decarboxylase (ODC) have also been studied at the level of their mRNA [Catterall and Leary, 1983; Palmer et al., 1983; Kontula et al., 1984; McConlogue et al., 1984; Berger et al., 1984]; these are the subject of the present review. Other mRNA markers for androgen action in mouse kidney have also been reported (Table I). These mRNAs were identified by their induction after testosterone administration, but their protein products and their functions are unknown. Included in this group are the mRNA for kidney androgen-regu-

TABLE I. Markers* for Androgen Action in Mouse Kidney

Protein	mRNA
Alcohol dehydrogenase	β-Glucuronidase
D-amino acid oxidase	KAP†
Arginase	MK908†
Galatosyltransferasse	Ornithine decarboxylase
β-Glucuronidase	
Glutamic-pyruvic acid transaminase	
Glutamate-oxaloacetate transaminase	
Glutamate dehydrogenase	
3-Ketoreductase	
α-Mannosidase	
Ornithine decarboxylase	
T proteins†	

*The proteins listed in this table increase (in activity or amount) at least twofold in response to androgens [see Bardin et al., 1978; Swank et al., 1978; and references therein].
†T proteins, testosterone-induced proteins [Bardin et al., 1978]; KAP, kidney androgen-regulated protein [Toole et al., 1979]; MK908, see Berger et al. [1981].

lated protein (KAP) [Toole et al., 1979; Watson et al., 1984] and the mRNA designated MK908 [Berger et al., 1981].

Several androgen-responsive genes in murine kidney have also been shown to be modulated by other hormones, in particular, progestins that interact with the androgen receptor to produce a range of androgenic, synandrogenic (potentiation of androgen action), and antiandrogenic effects [Mowszowicz et al., 1974; Gupta et al., 1978]. In addition, we have recently observed that three androgen-regulated renal genes (β-GLUC, ODC, and kidney androgen-regulated protein [KAP]) respond very differently to androgens and to the nonsteroidal antiandrogen flutamide [Catterall et al., 1985]. Studies of such multiple hormonal control using specific gene probes should help in elucidation of the function of androgen receptor complexes and their possible interactions with defined DNA regions.

Finally, genetic variation in inbred mouse strains modifies the response to androgen treatment. Two well defined genetic loci illustrate this point. The first is the Tfm/Y mutation that affects the androgen receptor gene or a regulator thereof. Two alleles of this locus have been shown to produce aberrant receptor populations that are functionally separable on the basis of their DNA-binding characteristics [Fox and Politch, 1983]. The other well defined genetic locus is Gus-r, the cis-acting regulator of the [Gus] genetic complex. The several alleles of Gus-r determine the ability of the mouse renal β-GLUC gene to respond to androgen stimulation [Palmer et al., 1983; Pfister et al., 1984; Watson and Catterall, 1986; Watson and Paigen, 1986].

Gus-r effects only the androgenic stimulation of the β-GLUC gene. However, similar genetic variation is also characteristic of the expression of other androgen-responsive genes in this tissue. For instance, the induction of ODC activity and ODC mRNA accumulation by androgen was shown to vary severalfold among genetically inbred strains of mice [Bullock, 1983; Melanitou et al., 1987]. In addition to these data, we have shown that the ratio of the two distinct forms of ODC mRNA (see below) varies in testosterone-treated females of different inbred strains. Moreover, the MK908 mRNA mentioned above shows strain-dependent size heterogeneity, the expression of which seems to be controlled by a genetic determinant that is tightly linked to the 908 structural gene [Elliott and Berger, 1983].

III. CHARACTERISTICS OF ODC AND β-GLUC ENZYMES

ODC is one of the rate-controlling enzymes in the biosynthesis of polyamines and catalyzes conversion of L-ornithine to putrescine. It is a constitutive enzyme probably present in all cells and tissues and requires pyridoxal 5′-phosphate for the activity [Pegg and McCann, 1982]. ODC concentration is very low in quiescent cells, but its activity is increased manyfold within a few hours of exposure to trophic stimuli, including hormones, drugs, and tissue regeneration and growth factors [Jänne et al., 1978]. Even after maximal stimulation, ODC forms only a minute fraction of total cellular protein, ranging from 0.0001 to 0.04% of the soluble cytosol protein. Interestingly, renal tissue of androgen-treated mice seems to be the richest source of ODC [Pegg and McCann, 1982], although the physiological significance of its high concentration in murine kidney is not yet understood.

One of the unique characteristics of ODC in virtually all cells is its remarkably short half-life of less than 1 hr; in mouse kidney, it is approximately 15 min [Seely and Pegg, 1983; Isomaa et al., 1983]. In this respect, ODC is similar to two other rate-controlling enzymes in polyamine biosynthesis (S-adenosylmethionine decarboxylase and spermidine/spermine-acetylase), which also have very rapid turnover rates [Pegg and McCann, 1982]. The enzyme protein has a subunit molecular weight of about 50,000 and is supposedly a dimeric molecule under physiological conditions. Each subunit appears to be catalytically active, as judged by its ability to bind [^{3}H]α-difluoromethylornithine, and enzyme-activated irreversible inhibitor [Pritchard et al., 1981; Seely and Pegg, 1983; Isomaa et al., 1983]. The catalytic activity of ODC in different tissues has been reported to be regulated by a variety of posttranslational mechanisms [Pegg and McCann, 1982]. However, data from our laboratory and other laboratories have indicated that, in

mouse kidney, the catalytic activity is regulated strictly via modulation of the enzyme protein concentration [Seely and Pegg, 1983; Isomaa et al., 1983].

β-Glucuronidase is an acid hydrolase that is present in the lysosomes of virtually all tissues. In mammalian liver and kidney cells, the enzyme is also present in membranes of the endoplasmic reticulum. The richest sources of the enzyme are the female rat preputial gland and the urine of androgen-treated mice [Beyler and Szego, 1954; Tulsiani and Keller, 1975; Lusis and Paigen, 1978; Mills et al., 1978]. Its physiological function is in the metabolism of mucopolysaccharides and possibly the modification of certain glucuronide conjugates such as those of steroid hormones. In humans, β-glucuronidase deficiency results in polysaccharide storage disease [Sly et al., 1973; Gehler et al., 1974].

Several forms of β-glucuronidase have been described that correlate with its dual localization. The most common is the lysosomal (L) form of the enzyme; its counterpart in microsomal membranes, designated X, give rise to four other forms (M1–4) by binding one to four molecules of egasyn that anchors X to the membrane. All six forms of β-glucuronidase can be separated by polyacrylamide gel electrophoresis [Swank and Paigen, 1973].

Mouse β-glucuronidase has been purified from both kidney [Lin et al., 1975] and liver tissue [Tomino et al., 1975]. The enzyme from both sources has a molecular weight of 280,000–300,000 and is a homotetramer of 70,000–75,000-dalton subunits. The enzyme has an isoelectric point of 5.8 ± 0.5, and it is unusually stable on storage [Tomino et al., 1975]. Carbohydrate analysis has showed the presence of glucosamine and mannose as well as some galactose and glucose, but no fructose or sialic acid could be detected. Biochemically, the broad range of β-D-glucuronides that are hydrolyzed by β-glucuronidase has allowed the development of sensitive and convenient assays for enzyme activity. Intensive biochemical, physiological, and genetic studies employing such assays over the past two decades have helped to make the β-glucuronidase gene complex one of the best characterized genetic loci in mammals [Paigen, 1979].

IV. IDENTIFICATION OF ODC AND β-GLUC mRNAs

To study in detail the molecular mechanism of androgen action in mouse kidney, we prepared cloned complementary DNA (cDNA) probes for several renal genes that code for the androgen-inducible proteins. Since a variety of kinetic responses to androgens had been described suggesting differential gene regulation, cDNAs for ODC and β-GLUC mRNAs were considered to be particularly useful because they represented examples of rapidly and relatively slowly responding genes.

Purification of the two proteins from mouse urine (β-GLUC) or renal cytosol (ODC) of androgen-treated female mice was accomplished essentially as previously described [Seely et al., 1982; Isomaa et al., 1983; Mills et al., 1978; Lusis and Paigen, 1978; Pajunen et al., 1982]. Each of the two proteins was judged to be greater than 95% pure by polyacrylamide gel electrophoresis under denaturing conditions and was used to raise monospecific antibodies in rabbits. Native ODC and heat-denatured β-GLUC (20–50 μg/ml) were injected at multiple subcutaneous sites at 2 week intervals for 6 weeks, after which they were given monthly [Isomaa et al., 1983]. Serum was prepared from blood samples taken 7–10 days following each booster injection and stored at $-20°C$. The monospecificity of these polyclonal antibodies was established by crossed immunoelectrophoresis and "Western" blotting.

Mouse kidney poly(A)-containing mRNA was isolated from total polysome preparations by oligo(dT)-cellulose chromatography [Catterall and Leary, 1983; Kontula et al., 1984]. After various treatments with testosterone, the mRNA was translated in vitro in the presence of L-[^{35}S]methionine, and the peptide products were immunoprecipitated with antiserum for β-GLUC or ODC. Specifically immunoprecipitated radioactive peptides were not detected in this assay for β-GLUC, and translation of ODC mRNA gave a barely detectable signal after a long exposure of the film. In that both β-GLUC and ODC mRNA were estimated to represent $<0.1\%$ of the total mRNA even under chronic androgen treatment, it was not surprising that further purification of the two mRNAs was required to detect clear signals in the cell-free translation assay.

To enrich these mRNAs, we chose to employ a modification of the polysome immunoprecipitation method [Shapiro and Young, 1981; Kraus and Rosenberg, 1982] (Fig. 2), since purification only on the basis of size of the mRNAs encoding these two enzymes with molecular weights fairly average for cellular proteins (β-GLUC 69,000 and ODC 53,000) was expected to be of limited value and since identification of cDNA clones from low-abundance mRNAs was expected to be difficult without a prior enrichment of the mRNA preparations. Prior to their use of polysome immunoadsorption, antisera for each protein were adsorbed to protein A-Sepharose, and bound IgG was eluted with 0.1 M glycine at pH 3. This purification step was sufficient to render the IgG fraction free of ribonuclease activity, at least in the presence of a relatively high concentration of heparin (1–2 mg/ml). The column used for antiserum purification was subsequently stripped with acetic acid (1 M) and equilibrated with polysome buffer [25 mM Tris, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% (v/v) Nonidet p-40, 5 μg/ml cycloheximide, and 2 mg/ml heparin]. Total renal polysomes were prepared as previously described

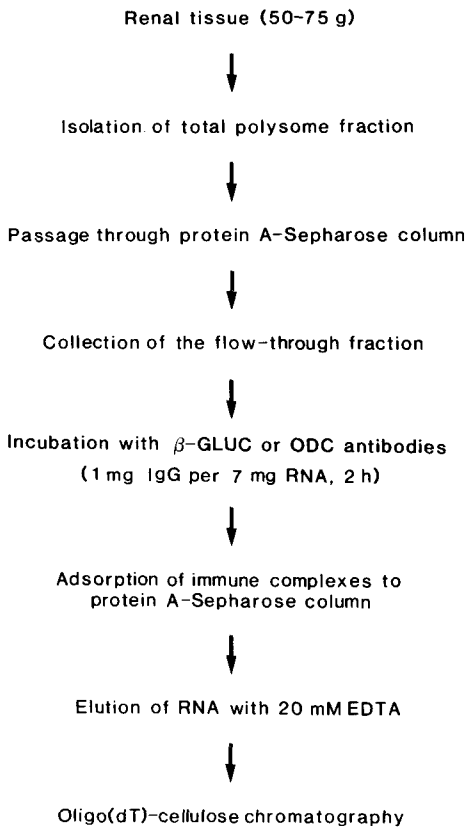

Fig. 2. Diagram of the method by which β-glucuronidase (β-GLUC) and ornithine decarboxylase (ODC) mRNAs were partially purified from kidneys of androgen-treated mice.

[Catterall and Leary, 1983; Kontula et al., 1984] and were passed over the protein A-Sepharose column at flow rate of 15 ml/hr to remove material that adsorbs nonspecifically to the matrix. β-GLUC IgG (8–10 mg protein) was added to the flow-through fraction and incubated at 4°C for 2 hr. In the meantime, the column was stripped with EDTA (20 mM in 25 mM Tris, pH 7.6, 0.2 mg/ml heparin) and again equilibrated with polysome buffer. Polysomes complexed to IgG via β-GLUC nascent chains were adsorbed to the matrix by passing the IgG-polysome mixture over the column at a flow rate of 6–8 ml/hr. The flow-through fraction was frozen and stored at −70°C unless used immediately for purification of ODC mRNA. The column was washed extensively with polysome buffer, unpacked, and pipetted repeatedly

to break up clumps of the resin. After repacking, β-GLUC mRNA along with ribosomal RNA was released from the matrix with 20 mM EDTA as described above. Fractions containing eluted RNA were pooled, precipitated with ethanol, and enriched for poly(A)-containing RNA by chromatography on oligo(dT)-cellulose. The protein A-Sepharose column was once again stripped with acetic acid and equilibrated with polysome buffer.

The flow-through fraction was mixed with ODC IgG, and the procedure was repeated. β-GLUC and ODC mRNA preparations that were routinely achieved were 10–20% pure at this stage as determined by translation in vitro followed by immunoprecipitation (Fig. 3). The order in which the two mRNAs were isolated did not affect their purity or yield. As a matter of covenience, we have purified β-GLUC and ODC mRNAs employing separate protein A-Sepharose columns, which have been used repeatedly for 1.5 years without apparent deterioration.

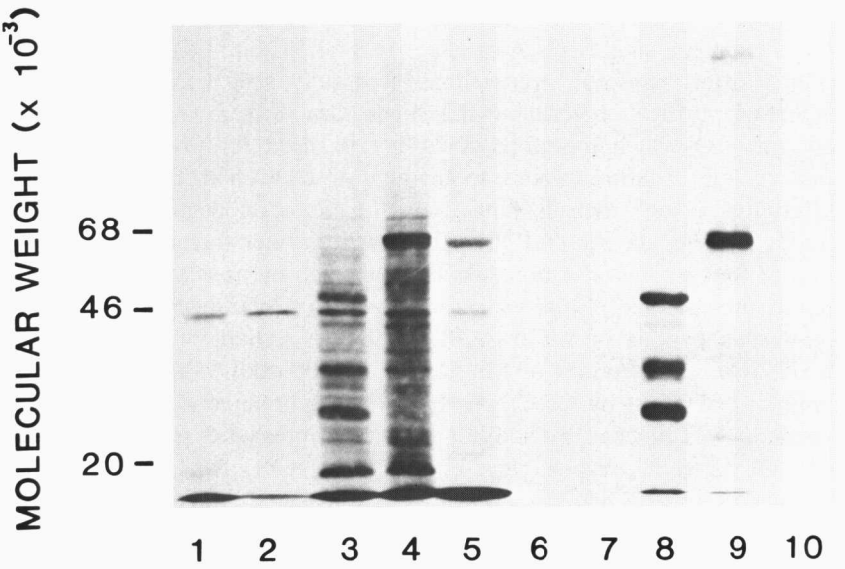

Fig. 3. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of immunopurified mRNA samples after translation in vitro using a rabbit reticulocyte lysate and L-[^{35}S] methionine. Lanes 1–5 contain total translation products of the various samples; lanes 6–10 the respective immunoprecipitated peptides. Lanes 1, 2 and 6, 7 no exogenous mRNA; lanes 3 and 8, ODC mRNA purified by scheme shown in Figure 2; lanes 4, 9, β-GLUC mRNA purified from the same polysome preparation as the ODC mRNA in lanes 3 and 8; lanes 5 and 10, rabbit globin mRNA. Autoradiographic exposure was for 40 hr at −70°C.

V. CLONING OF β-GLUC AND ODC cDNAs

After the purification of each mRNA, the major obstacles to producing cDNA clones were the amounts of purified mRNA available for cDNA synthesis and unambiguous identification of the clones of interest. Initially, conventional methods were used for cDNA synthesis from 1 μg of each mRNA [Stein et al., 1978]. The double-stranded cDNAs were treated with nuclease S1, tailed with dC residues, and inserted into dG-tailed pBR322 cleaved with *Pst* I. Clones were produced in *Escherichia coli* strain LE392 using the transformation procedure of Dagert and Ehrlich [1979].

The initial step in the identification of the cDNA clones exploited the methods used for the isolation of the mRNAs. Radioactively labeled cDNA was prepared from four mRNA fractions produced during the purification procedure. Each purified mRNA ("positive" hybridization probe) and the flow-through from each immunoaffinity column ("negative" hybridization probe) were used. The radiolabeled cDNAs were hybridized with clones produced from the purified mRNAs. To prepare "negative" hybridization probes lacking a single cDNA of interest, β-GLUC and ODC mRNAs were isolated from polysome preparations separately rather than sequentially. Sequential purification resulted in a single flow-through fraction devoid of both mRNAs which therefore could not be used for preparation of the negative hybridization probes to distinguish ODC and β-GLUC clones. Differential colony hybridization using these probes identified a group of candidate clones for each mRNA. These were further screened in groups of three or four by hybridization selection followed by translation in vitro and immunoprecipitation. Clones were identified that hybridized to mRNA that produced a peptide of Mr 69,000, which was immunoprecipitated by β-GLUC IgG, and peptides of Mr 54,000, 37,500, and 33,000, which were immunoprecipitated by ODC IgG (Fig. 4). The immunoprecipitation of the peptide(s) was blocked by addition of excess, unlabeled purified β-GLUC and ODC proteins, respectively. Final identification of β-GLUC and ODC cDNA clones was accomplished by different means. In the case of β-GLUC, sequencing of the cDNA [Watson et al., 1984] provided a predicted amino-acid sequence for a portion of the protein that matched peptides from rat β-GLUC that had been reported previously [Leighton et al., 1980]. In the absence of any amino acid sequence data for ODC, radioimmunoassay of β-lactamase-ODC fusion peptides in the media from cultures of positive clones corroborated results from differential colony hybridization and hybridization selection [Kontula et al., 1984]. Finally, other laboratories have described clones for β-GLUC [Palmer et al., 1983; Watson et al., 1985] and ODC

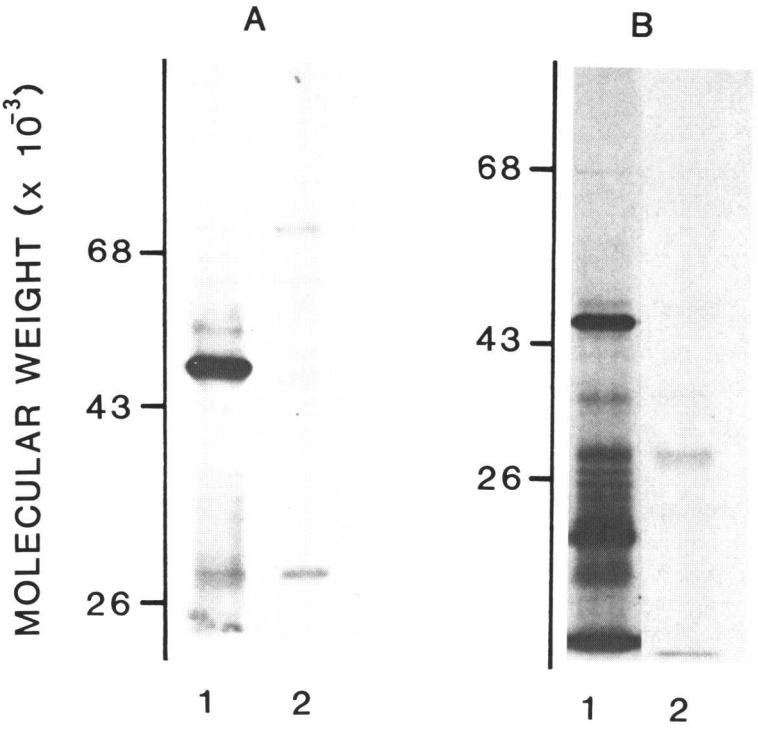

Fig. 4. Translation in vitro of hybridization-selected mRNA. **A:** lane 1, translation products of mRNA that hybridized to β-GLUC cDNA plasmid; lane 2, immunoprecipitated peptides from the sample in lane 1. B: lane 1, translation products of mRNA that hybridized to an ODC cDNA plasmid; lane 2, immunoprecipitated peptides from the sample in lane 1.

[McConlogue et al., 1984; Kahana and Nathans, 1985; Berger et al., 1984] that exhibit characteristics identical to those we have described.

Since these procedures did not produce full-length cDNA clones for either mRNA, other methods were used to complete the cloning of each cDNA. Specific primers derived from the initial isolates were annealed to mRNA templates and extending using reverse transcriptase. Second strand synthesis was carried out by the method of Okayama and Berg [1982] as modified by Gubler and Hoffman [1983]. Full-length cDNAs were obtained by these procedures and mapped by restriction endonuclease digestion. Nucleotide sequences of each of the cDNAs from mouse kidney have recently been determined [Hickok et al., 1986] (also, J.F. Catterall and S.L. Leary, unpublished data). Sequences of these two cDNAs have also been determined from