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Androgen Induction of Messenger RNA Concentrations in Mouse Kidney Is Posttranscriptional†

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ABSTRACT: The concentrations of several mRNAs in mouse kidney increase in response to testosterone. To determine if the increases are generated at the level of gene transcription, we have assayed transcription rates for several androgen-inducible mRNAs in kidney nuclei in vitro. No significant changes were found in the synthesis of three mRNAs whose concentrations increase 10–20-fold during testosterone treatment. Kinetic analysis of changes in transcript levels after testosterone administration and withdrawal suggests that mRNA stabilization is a major factor in the inductions. Thus, the androgen-mediated induction of these kidney mRNAs is generated predominantly at the posttranscriptional level.

Several studies indicate that steroid hormone induction of specific mRNA concentrations occurs at the level of gene transcription (Ringold et al., 1977; McKnight & Palmiter, 1979; Brock & Shapiro, 1983a). In a number of cases, the inductions have been shown to be mediated by specific DNA elements that are located in the immediate vicinity of the modulated genes and that have strong affinities for hormone–receptor complexes (Chandler et al., 1983; Karin et al., 1984; Renkawitz et al., 1984; Moore et al., 1985). However, marked alterations of mRNA stability in response to steroids have also been observed (Wiskocil et al., 1980; Brock & Shapiro, 1983b; Vannice et al., 1984), indicating that steroids function at multiple levels.

Androgenic steroids have profound effects on the mouse kidney (Bardin & Catterall, 1981); these effects, which are primarily exerted on proximal tubule cells, include inductions of a number of specific gene products. Treatment of female

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mice with testosterone elicits increases in the levels of β-glucuronidase (Swank et al., 1973; Watson et al., 1981), ornithine decarboxylase (Seely & Pegg, 1983), alcohol dehydrogenase (Ohno et al., 1970), and others. The increases in β-glucuronidase and ornithine decarboxylase have been shown to be generated in large part by changes in the respective mRNA concentrations (Paigen et al., 1979; Palmer et al., 1983; Kontula et al., 1984; Berger et al., 1984). In addition, several mRNAs whose translation products have not been completely characterized are under androgen control (Toole et al., 1979; Berger et al., 1981).

It is important to determine if the androgen-mediated increases in specific mRNA levels reflect alterations at the transcriptional level. Transcription rates can be measured in vitro by incubating isolated nuclei under conditions that allow incorporation of radioactive nucleotides into nascent transcript; radioactivity incorporated into a specific mRNA relative to that incorporated into total RNA is a measure of the rate of synthesis of that mRNA. RNA synthesis in such a system results from elongation of previously initiated transcripts with little or no reinitiation (Evans et al., 1977). Several studies with a variety of mRNAs indicate that in vitro RNA synthesis does reflect in vivo synthesis in the cells from which the nuclei were isolated (Evans et al., 1977; Weber et al., 1977; Derman et al., 1981).

In the present work, we have measured the transcription rates of three testosterone-inducible mRNAs in kidney nuclei. Our results indicate that during testosterone treatment, little or no changes occur in the transcription of the genes for three mRNAs whose steady-state concentrations are increased 10–20-fold. Thus, the hormone-mediated increases in these mRNA concentrations are generated predominantly at the posttranscriptional level, a conclusion supported by detailed analysis of induction kinetics. The implications of these results to current notions of steroid hormone regulation are discussed.

Experimental Procedures

Animals. Female mice of inbred strains DBA/2J and A/J were purchased from the West Seneca Laboratories, West Seneca, NY, and were used at 8–10 weeks of age. Testosterone induction was by subcutaneous implantation of 30-mg pellets for 7 days, unless stated otherwise. Deinduction was by surgical removal of the pellets.

Extraction and Analysis of RNA. Total kidney RNA was extracted by the method of Cox (1968) as described by Labarca & Paigen (1977). For dot blot analysis, 2-μL aliquots containing no more than 2 μg of RNA in water were spotted and baked onto nitrocellulose, hybridized to the appropriate ^{32}P-labeled probes, and visualized by autoradiography (Berger et al., 1984). Probes were radiolabeled by nick translation (Rigby et al., 1977). For the kinetic analysis, enhanced sensitivity was achieved with RNA probes generated by in vitro transcription of subcloned cDNA fragments using SP6 RNA polymerase. A 0.33-kb *Pst*I fragment of plasmid pODC934 (Berger et al., 1984) was inserted into the *Pst*I site of pSP64 (Melton et al., 1984), while a 1.0-kb *Pst*I–*Hae*III fragment of pMK908 (Berger et al., 1981) was inserted into the *Pst*I and *Hinc*II sites of pSP65 (Melton et al., 1984); the pMK908 subclone was prepared and supplied by J. Tseng-Crank and R. McGowan of Roswell Park Memorial Institute. Following hybridization, quantitation of dot blots was by scintillation counting, as described previously (Berger et al., 1984).

Nuclear RNA Synthesis. RNA synthesis in nuclei was measured as described in detail previously (Lamers et al., 1982), with certain modifications. Briefly, (2–3) × 10^7 nuclei in a total volume of 200 μL were incubated in 150–200 μCi of [α-^{32}P]UTP[1] for 20 min at 22 °C. After DNase treatment and phenol extraction, the newly synthesized [^{32}P]RNA was isolated and hybridized for 36–40 h at 68 °C to the appropriate cDNA-containing plasmids immobilized on nitrocellulose filters. The hybridization solution was 10 mM HEPES,[1] pH 7.5, 0.5 M NaCl, 10 mM Na_2EDTA,[1] 0.2% SDS,[1] 1× Denhardt's solution [0.02% poly(vinylpyrrolidone) and 0.02% Ficoll], and 0.1 mg/mL salmon sperm DNA. After washing, RNase digestion, and ethanol precipitation, hybridizing RNA was eluted from the filters and counted. Filters containing pBR322 DNA were included in each hybridization mixture and were used to measure nonspecific background radioactivity, which was never more than 0.001% of the radioactivity added. Specific hybridization represents the cpm bound to the cDNA-containing filter minus the cpm bound to the pBR322-containing background filter. Hybridization efficiency, which was always in the range of 0.45–0.60, was monitored in several experiments using [^{3}H]cRNA prepared from cDNA fragments (Lamers et al., 1982). The transcription rate, expressed as parts per million (ppm), was calculated from the equation

$$\text{ppm} = \left(\frac{\text{specific cpm}}{\text{input cpm}}\right)\left(\frac{\text{kb in mRNA}}{\text{kb in cDNA}}\right) \times 10^6$$

Results

Effect of Testosterone on Kidney RNA Synthesis. Kidney nuclei were isolated at various times after implantation of testosterone pellets and incubated in the presence of [α-^{32}P]UTP under conditions allowing completion of preinitiated RNA chains. Labeled RNA was extracted and hybridized to nitrocellulose filters affixed with cDNA plasmids corresponding to three different androgen-regulated mRNAs. Plasmid pMK908 contains a 1.2-kb cDNA that hybridizes to RNA, denoted 908 mRNA, encoding a 43 000-dalton polypeptide (Berger et al., 1981; Elliott & Berger, 1983); pODC934 contains a 1.4-kb cDNA corresponding to ornithine decarboxylase (ODC)[1] mRNA (Berger et al., 1984); and pKAP contains a 180-bp cDNA corresponding to KAP[1] mRNA (Toole et al., 1979). The cDNAs in plasmids pMK908 and pKAP correspond to single-copy genes (Toole et al., 1979; Elliott & Berger, 1983) while that in plasmid pODC934 corresponds to some 10–12 genes (Berger et al., 1984; B. Richards-Smith and R. Elliott, unpublished results).

In response to testosterone, total RNA synthesis reproducibly underwent a slight (i.e., ≤2-fold) increase within 24 h, which is consistent with the known effects of androgen on total RNA and protein synthesis in the mouse kidney (Bardin & Catterall, 1981). Of greater interest is the observation that relative rates of synthesis of both 908 and ODC mRNAs increased barely, if at all, during the 3 days of androgen treatment (Table I). Transcription of KAP mRNA was also measured (Table II) and, like the others, was not significantly altered by the hormone. As expected on the basis of previous studies (Berger et al., 1981, 1984), the concentrations of the 908 and ODC mRNAs increased 10–20-fold during androgen treatment (Figure 1). Others have reported that the level of KAP mRNA is increased 10-fold by testosterone (Toole et al., 1979). These mRNA inductions are clearly not accounted

[1] Abbreviations: UTP, uridine 5′-triphosphate; ODC, ornithine decarboxylase; MUP, major urinary protein; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; kb, kilobase; KAP, kidney androgen-regulated protein.

Table I: Effect of Testosterone on Kidney mRNA Synthesis[a]

time of androgen treatment (h)	total [^{32}P]RNA added (cpm × 10^{-6})	specific hybridization (cpm)		transcription rate (ppm)	
		pMK908	pODC934	pMK908	pODC934
untreated	16.9	203	323	26	29
	17.8	256	287	31	24
6	18.2	206	376	25	31
	16.6	251	364	33	33
12	20.2	279	582	30	43
	17.4	260	435	32	38
24	28.8	344	751	26	39
	26.7	307	606	25	34
72	34.8	491	665	31	29
	21.3	273	544	28	38

[a] Kidney nuclei were isolated from DBA/2J female mice at various times after initiation of testosterone treatment. Synthesis of RNA specific to cDNA plasmids pMK908 or pODC934 was assayed as described under Experimental Procedures.

for by changes in the respective transcription rates.

Several control experiments were performed. As a test of the fidelity of the transcription assay, we examined the α-amanitin sensitivity of transcription in kidney nuclei from normal and androgen-treated mice. Low α-amanitin concentrations selectively inhibit RNA polymerase II, which is responsible for most of the mRNA synthesis in vivo (Derman, 1981). As shown in Table II, specific mRNA synthesis was almost completely abolished for all three mRNAs. Just as importantly, the fraction of RNA synthesis due to RNA polymerase II was unchanged in androgen-treated mice.

As a further control, we varied the input of ^{32}P-labeled RNA in the hybridization mixtures to ensure that our measurements were being made within the linear range of the assay. This was especially critical for analysis of KAP. In kidneys of androgen-treated mice, KAP mRNA, which is 5% of the total mRNA, reaches a concentration that could potentially saturate the hybridizable cDNA on the filter and lead to an underestimation of the transcription rate (Toole et al., 1979). The experiment depicted in Figure 2 shows that for both pMK908 and pKAP, the level of hybridizing radioactivity was a linear function of the [^{32}P]RNA input. The calculated relative rate of transcription was identical at both inputs. The lack of measurable induction of transcription by androgen is not, therefore, a trivial consequence of saturation of the filter-bound cDNA.

Effect of Testosterone on Major Urinary Protein Gene Transcription. To test whether our experimental manipulations permit detection of androgen-regulated transcription, we measured the transcription rate for mRNAs encoding the major urinary proteins (MUPs)[1] in mouse liver; synthesis of these mRNAs has been shown to be induced by testosterone (Derman, 1981). Liver nuclei from normal and testo-

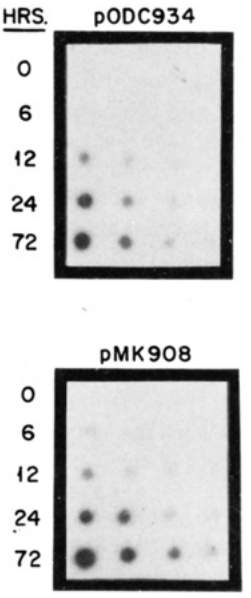

FIGURE 1: Effect of testosterone on specific mRNA concentrations in mouse kidney. Total RNA was extracted from the kidneys of DBA/2J female mice at various times after testosterone administration. The RNAs were spotted and baked onto nitrocellulose paper, hybridized to nick-translated probes, and visualized by autoradiography (see Experimental Procedures). The time, in hours, after hormone administration is indicated on the figure; horizontal rows represent a 2-fold dilution series containing 2, 1, 0.5, and 0.25 μg, respectively, of each RNA.

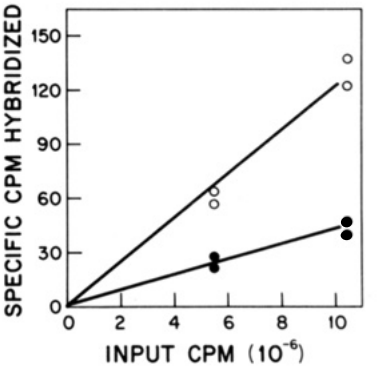

FIGURE 2: Linearity of the nuclear transcription assay with respect to different RNA inputs. Nuclei were isolated from the kidneys of female mice after 7 days of androgen treatment, and specific RNA synthesis was measured (see Experimental Procedures), in duplicate, at two [^{32}P]RNA inputs (5.4×10^6 cpm and 10.9×10^6 cpm). For all points, background counts per minute hybridizing to a pBR322 DNA-containing filter were subtracted out. (○) 908 mRNA synthesis; (●) KAP mRNA synthesis.

Table II: Effect of α-Amanitin on in Vitro RNA Synthesis by Kidney Nuclei[a]

testosterone treatment	α-amanitin	total [^{32}P]RNA added (cpm × 10^{-6})	specific hybridization (cpm)			transcription rate (ppm)		
			pMK908	pODC934	pKAP	pMK908	pODC934	pKAP
−	−	14.7	182	199	94	27	20	36
		22.9	202	217	71	20	14	17
−	+	8.8	20	0	0	5	0	0
+	−	37.0	292	364	151	17	15	23
		31.2	295	345	232	21	17	41
+	+	24.1	92	0	0	8	0	0
		17.8	40	0	0	5	0	0

[a] Kidneys from five to six DBA/2J female mice either before or after treatment with testosterone were pooled, nuclei were isolated, and specific RNA synthesis was measured (see Experimental Procedures). Where indicated, nuclei were treated with 1 μg/mL α-amanitin for 3 min before addition of [α-^{32}P]UTP.

Table III: Effect of Testosterone on MUP mRNA Synthesis in Liver[a]

testosterone treatment	total [^{32}P]RNA added (cpm × 10^{-6})	specific hybridization (cpm)	transcription rate (ppm)
−	5.1	269	56
	4.6	172	40
+	4.6	860	199
	4.8	951	211

[a] Liver nuclei from both normal and testosterone-treated DBA/2J female mice were isolated, and MUP-specific RNA synthesis was measured with cDNA plasmid p499 (Kuhn et al., 1984) according to the protocol described under Experimental Procedures.

sterone-treated female mice were incubated under standard transcription assay conditions, and MUP mRNA synthesis was quantitated with plasmid p499, which contains an 800-bp cDNA complementary to MUP mRNA (Kuhn et al., 1984). As seen in Table III, MUP mRNA synthesis was increased 4-fold by testosterone. Both the level of MUP mRNA synthesis and its induction were very similar to that reported previously (Derman, 1981). Thus, our conditions allow measurement of androgen-regulated transcription.

Analysis of Induction Kinetics. The kinetics of changes in RNA levels during androgen administration and withdrawal offer an alternative way of assessing the roles of RNA synthesis and turnover in the testosterone response (Watson et al., 1981). After initiation of hormone treatment, there is usually a short lag followed by an increase in RNA concentration until the induced level is reached; the kinetics of this induction can be described by the equation

$$dR/dt = k_s - k_d R \quad (1)$$

where R is the concentration of RNA, k_s is the zero-order rate constant for RNA accumulation, and k_d is the first-order rate constant for RNA loss. Equation 1 is basically the classical rate equation originally used by Schimke et al. (1964) to describe the kinetics of protein induction. The simplest interpretation of the kinetic constants assumes that k_s and k_d describe RNA synthesis and turnover, respectively, in the presence of hormone. The kinetics of deinduction, i.e., the decrease to the basal level of RNA after cessation of hormone treatment, can be described by a similar rate equation:

$$dR/dt = k'_s - k'_d R \quad (2)$$

Here, rate constants k'_s and k'_d describe RNA synthesis and turnover, respectively, in the absence of hormone. The RNA turnover rate constants k_d and k'_d can be evaluated by integrating and rearranging eq 1 and 2 to give the following (Watson et al., 1981):

$$\ln \frac{R_f - R_t}{R_f - R_0} = -k_d t \text{ for induction} \quad (3)$$

$$\ln \frac{R_f - R_t}{R_f - R_0} = -k'_d t \text{ for deinduction} \quad (4)$$

In eq 3 and 4, R_f is the final RNA concentration, R_0 is the initial RNA concentration, and R_t is the RNA concentration at time = t. The slopes of linear plots of these equations provide estimates of k_d and k'_d. Synthesis rate constants k_s and k'_s can, in turn, be determined from the general steady-state equation $R = k_s/k_d$ (Watson et al., 1981).

The kinetics of ODC and 908 mRNA induction and deinduction were followed by RNA dot blotting (Berger et al., 1984) and are shown in Figure 3. When the data were

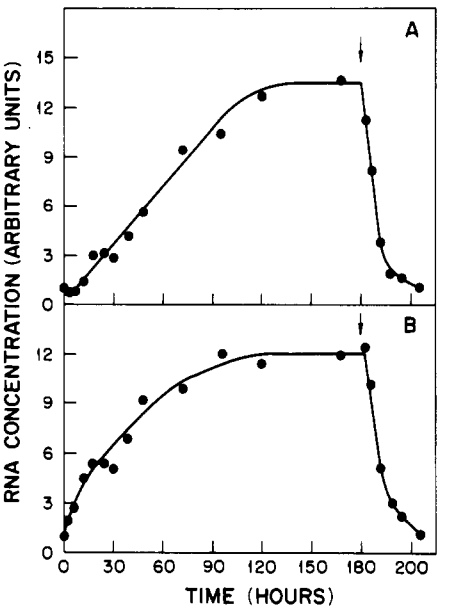

FIGURE 3: Induction and deinduction kinetics for androgen-regulated mRNAs. Testosterone pellets were administered subcutaneously to A/J female mice at time zero, kidney RNA from three mice was extracted and pooled for each of several time points thereafter, and the levels of ODC (panel A) and 908 (panel B) mRNAs were determined by dot blotting (see Experimental Procedures for details). After full induction was reached (arrows), hormone pellets were removed and RNA levels during the deinduction were measured similarly. RNA concentrations are expressed in arbitrary units, with the basal level set at 1.0.

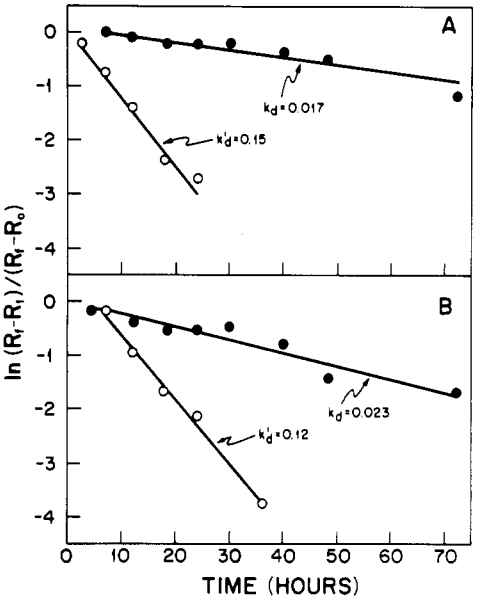

FIGURE 4: Evaluation of turnover rate constants k_d and k'_d. The RNA induction profiles of Figure 3 were replotted according to eq 3 and 4 for ODC mRNA (panel A) and for 908 mRNA (panel B). k_d, determined from the induction data, is the slope of the straight line generated by plotting ln $[(R_f - R_t)/(R_f - R_0)]$ vs. time (●); k'_d, determined from the deinduction data, is the slope of the straight line generated by plotting ln $[(R_f - R_t)/(R_f - R_0)]$ vs. time (○). See text for a detailed explanation of the origin and interpretations of the kinetic constants.

replotted according to eq 3 and 4, straight lines were obtained (Figure 4), the slopes of which represent k_d and k'_d for each mRNA. The results are summarized in Table IV. For both mRNAs, there were significant differences between k_d and k'_d. For ODC mRNA, k_d and k'_d correspond to half-lives of 41 h in the presence of androgen and 4.6 h in its absence; for

Table IV: Kinetic Constants for Induction of Kidney mRNAs by Testosterone[a]

RNA	steady-state RNA concn		turnover rate constants (h^{-1})		synthesis rate constants (units/h)	
	basal	induced	k_d	k'_d	k_s	k'_s
ODC	1.0	13.5	0.017	0.15	0.23	0.15
908	1.0	12	0.023	0.12	0.28	0.12

[a] Rate constants describing induction and deinduction of ODC and 908 mRNAs were determined from the data in Figures 3 and 4. The steady-state RNA concentrations are expressed in arbitrary units, setting the basal level at 1.0 for each mRNA. Rate constants k_d and k'_d, representing RNA turnover in the presence and absence, respectively, of hormone, were defined by the slopes of the plots in Figure 4. Rate constants k_s and k'_s, representing RNA synthesis in the presence and absence, respectively, of hormone, were calculated from the general steady-state rate equation $R = k_s/k_d$ (Watson et al., 1981).

908 mRNA, the two rate constants represent half-lives of 30 h in the presence of hormone and 5.8 h in its absence. The RNA synthesis rate constants k_s and k'_s differ by about 2-fold or less for each mRNA. Thus, on the basis of kinetic analysis, we can conclude that testosterone induces these mRNAs primarily by an effect on their stability with little, if any, effect on their synthesis. This supports the conclusions drawn from nuclear runoff experiments described above.

Discussion

The present studies show that androgens have little or no effect on the synthesis of three kidney mRNAs whose concentrations are greatly induced by the hormone. Thus, induction of these mRNAs is not fully accounted for by stimulation of transcription but must be generated predominantly at the level of mRNA processing or turnover. We cannot rule out a small (i.e., 2-fold or less) increase in transcription, owing to experimental variation encountered when measuring rates of mRNA synthesis as low as those in this study.

It is rather striking that transcription rates for the three mRNAs are quite similar while their concentrations differ dramatically; this is especially so for KAP mRNA, which represents 5% of the kidney mRNA in androgen-treated animals (Toole et al., 1979). These results suggest that relative mRNA levels within cells are governed to a large extent by posttranscriptional events exerted differentially upon different mRNAs. A similar conclusion was reached by Carneiro and Schibler (1984) in their studies of the synthesis and accumulation of mRNAs in mouse L-cells.

To verify our conclusions, we performed a detailed study of the 908 and ODC mRNA induction kinetics (Watson et al., 1981), the results of which are consistent with a testosterone-mediated stabilization of these transcripts. It should be emphasized that kinetic analysis makes several assumptions, a major one being that the rate constants k_d and k'_d describe RNA turnover in the presence and absence, respectively, of testosterone. This assumption has not been rigorously tested and may, in fact, be an oversimplification when applied to whole animals. Clearly, direct measurement of mRNA degradation rates is obligatory in extending our present results. Unfortunately, current techniques for pulse-labeling kidney RNA, either in whole animals or in organ culture, are inadequate for measuring the synthesis and turnover of specific mRNAs that are in low abundance.

Effects of steroid hormones on mRNA degradation have been noted previously and, in some cases, play a major role in the alteration of mRNA levels (Brock & Shapiro, 1983b; Vannice et al., 1984). Androgens have been shown to act at both the transcriptional and posttranscriptional levels. Inductions of major urinary protein mRNAs in mouse liver (Derman, 1981) and of ovomucoid mRNA in chick oviduct (Compere et al., 1981) are consequences of androgen-mediated increases in transcription. In contrast, stimulation of mRNA concentrations in the rat prostate appears to occur predominantly via transcript stabilization (Page & Parker, 1982). Thus, similar to other steroids, androgen effects occur at several levels.

An important question is whether the posttranscriptional effects of steroid hormones are direct or indirect. Several investigators have reported interaction between steroid receptors and RNA (Liao et al., 1980; Feldman et al., 1981; Economidis & Rousseau, 1984). Such interactions may account for the direct modulation of mRNA stability or processing by steroids. Alternatively, the posttranscriptional effects may be indirect. It is possible that steroids function primarily by changing transcription of genes other than the ones being studied. Alterations in mRNA stability or processing could be a secondary consequence of the enhanced transcription of these other genes.

Complex organs such as mammalian kidney contain a variety of cell types; it is feasible that transcriptional and posttranscriptional effects of steroid hormones in one cell type may result from events initiated in another. A large body of experimental evidence has suggested, for example, that estrogen- and testosterone-dependent growth and differentiation of gonadal epithelium are elicited through factors produced by nearby mesenchymal cells (Cunha et al., 1983). It is the latter cells that directly respond to hormone, presumably by increasing the production of these factors. For several steroid-regulated genes, molecular studies have clearly implicated direct modulation of transcription via critical DNA sequences that interact with hormone–receptor complexes (Chandler et al., 1983; Karin et al., 1984; Renkawitz et al., 1984; Moore et al., 1985). Similar mechanisms for androgen-regulated genes have not been demonstrated thus far (Parker et al., 1984; Williams et al., 1985). It may be pertinent to consider the possibility that androgen effects upon these genes are a consequence of primary events elicited at other genes or in other cell types, or both.

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Registry No. Testosterone, 58-22-0; ornithine decarboxylase, 9024-60-6.

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