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ANDROGENIC REGULATION OF POLY(A)-CONTAINING RNA SEQUENCES IN RAT VENTRAL PROSTATE

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SUMMARY

The effect of androgens on RNA production in rat ventral prostate has been studied by analysing the base sequence complexity of cytoplasmic poly(A)-containing RNA from normal and castrated rats. The kinetics of hybridization of RNA from normal animals with its complementary DNA suggests that approximately 50% comprises 2–3 sequences, about 20% comprises 36 sequences while the remainder is a scarce class comprising about 8,000 sequences. Following a three-day castration period the total number of sequences is about the same but there is a marked alteration in the relative concentrations of the sequences. In particular the abundant class of RNA in normal rats was not detected after castration. In heterologous hybridization experiments using normal prostatic complementary DNA as a probe it was shown that the abundant sequences were reduced 10-fold after 3 days. The usefulness of this technique in studying hormone dependent mRNA production in target tissues including the brain will be discussed.

INTRODUCTION

Androgens control the function of a wide variety of cell types [1]. In tissues such as the sex accessory glands both their growth and differentiation are dependent on the presence of the steroid hormone. In many others such as liver and kidney the hormone does not affect growth but rather the expression of certain specific genes thereby regulating protein synthesis within the organ. The brain probably falls into this second class of tissues, though specific proteins, regulated by androgens, have not yet been identified.

That protein synthesis is regulated by cellular mRNA concentration is suggested by the process of nuclear uptake of steroids in target cells. By altering genetic transcription steroid hormones may regulate specific cytoplasmic mRNA molecules and thence regulate protein synthesis. In the rat sex accessory tissues, ventral prostate, seminal vesicle, and in rat liver, the regulation of specific proteins appears to be mediated *via* the production of cellular mRNA [2–7].

It is now possible to examine the effects of hormones on RNA production in target cells even when proteins regulated by the hormone have not been identified. By measuring the kinetics of hybridization of poly (A)-containing RNA with its own complementary DNA as first described by Bishop and his coworkers[8], it is possible to estimate the number of different mRNA sequences together with their relative concentrations in tissues and cells [9]. We have therefore applied the technique to examine the effects of androgens on mRNA sequences in rat ventral prostate. The possibility of applying the technique to studies of androgen action in the brain will also be discussed.

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MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (200–250 g) from this Institute were used. Castration was carried out by the scrotal route under fluothane anaesthesia and where indicated testosterone propionate (1.25 mg) in 0.25 ml of arachis oil was injected subcutaneously in the flank region.

Materials

Most chemicals used were of the highest available purity and supplied by either Sigma (London) Chemical Co. or British Drug Houses. Purified AMV reverse transcriptase was supplied by Dr. J. W. Beard (Life Sciences, Inc.). Other chemicals used with their suppliers in parentheses were as follows: testosterone propionate or 'Androject' (Intervet Labs. Ltd.); [^{3}H]-dCTP (20–25 Ci/mmol, The Radiochemical Centre, Amersham); [^{3}H]-poly(U) (75 μCi/μmol Pi) and oligo(dT_{12-18}) (Miles-Seravac Ltd.); oligo dT-cellulose (Collaborative Research) and butyl-PBD (Ciba ARL).

Isolation of poly(A)-rich RNA

Ventral prostates were removed from rats and frozen immediately on solid CO_2. Frozen tissue reduced to a fine powder with an impact homogenizer was transferred into 10 vols (with respect to original tissue weight) of 0.25 M sucrose, 3 mM $MgCl_2$, 0.1% Triton X-100 and 0.1% diethyl-pyrocarbonate and homogenized with a motor-driven homogenizer (Silverson Machines Ltd.) at full-speed for 30 s. The homogenate was then centrifuged at 6000 *g* for 1 min and the supernatant made up to 40 vol in a medium containing 0.5% SDS, 0.01 EDTA pH 7.0 and 0.1 M NaCl. The RNA was then extracted at room tempera-

ture with phenol–chloroform as described by Penman *et al.*[10] and precipitated from aqueous solution with ethanol at −20°C overnight. RNA was assayed by absorbance at 260 nm as described by Higgins *et al.*[4] and recoveries of 30–40% were routinely obtained. The poly(A)-containing RNA was separated by oligo dT-cellulose chromatography using the method of Aviv and Leder[11] as modified by Rosen *et al.*[12]. The poly (A)-rich RNA was concentrated by precipitation with ethanol and stored in 0.01 M Tris–HCl pH 8.3 at −70°C.

Synthesis of cDNA

The synthesis of cDNA to poly(A) RNA was essentially as described for ovalbumin cDNA [13]. [^{3}H]-dCTP (100 μCi) and 2.5 μl of 720 μg/ml actinomycin D were dried down under N_2 and resuspended in reaction mixture consisting of 50 mM Tris–HCl pH 8.3, 10 mM dithiothreitol, 6 mM $MgCl_2$, 35 μM dATP, 35 μM dGTP, 0.2 mM dTTP, 5 μg/ml oligo (dT_{12-18}) and 25 μg/ml poly(A) RNA in a total vol. of 50 μl. The mixture was cooled on ice and then 0.5 μl avian myoblastosis virus reverse transcriptase was added to a final concentration of 100 units/ml. After incubation at 46°C for ½ h the cDNA was isolated as previously described [14]. Briefly RNA was hydrolysed with alkali and the cDNA separated from unincorporated [^{3}H]-dCTP by gel filtration through Sephadex G50. After precipitation with ethanol, the cDNA was stored in 0.01 M Tris–HCl pH 7.5 at −70°C.

The cDNA was analysed in alkaline sucrose gradients [15]. The [^{3}H]-cDNA was mixed with ^{32}P-labelled polyoma DNA Hpa II fragment 6 consisting of approximately 350 base pairs (provided by Dr. B. Griffin), treated with alkali, and then centrifuged in a 5–20% sucrose gradient at 45,000 rev/min for 15 h at 4°C in an SW 50.1 rotor. The cDNA sedimented as a broad peak coincident with the 350 nucleotide marker.

The hybridization of poly(A) RNA with cDNA

Hybridization was carried out in 0.24 M sodium phosphate buffer (equimolar) containing 0.05% SDS and 1 mM EDTA at 70°C in vols of 120 μl in siliconised conical tubes. Excess poly(A) RNA at concentrations described in the figure legends was mixed with sufficient cDNA to give about 800 c.p.m. per sample and overlaid with paraffin oil[16]. Portions of 5–10 μl were removed at intervals ranging from 5 min to 24 h and pipetted into 1.1 ml of S1 nuclease buffer [14] which was subsequently divided into duplicate portions (0.5 ml). To one was added S1 nuclease to hydrolyse unhybridized cDNA and then both were incubated at 46°C for 2 h. The samples were cooled in ice and adjusted to contain 10 μg/ml bovine serum albumin and 5% trichloroacetic acid. They were then collected on millipore filters and counted in scintillation fluid consisting of 6 g of butyl-PBD per litre of toluene. The percentage S1 nuclease resistance is equivalent to the percentage hybridization. The data was analysed by Dr. J. Bishop using a computer programme designed to solve from 1 to 4 components [17].

Characterization of prostate poly(A) RNA

Estimation of number average molecular weight. Samples of total cellular poly(A) RNA (10–40 μg) in a buffer consisting of 0.5% SDS, 0.1 M NaCl, 0.01 M Tris–HCl pH 7.5 and 1 mM EDTA were heated at 70°C for 30 s and then cooled quickly to 0°C. The RNA was then centrifuged in 15–30% sucrose gradients containing the above buffer at 20,000 rev/min at 20°C for 15 h. After fractionation of the gradients 50 μl aliquots of each fraction was assayed for poly(A) by hybridization with excess [^{3}H]-poly(U) in 2 × SSC containing 50% formamide. Fractions were incubated at 45°C for 30 min and then treated with pancreatic ribonuclease for 2 h at 30°C to hydrolyze unhybridized [^{3}H]-poly(U). The hybridized material was precipitated with 5% trichloracetic acid, collected on millipore filters and counted as described for [^{3}H]-cDNA. The distribution of hybridizable [^{3}H]-poly(U) across the gradient gives the molecular weight distribution of the poly(A) RNA sample. Assuming that there were no size variations in poly(A) tail present in the poly(A)-containing RNA across the gradient, it was possible to calculate its number average molecular weight from a cumulative plot of the fraction of poly(A) RNA molecules against their length [18].

RESULTS AND DISCUSSION

Hybridization of cDNA with cytoplasmic poly(A)-containing RNA

Cytoplasmic poly(A)-containing RNA was isolated from the prostates of normal and castrated animals and cDNA to each RNA sample was prepared. In both cases the cDNA sedimented on alkaline sucrose gradients as a broad peak coincident with a 350 nucleotide marker. Hybridization between each RNA and its complementary DNA is shown in Fig. 1. A very marked difference in the rate of hybridization obtained with the two RNA preparations is clearly evident. Approximately half of the normal cDNA had hybridized to its complementary RNA before any of the castrate cDNA had started to hybridize. This indicates that a considerable proportion of the RNA from normal animals is much more abundant than is the RNA from castrated animals.

Hybridization of globin mRNA with its cDNA has been used as a kinetic standard for the hybridization reactions of poly(A)-containing RNA from rat prostate. Figure 2 shows that about 95% of the cDNA molecules hybridized to the globin mRNA with a Rot ½ of $5–10^{-4}$ mol/litre-second which is similar to that obtained by Bishop *et al.*[8]. The globin mRNA was assumed to consist of an equal number of α and β chains giving a rate constant of 2.5×10^{-4} mol/litre-second for the monomolecular reaction.

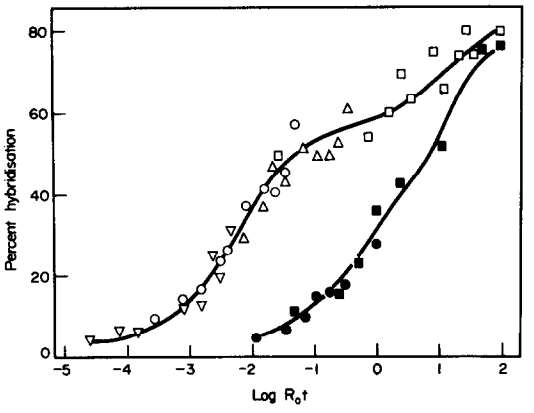

Fig. 1. Hybridization of cytoplasmic RNA, prepared from prostates of normal and castrated rats, to their complementary DNA. Cytoplasmic RNA, isolated from prostates of normal and castrated (3 days) animals, was hybridized to its homologous cDNA (10,000 c.p.m.). At intervals the extent of hybridization of cDNA was determined (resistance to S1 nuclease). Rot values (abscissa) are the product of concentration of RNA, in moles of nucleotide per litre, × time of reaction in seconds. Open symbols are for RNA from normal rats and closed symbols are for RNA from castrated animals. The solid lines represent the best fit to the data derived by computer analysis.

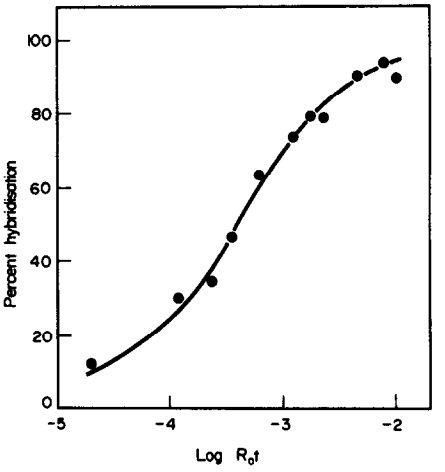

Fig. 2. Hybridization of globin mRNA to its complementary DNA .Globin mRNA (72 ng) was hybridized to its cDNA (20,000 c.p.m.).

Determinations of the numbers of different prostate poly(A) RNA sequences require the number average size of the RNA compared with that of the kinetic standard, namely globin mRNA. The size of prostatic RNA samples was obtained by sucrose gradient centrifugation of total cellular poly(A) RNA followed by [^{3}H]-poly(U) hybridization of each fraction across the gradient. Plots of the cumulative fraction of the total poly(A) RNA against the nucleotide chain length of the RNA obtained from the sedimentation value indicated that the number average nucleotide chain lengths ranged from 1500–1600 nucleotides for RNA from normal animals to 2000 nucleotides for RNA from castrated animals. Since globin mRNA contains about 650 nucleotides the Rot $\frac{1}{2}$ of an RNA molecule of 1500 nucleotides would be 6×10^{-4} mol/litre-second whereas that for molecules of 2000 nucleotides would be 8×10^{-4} mol/litre-second. The numbers of molecules of RNA in each abundance class can then be calculated by dividing the Rot $\frac{1}{2}$ obtained for each class of normal prostatic RNA by 6×10^{-4} and of castrated rat prostate RNA by 8×10^{-4}.

The Rot $\frac{1}{2}$ values of the prostatic RNA were estimated by computer analysis. The best fit for the data for RNA from normal animals is obtained with three discrete abundance classes of poly(A)-containing RNA. About 2–3 sequences are present at very high abundance, approximately 36 sequences are of moderate abundance while 7500 sequences are rather scarce. This latter value is a minimum estimate since hybridization was not quite complete at Rot values of 100, the maximum utilized. In contrast, analysis of the data with RNA from castrated animals showed there to be only two abundance classes of sequences in the RNA (Table 1). A very high abundance class was not observed but rather a moderate abundance class consisting of about 210 sequences and a low abundance class of approximately 10,000 sequences. Thus RNA sequences present in great abundance (Class 1) in RNA from normal rats are either completely absent in RNA from castrated rats or, because they are

Table 1. Sequence complexity of cytoplasmic poly(A)-containing RNA from normal and 3-day castrated rat prostate

Tissue	Transition	P (% cDNA Hybridized)	Rot $\frac{1}{2}$ (observed)	Rot $\frac{1}{2}$ (corrected)	No. of sequences
Normal	1	49	3.3×10^{-3}	1.6×10^{-3}	2–3
Prostate	2	21	1.1×10^{-1}	2.2×10^{-2}	36
	3	30	1.5×10^{1}	4.5×10^{0}	7,500
3-day Castrated	1	43	4.0×10^{1}	1.7×10^{-1}	210
Prostate	2	57	1.4×10^{1}	8.3×10^{0}	10,000

The data was obtained by computer analysis of the data shown in Fig. 1. The observed Rot $\frac{1}{2}$ values were corrected for the fraction of RNA(P) they represent and the number of sequences was calculated by dividing the corrected Rot $\frac{1}{2}$ value by 6×10^{-4} in the case of RNA from normal prostate and 8×10^{-4} for RNA from castrated rat prostate.

reduced in abundance, are now included in the classes of moderate or low abundance.

Hybridization of cDNA with heterologous RNA

The fate of the rapidly annealing RNA in rat prostate following castration could best be studied by carrying out heterologous hybridization reactions using cDNA prepared to normal prostatic RNA. If the highly abundant sequences were absent in castrated rat prostate then approximately one half of the normal cDNA, namely that corresponding to the abundant sequences, would fail to hybridize with the castrate RNA. On the other hand, if the sequences are present but reduced in abundance then hybridization would occur but at increased Rot values. From Fig. 2 it may be seen that normal cDNA hybridizes with cytoplasmic poly(A)-containing RNA from castrated animals to essentially the same level as observed with normal RNA. This indicates that the highly abundant sequences in normal RNA are still present in RNA from castrated animals but the shift in the Rot curve of about 1.0 log units suggests that the highly abundant sequences are no longer present at the same concentration but are reduced to at least one-tenth following a three-day castration period.

Androgen dependence of abundant poly(A) RNA sequences

It seems reasonable to predict that androgen treatment of castrated rats would abolish the differences in hybridization kinetics of RNA from normal and castrated rats. RNA from castrated animals treated with testosterone propionate was hybridized to its homologous cDNA. Treatment for 72 h shifted the hybridization curve to a position intermediate between those for normal and castrated animals (Fig. 4). This suggests that the synthesis of the abundant sequences is regulated by testosterone.

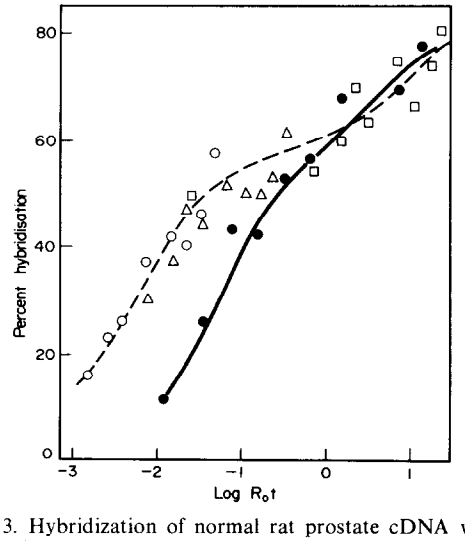

Fig. 3. Hybridization of normal rat prostate cDNA with prostatic RNA from normal and castrated animals. Cytoplasmic RNA from prostates of normal and castrated (3 days) animals was hybridized to cDNA prepared from normal prostate RNA. The open symbols represent the homologous hybridization reaction whereas the solid symbols represent the heterologous hybridization reaction.

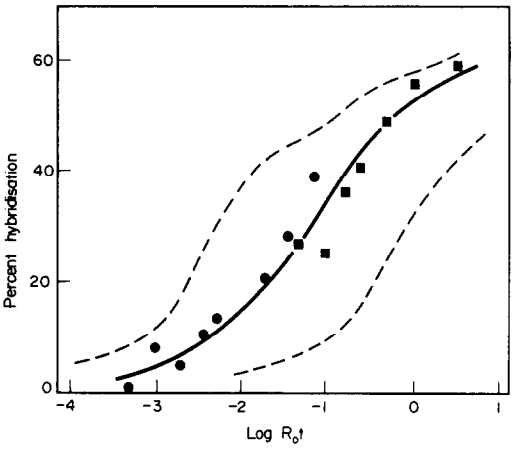

Fig. 4. Effect of testosterone on the complexity of cytoplasmic RNA. Cytoplasmic RNA from prostates of castrated (3 days) animals, injected daily with testosterone for 3 days, was hybridized to its complementary DNA (10,000 c.p.m.). Dotted lines to the left and right represent best fit lines for the data obtained from the homologous hybridizations of normal and castrated rat prostate RNA.

Providing the RNA sequences are mRNA molecules we predicted that it should be possible to identify major prostatic proteins regulated by androgens. When we examined the synthesis of prostatic proteins using [^{35}S]-methionine we found that was the case. Four proteins accounting for 30–40% of the total protein synthetic capacity of the gland were acutely dependent on androgens for their synthesis [19].

Brain mRNA complexity

Although nothing is known about the role hormones play in the brain, a number of laboratories [17, 20, 21] have analysed the complexity of mouse brain mRNA sequences. The hybridization curves reported by Hastie and Bishop[17] indicate that brain RNA consists of three abundance classes. From the Rot $\frac{1}{2}$ values and the number average molecular weight of the RNA it was possible to calculate that brain mRNA consists of about 4 relatively abundant sequences, 480 moderately abundant sequences and some 11,000 scarce sequences. However, these values may represent underestimates of the total number of brain mRNA sequences because of the fact that brain contains many different cell types. Sequences present in low concentrations in only a fraction of the cells of the brain may not be detected by the complementary DNA hybridization method. That this may be the case is suggested by the results of Bantle and Hahn[22] using single-copy saturation hybridization analysis. They concluded that there may be as many as 10^5 different mRNA sequences in mouse brain not necessarily in all cells but resulting from the heterogeneity of cell types.

Nevertheless, it should be possible to use complementary DNA hybridization methods to examine effects of androgens on brain mRNA especially if the complementary DNA is fractionated so that only a portion of the RNA was examined. This would be done in two ways. Firstly, it may be preferable to examine the effects of androgens on abundant sequences only since in other tissues this class of RNA has been shown to be hormone dependent. For example, in addition to those described in the rat ventral prostate the abundant sequences in the chick oviduct probably coding for egg-white proteins have been shown to be oestrogen dependent [23]. Complementary DNA to the abundant brain mRNA sequences could be isolated by hybridizing total cDNA to a Rot value at which, say 10%, had hybridized. This could then be separated from the unhybridized material by hydroxyapatite chromatography. Single stranded DNA is eluted with 0.14 M sodium phosphate whereas the hybridized DNA requires the use of 0.4 M sodium phosphate. This cDNA, enriched for the abundant sequences, could then be used as a probe to look at the effect of androgens on the abundant class of RNA.

Secondly, use could be made of the observation that a significant proportion of cellular mRNA sequences are present in all tissues of a single organism presumably required for growth and maintenance of the organ. Heterologous hybridization experiments in which the cDNA prepared to the RNA of one tissue has been used to analyse the RNA in another tissue [17, 20, 21].

In mouse brain, liver and kidney, about 8000 sequences are common to all three tissues and presumably encode "housekeeping" functions shared by all cell types. Another 1–2000 are common to any two of the tissues and a further 1–2000 sequences are unique to each of the tissues. It is these tissue specific sequences that are probably regulated by factors such as hormones. Thus, by hybridizing brain cDNA to either liver or kidney RNA, cDNA homologous to RNA in these tissues could be separated from brain specific cDNA which will not hybridize. The brain specific cDNA would then be a useful probe to analyse brain specific RNA sequences and may increase the sensitivity of detecting hormonally regulated mRNA sequences.

CONCLUSIONS

Steroid hormones regulate protein synthesis in target tissues. When nuclear uptake of the steroid has been demonstrated, subsequent studies have usually indicated the regulation of the cellular mRNA concentration. The hormonal regulation of specific mRNA sequences can be investigated by analysing the kinetics of hybridization of complementary DNA and RNA isolated from animals of different hormonal status. This approach suggests that about 50% of prostatic RNA comprising 2–3 sequences is under androgenic control. Similar studies could be applied to any target tissue to investigate the effects of hormones on mRNA populations.

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