Studies on the Form and Synthesis of Messenger Ribonucleic Acid in the Rat Ventral Prostate Gland, including its Tissue-Specific Stimulation by Androgens

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1. When prostate polyribosomes are labelled with radioactive precursors *in vivo* and subsequently dissociated with sodium dodecyl sulphate, a heterogeneous 6–15S RNA species may be identified that possesses all of the distinctive properties of mRNA. 2. Apart from the selective incorporation of 5'-fluoro-orotic acid into this 6–15S RNA component, it is bound by nitrocellulose filters under experimental conditions where only poly(A)-rich species of RNA are specifically retained. Most importantly, however, only the 6–15S RNA fraction is capable of promoting the incorporation of amino acids into peptide linkage in an mRNA-depleted cell-free system derived from ascites-tumour cells. 3. With the development of a simpler method for labelling the total RNA fraction of the prostate gland *in vitro*, the poly(A)-enriched RNA fraction may be readily isolated by adsorption and elution from oligo(dT)-cellulose. The synthesis of the poly(A)-enriched 6–15S RNA fraction is stringently controlled by androgens in a highly tissue- and steroid-specific manner. 4. From an analysis of the proteins synthesized in the ascites cell-free system in the presence of the poly(A)-rich RNA fraction, it appears that protein synthesis in the prostate gland is stimulated in a rather general way, even during the earliest phases of the androgenic response. This conclusion may require modification when more specific means of analysis are available than those used in the present investigation. 5. The implications of these findings to the mechanism of action of androgens are discussed.

The last few years have seen the innovation of many novel methods for the study of the mRNA of eukaryotic cells. Until recently, the isolation and assay of the mRNA of higher organisms had been based on unsatisfactory or, at best, tenuous criteria, and impressive advances have been made in its isolation and characterization. Apart from the development of relatively selective means for labelling mRNA with such radioactive precursors as 5'-fluoro-[¹⁴C]orotic acid (Wilkinson *et al.*, 1971), considerable effort has been directed towards the preparation of protein-synthesizing systems from eukaryotic organisms (Anderson & Gilbert, 1969; Mathews & Korner, 1970; Aviv *et al.*, 1971) in which a putative mRNA can be assayed with the facility and authenticity formerly possible only with cell-free systems derived from bacteria (Nirenberg, 1963). Perhaps the most striking advance, however, has been the development of methods for purifying the small amounts of mRNA contained in complex mixtures with other species of RNA. Although poly(A) has long been recognized as a constituent of eukaryotic cells (Edmonds & Abrams, 1960, 1963; Hadjivassiliou & Brawerman, 1966, 1967), it has only been recently identified as a

characteristic component of the mRNA of animal viruses (Kates & Beeson, 1970) and tumour cells (Darnell *et al.*, 1971; Lee *et al.*, 1971) but seemingly not in the mRNA of bacterial origin (Perry *et al.*, 1972). The presence of the poly(A) sequences imparts characteristic binding properties to eukaryotic mRNA that may be profitably exploited in its isolation. In chromatography on oligo(dT)-cellulose (Aviv & Leder, 1972) and poly(U)-cellulose (Sheldon *et al.*, 1972) the selective retention of mRNA depends on the formation of stable duplexes between the poly(A) sequences and the immobilized polyribo- (or polydeoxyribo-) nucleotide; in the nitrocellulose procedure (Lee *et al.*, 1971) the precise basis for the binding of mRNA remains unknown.

These advances enable a new insight to be made into the mechanism of action of steroid hormones expressed at a molecular level, particularly with respect to the part played by mRNA synthesis during the early phase of the hormonal response. However, with the notable exception of the induction of ovalbumin (Rosenfeld *et al.*, 1972) and avidin (Tuohimaa *et al.*, 1972) in chick oviduct, few attempts have been made at the isolation of the mRNA from steroid-

sensitive cells. To some extent this is due to the fact that, unlike chick oviduct, hormonal stimulation in other experimental systems is probably reflected in the synthesis of a diversity of cellular components rather than the induction of a limited number of well-characterized proteins. In a previous publication from this laboratory (Mainwaring & Wilce, 1973), it was demonstrated that androgens promote a large and tissue-specific increase in the polyribosome content of rat ventral prostate gland. If there are few prostate mRNA molecules of exceedingly long existence in the absence of androgenic stimulation, then a reasonable interpretation of these findings is that extensive synthesis of mRNA *de novo* occurs during the androgenic response. The major objective of the present investigation was to characterize fully the mRNA or poly(A)-rich fraction of the prostate gland and particularly to investigate its synthesis during the early phase of androgenic stimulation. Justification for the common identity of prostate-gland mRNA with the poly(A)-rich RNA fraction will be made apparent during the presentation of the experimental results.

Materials and Methods

Animals

Bilateral orchidectomy of male Sprague-Dawley rats (250–300 g body wt.) was performed by the scrotal route under fluothane anaesthesia. Animals were generally killed 72 h after castration, by cervical dislocation. Where indicated, testosterone phenylpropionate was administered subcutaneously into the flank region in oil (0.25 ml); control animals received oil only. The doses of androgen administered, together with details of the time-course of individual experiments, are given in legends to the tables.

Chemicals

[5-³H]Orotic acid (2.1 Ci/mmol), [5-³H]uridine (29 Ci/mmol), L-[U-³H]methionine (9.0 Ci/mmol) and L-[³⁵S]methionine (180 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 5'-Fluoro-[¹⁴C]orotic acid (4.1 mCi/mmol) was supplied by Tracer Laboratories Ltd., Weybridge, Surrey, U.K. Tris base (99.5% pure), L-amino acids, creatine phosphate, creatine kinase, dithiothreitol, calf thymus DNA, yeast tRNA and thymidine 5'-phosphate were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Acrylamide, *NN'*-methylenebisacrylamide, (NH₄)₂S₄O₈ and dimethylaminopropionitrile were supplied by British Drug Houses Ltd., Poole, Dorset, U.K. Poly(A), poly(C) and poly(U), in both their non-radioactive and tritiated forms, were purchased from Miles-Seravac Ltd., Maidenhead, Berks., U.K. Agarose

(type A-45) was obtained from L'Industrie Biologique Française, Gennevilliers, France. Highly purified Serva sodium dodecyl sulphate (low extinction at 260 nm) was obtained through Camlab Ltd., Cambridge, Cambs., U.K. Pancreatic ribonuclease and ribonuclease-free deoxyribonuclease were supplied by Worthington Chemical Corp., Freehold, N.J., U.S.A. Other chemicals were of the highest available purity and all solutions were made up in glass-distilled water. It should be emphasized that all glassware and solutions used in this study were autoclaved before use; without this provision, adsorbed ribonuclease led to spurious results.

Labelling of prostate RNA

(a) *In vivo*. Animals were subjected to complete surgical anaesthesia by the intraperitoneal injection of Nembutal (60 mg/ml of solution; 0.15 ml/100 g body wt.) and radioactive RNA precursors were injected directly into the prostate gland as described by Mainwaring & Wilce (1972). Injection of labelled precursors by other routes led to insignificant labelling of prostate RNA.

(b) *In vitro*. After rapid removal from the animal, each lobe of the prostate gland was cut approximately in half and promptly placed in Eagle's minimal medium at 37°C. Tissue pooled from four glands was drained, resuspended in 10 ml of Eagle's medium containing 50 or 100 μ Ci of [³H]uridine and shaken in a Grant bath operating at 120 cycles/min for 1 h at 37°C in an atmosphere of O₂ + CO₂ (95:5). The glands were drained, rinsed twice in 25 ml of ³H-free Eagle's medium and used immediately for the preparation of labelled prostate RNA, as described in detail below. The extensive incorporation of radioactivity into RNA demanded that the prostate glands were initially processed as quickly as possible at temperatures close to 37°C; cooling led to variable and lower rates of incorporation of radioactive precursors.

Isolation of prostate RNA fractions

(a) *Polyribosomal RNA*. Full details of the isolation of prostate polyribosomes have been described by Mainwaring & Wilce (1972). For subsequent analysis in sucrose density gradients, polyribosomes were resuspended in medium A (25 mM-sodium acetate-acetic acid buffer, pH 5.2, containing 0.1 M-NaCl) to give a suspension containing 20 E₂₆₀ units of RNA/ml. Then one-ninth vol. of 10% (v/v) sodium dodecyl sulphate in medium A was added, and after 30 s incubation at 37°C samples (0.5 ml) were layered directly on to 4.0 ml linear 8–40% (w/v) sucrose gradients in medium A and centrifuged at 190 000 g_{av} for 3 h at 4°C in the SWTi52 rotor of a Christ Omega ultracentrifuge. Gradients were ana-

lysed as described by Mainwaring & Wilce (1972). Alternatively, total polyribosomal RNA was extracted by the method of Aviv & Leder (1972) and stored at -70°C . For the preparation of rRNA, depleted of mRNA, polyribosomes were incubated for 20 min at 0°C with $0.5\text{ }\mu\text{g}$ of ribonuclease/ml before extraction of the RNA.

(b) *Total prostate RNA*. Many methods were initially used for the extraction of poly(A)-enriched RNA from the prostate gland. The method of choice was a modification of that proposed by Rosenfeld *et al.* (1972). Provided that highly selective methods are subsequently used for the isolation of the poly(A)-enriched RNA, the principal advantage of this method is the speed and ease with which many samples of tissue may be processed concomitantly. Labelled tissue (1.2–1.5 g wet wt.) was minced finely with scissors and placed in 10 ml of extraction medium [1% (w/v) sodium dodecyl sulphate, 0.15 M-NaCl and 25 mM-EDTA] and 10 ml of water-saturated phenol. After thorough disruption of the tissue by blending in a motor-driven homogenizer (Silverson Machines Ltd.) operating at half-speed for 30 s, the emulsions were vigorously shaken at room temperature for 20 min and cooled in an ice bath. After centrifugation at $10000g$ for 10 min, the aqueous phase was aspirated and kept in an ice bath, whereas the phenol phase (plus interphase) was supplemented with 5 ml of extraction medium and vigorously shaken in a water bath at 55°C for 90 s. After cooling and centrifugation, the aqueous phases from each extraction were combined, made 0.15 M with respect to NaCl, treated with 2 vol. of ethanol and stored at -20°C for 16 h. The RNA precipitate was collected by centrifugation at $10000g$, washed twice in 30 ml of cold ethanol, dissolved in 3.0 ml of water and stored at -70°C . The initial extraction at room temperature releases 75–80% of the poly(A)-enriched RNA and the remainder is only recovered on further extraction at 55°C . This method releases considerable quantities of DNA, and this was partially hydrolysed before the isolation of the poly(A)-enriched RNA by certain procedures, particularly oligo(dT)-cellulose chromatography. Samples were made 10 mM with respect to Tris-HCl buffer, pH 7.4, and 1 mM with respect to MgCl_2 , and after addition of $10\text{ }\mu\text{g}$ of ribonuclease-free deoxyribonuclease/ml the samples were incubated at 30°C for 10 min.

(c) *Transfer RNA*. This was prepared from the $105000g$ supernatant fraction of prostate homogenates as described in detail by Mainwaring & Wilce (1973) for rat liver tRNA.

Selective isolation of poly(A)-rich RNA

Three methods were used for the isolation of this RNA constituent. Two of these methods utilize the selective adsorption of poly(A) to materials in the

form of discs and were used essentially for analytical purposes only; the third procedure, oligo(dT)-cellulose chromatography, possesses the additional advantages that it may be used as both a preparative and a quantitative analytical means for isolating poly(A)-rich RNA.

(a) *Analytical procedures based on the use of immobilized adsorbents*. Poly(U) was attached to glass-fibre discs (Whatman GF/A; 2.5 cm diam.) by the photochemical procedure devised by Sheldon *et al.* (1972). Poly(U) solution (0.15 ml; 1 mg/ml) was pipetted on to the centre of the discs which were then dried in an oven at 37°C for 2 h. Each disc was irradiated with a Minivis u.v. lamp (Desaga Ltd., Heidelberg, W. Germany) at a distance of 6 cm, 2.5 min on each side at a wavelength of 366 nm. From studies with $[^3\text{H}]$ -poly(U), it was established that $52\text{ }\mu\text{g}$ of poly(U) was firmly associated with each disc under these conditions. Nitrocellulose filters (Millipore; code HAWP 02500) were used as supplied by the manufacturers according to the recommendations of Lee *et al.* (1971). Both types of discs were thoroughly soaked in the appropriate analytical medium before use; medium B, for poly(U) discs, 25 mM-Tris-HCl buffer, pH 7.4, containing 0.12 M-NaCl; or medium C, for nitrocellulose discs, 25 mM-Tris-HCl buffer, pH 7.4, containing 0.50 M-KCl. The discs were mounted in a filtration assembly and gentle suction was applied until they were just dry. RNA samples for analysis (0.25–0.50 ml) were diluted with 5.0 ml of the appropriate medium and filtered at a rate of 1.5–2.0 ml/min. The discs were finally washed three times with 2.5 ml of medium B or C, depending on the method used.

(b) *Oligo(dT)-cellulose chromatography*. This adsorbent was prepared by the two-stage procedure of Gilham (1964) from thymidine 5'-phosphate and Whatman CF11 cellulose under anhydrous conditions and with *NN'*-dicyclohexylcarbodi-imide as the coupling agent. Columns containing 1 g wet wt. of oligo(dT)-cellulose were used for the isolation of poly(A)-rich RNA as described by Aviv & Leder (1972) with an input of up to $500\text{ }\mu\text{g}$ of RNA. To facilitate a reasonable rate of flow without the application of external pressure, the DNA in samples under analysis was partially hydrolysed by deoxyribonuclease digestion, as described above. Perhaps owing to minor differences in our preparation of oligo(dT)-cellulose, the only departure from the recommended chromatographic procedure (Aviv & Leder, 1972) was that the second wash medium contained 0.15 M-KCl rather than 0.10 M-KCl. Without this modification, both poly(A)-rich RNA and poly(A) itself were eluted prematurely.

Ascites protein-synthesizing system

These $30000g$ supernatant (S-30) preparations were isolated from Krebs II ascites-tumour cells and pre-

incubated to deplete the endogenous mRNA as described in detail by Mathews & Korner (1970). The final stage of the preparation was accomplished by gel-exclusion chromatography (Mathews & Korner, 1970) rather than dialysis (Metafora *et al.*, 1972). Each assay mixture (total vol. 50 μ l) contained the constituents recommended elsewhere (Mathews & Korner, 1970; Aviv *et al.*, 1971), 36 μ g of rRNA, 160 μ g of protein factors and mRNA in the range 0.5–8 μ g. All the naturally occurring L-amino acids were present, except for L-methionine, and 5 μ Ci of either [35 S]- or [3 H]-methionine was added as tracer. At the completion of the 40 min period of incubation at 37°C, the products were processed one of two ways. When simply an estimate of the extent of the incorporation of radioactive methionine into protein was required, each sample was made 0.2M with respect to KOH, incubated at 37°C for a further 5 min to degrade amino acid-tRNA complexes and the radioactivity insoluble in cold 1.0M-HClO₄ was collected as described by Mainwaring & Wilce (1972). Alternatively, the products were analysed in polyacrylamide gels. In this case, 50 μ l of 25mM-Tris-HCl buffer, pH 8.0, containing 1.0% (w/v) sodium dodecyl sulphate, 0.002% (w/v) Bromophenol Blue, 20mM-2-mercaptoethanol and 20% (v/v) glycerol was added to each 50 μ l incubation mixture and the tubes were heated in a boiling-water bath for 2 min (Laemmli, 1970).

Analytical procedures

Proteins were analysed in 7.5% (w/v) polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulphate as described by Weber & Osborn (1969). The gels (7.5 mm diam.) were sliced into 1.5 mm sections and the labelled proteins were eluted by placing individual slices in scintillation-counting vials and shaking overnight in 1.0 ml of 0.1% (w/v) sodium dodecyl sulphate. Samples of RNA (3–30 μ g) were analysed in either 2.5% (w/v) or 5.0% (w/v) polyacrylamide gels, supported in both cases with 0.5% (w/v) agarose, as described by Dingman & Peacock (1968).

Chemical determinations

Protein was determined by the Folin procedure (Lowry *et al.*, 1951), bovine serum albumin serving as the standard. DNA was determined by the diphenylamine reaction of Burton (1956) with calf thymus DNA as reference. As a routine RNA was determined by the spectrophotometric procedure developed by Munro & Fleck (1966). In certain instances, these RNA determinations were corroborated by the colorimetric procedure of Greenbaum & Slater (1957). Yeast tRNA was used as the standard.

Counting of radioactivity

This was done in a mark II Nuclear-Chicago liquid-scintillation spectrometer. Labelled proteins collected on Whatman GF/A discs (Mainwaring & Wilce, 1972) were counted in a phosphor containing 2.5 g of 2,5-diphenyloxazole/litre of toluene, with a counting efficiency of 50% for 3 H and 78% for 35 S. Aqueous samples (0.1–0.5 ml) were counted in a water-soluble phosphor (Mainwaring & Wilce, 1973) at a counting efficiency of 42% for 3 H. Eluted proteins from slices of polyacrylamide gel were counted in 6.0 ml of Aquasol type NEF-934 (New England Nuclear Corp., Boston, Mass., U.S.A.). In all counting procedures, the degree of quenching was calculated by the channels-ratio procedure. Calculation of counting data from double-labelling procedures, with 3 H and 35 S, was greatly facilitated by a computer program written for an Olivetti P602 computer, as suggested in the Nuclear-Chicago laboratory manual.

Results

(a) Studies on polyribosomal RNA

Polyribosomes labelled with [3 H]uridine in vivo. Since the structure of polyribosomes is essentially maintained by mRNA, these subcellular particles were a logical beginning for investigations into the nature of prostate mRNA. In the course of preliminary experiments, it was demonstrated that after the administration of [3 H]uridine directly into the prostate gland *in vivo*, the incorporation of radioactivity into the polyribosomes required between 3 and 4 h to attain a maximum.

Once the optimum conditions for labelling had been established *in vivo*, a more critical study of the individual polyribosomal RNA components was undertaken. In these experiments, maximally labelled prostate polyribosomes were dissociated in sodium dodecyl sulphate and analysed in sucrose density gradients (Fig. 1a). The 18S and 28S rRNA components were readily identified in the sedimentation profile of the dissociated polyribosomes by their prominent absorption at 254 nm, and a third, slower sedimenting but heterogeneous species of RNA was also present. This 6–15S polyribosomal RNA component was of particularly high specific radioactivity, for although considerable radioactivity was recovered in the upper region of the gradients, only trace amounts of RNA were revealed by extinction analysis at 254 nm. Several additional criteria provided the first indications that the 6–15S RNA could be equated with the poly(A)-rich or mRNA fraction of the prostate gland. First, the synthesis of this particular RNA component was relatively refractory to the concomitant administration of actinomycin D with [3 H]uridine *in vivo*, and secondly, incubation of

Fig. 1. Analysis of labelled prostate polyribosomes after their dissociation with sodium dodecyl sulphate

[^3H]Uridine (50 μCi) was injected directly into the prostate glands of normal (non-castrated) animals under conditions of surgical anaesthesia. Labelled polyribosomes were prepared 3 h later and samples equivalent to 10 E_{254} units of RNA were dissociated with sodium dodecyl sulphate just before analysis in sucrose density gradients. The direction of centrifugation is from left to right and completed gradients were fractionated with continuous monitoring at 254 nm. In both figures E_{254} (—) was essentially identical in all sedimentation profiles. (a) Radioactivity of polyribosomes prepared without any additional treatments (●); after the administration of 25 μg of actinomycin D/100 g body wt. concomitantly with the [^3H]uridine *in vivo* (○); after incubation with 0.1 μg of ribonuclease *in vitro* just before treatment with detergent (▲). (b) Radioactivity of polyribosomes prepared without additional treatments (controls, ●); after dilution of individual fractions with medium containing 0.5 M-KCl and collection of poly(A)-rich RNA on nitrocellulose filter (○). Full details of all procedures are given in the Materials and Methods section. Fractions (0.25 ml) were collected.

Fig. 2. Relatively selective labelling of the 6-15S RNA component of prostate polyribosomes by the administration of 5'-fluoro-[^{14}C]orotic acid *in vivo*

Either 10 μCi of [^3H]orotic acid (2.1 Ci/mmol) or 10 μCi of 5'-fluoro-[^{14}C]orotic acid (4 mCi/mmol) was injected directly into the prostate glands of normal rats (four animals/group) and labelling allowed to proceed for 3 h *in vivo*. The ^3H - and ^{14}C -labelled glands were pooled together and polyribosomes prepared. Samples of doubly labelled polyribosomes equivalent to 20 E_{254} units of RNA were dissociated and analysed in sucrose gradients. The direction of centrifugation is from left to right. E_{254} , —; ^3H , ●; ^{14}C , ○. To accommodate the radioactivity from each radioisotope on a convenient scale, the experimental values for ^3H were halved in the preparation of the figure. Fractions (0.25 ml) were collected.

widely documented, particularly the work of Georgiev *et al.* (1963) and Harel *et al.* (1964) on the relative insensitivity of mRNA synthesis to the inhibitory effects of actinomycin D and the investigations of Staehelin *et al.* (1964) on the acute sensitivity of mRNA to enzymic degradation. Thirdly, as shown in Fig. 1(b), only the 6-15S RNA component was selectively retained on nitrocellulose filters under conditions where only RNA species containing sequences of poly(A) are preferentially bound (Lee *et al.*, 1971).

Polyribosomes labelled with 5'-fluoro-[^{14}C]orotic acid *in vivo*. Particularly informative studies on the 6-15S RNA component of prostate polyribosomes were conducted where fluoro-[^{14}C]orotic acid served as radioactive precursor *in vivo* (Fig. 2). Whereas [^3H]orotic acid was extensively incorporated into all the RNA components of prostate polyribosomes, fluoro-[^{14}C]orotic acid was preferentially detected in only the heterogeneous 6-15S RNA component and less significantly in either of the rRNA components. In view of the low specific radioactivity of the 5'-fluoro-[^{14}C]orotic acid, the incorporation of this radioisotope was surprisingly extensive. This may be attributed either to its lower rate of degradation within the prostate gland or its lower degree of dilution by intracellular metabolic pools of RNA precursors. Wilkinson *et al.* (1971) have presented evidence that although 5'-fluoro-orotic acid may be incorporated into the 45S rRNA precursor, the presence of the halogen substituent severely impairs the maturation process whereby the 18S and 28S rRNA components are formed. Our observations on the

labelled polyribosomes with trace amounts of ribonuclease before analysis led to the complete elimination of the 6-15S RNA (Fig. 1a). The association of these distinctive properties with mRNA has been

Fig. 3. Stimulation of an ascites-tumour S-30 system by prostate polyribosomal RNA components

Polyribosomes were prepared from unlabelled prostate glands pooled from eight normal (non-castrated) animals, and after dissociation with sodium dodecyl sulphate, samples equivalent to 10 E_{254} units of RNA were processed further in one of two ways. First, the dissociated polyribosomes were analysed directly in sucrose density gradients or, secondly, the samples were cooled at 0°C to precipitate the detergent, and the supernatant, after low-speed centrifugation, was subjected to oligo(dT)-cellulose chromatography. The retained RNA was then analysed in sucrose gradients with the direction of centrifugation from left to right. At the completion of centrifugation, samples (25 μ l) from each gradient fraction were added to an ascites-tumour S-30 preparation and the incorporation of [3 H]methionine into protein was measured. E_{254} (—) only for polyribosomes analysed directly after dissociation; incorporation of [3 H]methionine from total polyribosomal RNA (●); incorporation of [3 H]methionine from RNA retained on columns of oligo(dT)-cellulose (○). The incorporation of radioactivity was corrected for the background of 550 c.p.m. found in the absence of added RNA. Fractions (0.25 ml) were collected.

selectivity of the incorporation of this halogenated precursor are not so clear cut, but these studies provided an additional inference that the prostate mRNA was probably contained within the heterogeneous 6–15S RNA fraction of polyribosomes.

Priming of protein synthesis by polyribosomal components. When the ability of the various RNA components of dissociated prostate polyribosomes to promote the incorporation of [3 H]methionine into peptide linkage was investigated, only the 6–15S RNA component provided any significant stimulation of a mRNA-depleted S-30 system derived from ascites-tumour cells (Fig. 3). In addition, the common identity of the mRNA fraction and the poly(A)-rich RNA component of polyribosomes was unequivocally determined. Only the 6–15S RNA was retained by oligo(dT)-cellulose and its ability to stimulate the protein-synthesizing system persisted after centrifugation in sucrose density gradients. These studies provided the culminating evidence in support of the common identification of the mRNA or poly(A)-rich

RNA in the heterogeneous 6–15S RNA fraction of the prostate gland.

(b) Studies on unfractionated (total) RNA

Despite the success of labelling prostate polyribosomes *in vivo*, several factors militated against the adoption of this experimental approach in other aspects of the present study. The principal disadvantage of this method is the relatively slow rate of labelling of polyribosomes, requiring 3 h to obtain maximum incorporation. This is incompatible with studies on the very early phase of the androgenic response that occurs within the first hours of hormonal stimulation. In addition, tissue-specificity experiments are difficult to conduct under conditions *in vivo* where the amount of available radioactive precursor is often too low to give significant incorporation. To circumvent these drawbacks, it was evident that a means must be found for labelling prostate RNA *in vitro* and for isolating the poly(A)-rich RNA (or mRNA) without recourse to the preparation of polyribosomes. Fortunately for our present purpose, several means are now available for selectively isolating poly(A)-rich RNA with a relative degree of practical facility.

Specificity of the methods used for isolating poly(A)-rich RNA. Commercially available 3 H-labelled polyribonucleotides facilitated these investigations, and on the grounds of the results presented in Table 1, three analytical procedures demonstrated acute specificity in the retention of poly(A), as reported by the innovators of the original methods (Gilham, 1964; Lee *et al.*, 1971; Sheldon *et al.*, 1972; Aviv & Leder, 1972).

Labelling of prostate RNA *in vitro*. Although a relatively specific incorporation of 5'-fluoro-[14 C]orotic acid into the 6–15S RNA fraction had been demonstrated (see Fig. 2), [3 H]uridine was used in the remaining experiments, largely on the grounds of expense and the higher degree of labelling obtained. Several methods were critically assessed for the extraction of 3 H-labelled poly(A)-rich RNA in an undegraded form. Despite its suitability for the isolation of mRNA from certain eukaryotic cells the procedure of Penman (1966) was not applicable to the prostate gland, perhaps owing to the high content of nucleases in the male accessory sexual glands. The technique developed by Joel & Hagerman (1969) was successful in extracting prostate poly(A)-rich RNA, although it was somewhat laborious when many samples were processed concurrently, and the yields were subject to extreme variation. A method based on that of Rosenfeld *et al.* (1972) was the best means of extracting the poly(A)-rich RNA fraction of prostate gland, with only the marginal disadvantage that appreciable amounts of DNA are also extracted at the same time. Our principal amendment of the published procedure

Table 1. *Specificity of three analytical procedures for retaining poly(A)-rich RNA*

Full details of each procedure are given in the Materials and Methods section and the results represent the binding of [³H]-polyribonucleotides to single discs or columns equivalent to 1 g wet wt. of adsorbent [oligo(dT)-cellulose, only]. Samples of [³H]polyribonucleotide in duplicate [0.25 ml; 20000 c.p.m. plus 100 µg of non-radioactive homopolymer, or 500 µg of non-radioactive homopolymer with oligo(dT)-cellulose columns] were analysed. The [³H]poly(A) controls include analyses conducted with glass-fibre discs alone [poly(U) disc procedure] or in the absence of KCl (other procedures) where no binding should occur.

Polyribonucleotide	Bound radioactivity (c.p.m.)		
	(a) Poly(U)-glass-fibre discs	(b) Nitrocellulose discs	(c) Oligo(dT)-cellulose columns
[³ H]Poly(C)	106	84	290
[³ H]Poly(U)	111	110	440
[³ H]Poly(A)	1235	1740	6800
[³ H]Poly(A) controls	103	60	380
Approx. saturation limit for poly(A)	5.7 µg/disc	8.1 µg/disc	170 µg/g wet wt. of adsorbent

Fig. 4. *Labelling of prostate RNA in vitro*

Prostate glands from normal (non-castrated) animals were pooled with four glands/experimental group. After timed intervals of incubation at 37°C in Eagle's medium containing 100 µCi of [³H]uridine, the total RNA fraction was extracted as described in the Materials and Methods section. Analysis for poly(A)-rich RNA was performed by two independent methods. Since the total amount of RNA was within the range 4.8–5.1 mg for all groups, the data are plotted simply in terms of ³H incorporated/preparation; no estimate was made of the RNA retained in the analytical procedures for poly(A)-rich RNA other than selectively retained ³H. Radioactivity is as total (unfractionated) RNA(●), as poly(A)-rich RNA, determined by binding to nitrocellulose discs (Δ) or to poly(U)-glass-fibre discs (▲).

of Rosenfeld *et al.* (1972) for the satisfactory extraction of the poly(A)-rich RNA of the prostate gland was the inclusion of a second extraction step at 55°C. Extensive incorporation of [³H]uridine into prostate RNA was detected after incubation *in vitro*, yet although the synthesis of other RNA fractions continued for some time, that of the poly(A)-rich RNA was complete after 1 h of incubation (Fig. 4). This rapid rate of labelling of the 6–15S poly(A)-rich RNA is consistent with other experimental findings from this laboratory (P. A. Wilce, unpublished work). From time-course studies on the labelling of polyribosomes *in vivo*, this RNA fraction was maximally labelled within 1–2 h, whereas the 18S and 28S rRNA components were maximally labelled only 3–4 h after the administration of [³H]uridine. On the basis of our former estimates of the total RNA content of the prostate gland of normal animals (Mainwaring & Wilce, 1973), only 48–54% of the RNA is extracted by the procedure used here under conditions where seemingly the poly(A)-rich RNA fraction is totally released into solution.

Although considerable amounts of prostate DNA were also extracted during these experiments, this did not present any insurmountable problems in the interpretation of the experimental results. First, specifically with [5-³H]uridine as precursor, the incorporation of radioactivity into DNA by its conversion into [³H]thymidine can be discounted, as the ³H atom is eliminated before methylation at C-5 (Hayhoe & Quaglino, 1965). Secondly, the presence of even non-radioactive DNA did not impair the binding of labelled poly(A)-rich RNA to nitrocellulose filters (Table 2). Two additional points from Table 2 warrant particular mention. The method of isolating RNA essentially inactivates all ribonuclease activities in the prostate gland, as the RNA may subsequently be briefly incubated at 30°C without degradation of the

poly(A)-rich RNA. In addition, the source of deoxyribonuclease must be carefully selected, for crystalline but not electrophoretically purified enzyme contains sufficient ribonuclease as a contaminant to degrade the poly(A)-rich RNA.

Tissue and steroid specificity of the stimulation of the synthesis of poly(A)-rich RNA in the prostate gland. As widely reported elsewhere, RNA synthesis in the prostate gland is markedly enhanced by the administration of testosterone *in vivo* (Mainwaring *et al.*, 1971; Mainwaring & Wilce, 1972, 1973). Investigations into the androgenic control of the synthesis of the important poly(A)-rich RNA fraction are a counterpart to these earlier studies, and the new data are summarized in Table 3. The androgen-mediated stimulation of the synthesis of poly(A)-rich RNA in the prostate gland attained its maximum after 4–8 h of hormonal stimulation and then declined. The latter is an important observation, since the androgenic response is temporally controlled by the overall retention time of androgens of approx. 12–16 h (Tveter & Attramadal, 1968; Mainwaring & Peterken, 1971). In these terms, the stimulation of synthesis of poly(A)-rich RNA may be reasonably described as an

early event in the androgenic response. The increase in prostate poly(A)-rich RNA is reflected not only in the enhanced rate of incorporation of [³H]uridine, but in an increase in the amount of this RNA fraction

Table 2. *Effect of nucleases on the retention of ³H-labelled poly(A)-rich RNA*

Prostate RNA was labelled with [³H]uridine *in vitro* and duplicate samples of RNA (containing 28000 c.p.m. of radioactivity, 500 µg of RNA and 90 µg of DNA) were incubated with 5 µg of various enzymes at 30°C for 10 min before isolation of the poly(A)-rich RNA by the nitrocellulose-disc procedure. Enzyme controls represent RNA samples incubated without added enzymes.

Conditions of incubation	Radioactivity retained/disc (c.p.m.)
None; samples as prepared	3680
Enzyme controls	3240
+ Pancreatic ribonuclease	40
+ Ribonuclease T ₁ (<i>Aspergillus oryzae</i>)	190
+ Ribonuclease-free deoxyribonuclease	3140
+ Deoxyribonuclease	1520

Table 3. *Tissue and steroid specificity of the synthesis of poly(A)-rich RNA in the prostate gland*

At 72 h after castration, animals were subdivided into experimental groups, each containing four animals. Two groups served as controls and received a subcutaneous injection of oil only. In the experiment on tissue specificity, animals received 2.5 mg of testosterone phenylpropionate and were killed at various times thereafter. In the study of steroid specificity, animals received 1.0 mg of testosterone, androsterone or antiandrogen (6 α -bromo-17 β -hydroxy-17 α -methyl-4-oxa-5 α -androstane-3-one) when these were administered alone or 1.0 mg of testosterone plus 10.0 mg of other steroids in the remaining experimental groups; animals were killed 8 h later. In all experiments, tissue pooled from four animals was incubated with 50 µCi of [³H]uridine for 1 h *in vitro*, and the total RNA fraction was extracted. After digestion with electrophoretically purified deoxyribonuclease, 10 *E*₂₅₄ units of ³H-labelled RNA were analysed by oligo(dT)-cellulose chromatography. Full details of all procedures are given in the Materials and Methods section.

Expt.	Tissue	Hormonal treatment	Input of ³ H-labelled RNA		Retained [poly(A)-rich] RNA	
			(<i>E</i> ₂₅₄ units)	(c.p.m.)	(<i>E</i> ₂₅₄ units)	(c.p.m.)
(1) Tissue specificity	(a) Prostate gland	None (castrated controls)	10.0	2840	0.46	230
		Testosterone, 2 h	10.0	4820	0.53	590
		Testosterone, 4 h	10.0	5370	0.71	780
		Testosterone, 8 h	10.0	9720	0.78	1250
		Testosterone, 16 h	10.0	1140	0.53	1040
	(b) Urinary bladder	None (castrated controls)	10.0	19660	0.41	1180
		Testosterone, 4 h	10.0	19320	0.42	1090
		Testosterone, 16 h	10.0	18490	0.40	1060
	(c) Diaphragm	None (castrated controls)	10.0	19620	0.27	780
		Testosterone, 4 h	10.0	20200	0.27	810
		Testosterone, 16 h	10.0	21070	0.29	830
(2) Steroid specificity	Prostate gland only	None (castrated controls)	10.0	2990	0.42	260
		Testosterone, 8 h	10.0	5790	0.74	680
		Androsterone, 8 h	10.0	3240	0.44	330
		Antiandrogen, 8 h	10.0	2840	0.40	240
		Testosterone+androsterone, 8 h	10.0	7920	0.78	770
		Testosterone+antiandrogen, 8 h	10.0	3720	0.46	390
(3) RNA controls	Prostate rRNA	—	10.0	20320	<0.02	80
	Prostate tRNA	—	10.0	4340	<0.02	70

relative to the total RNA present in the gland. The tissue specificity of this response was illustrated by the failure to demonstrate similar changes in the diaphragm and urinary bladder; these organs were chosen as controls because they may be dissected from animals relatively intact and the bladder has the particular advantage of anatomical juxtaposition to the prostate gland. The high rate of incorporation of [3 H]uridine into the diaphragm simply reflects its lower RNA content than the prostate gland; the high rate of labelling of bladder RNA is due to the incubation of much smaller amounts of bladder *in vitro* relative to the other tissues under comparison yet in the presence of a constant amount of [3 H]uridine. The acute steroid specificity of the response in poly(A)-rich RNA synthesis in the prostate gland is shown by its refractiveness to the administration of an androgen of low biological potency, androsterone, and to the anti-androgen, 6 α -bromo-17 β -hydroxy-17 α -methyl-4-oxa-5 α -androstane-3-one. The latter synthetic steroid, however, unlike androsterone, can suppress the response in RNA synthesis when administered concomitantly with testosterone *in vivo*. This finding is harmonious with previous studies on the effects of this anti-androgen on RNA synthesis in the prostate gland (Mangan & Mainwaring, 1972; Mainwaring & Wilce, 1973) and prompts the important inference that the stimulation of poly(A)-rich RNA is similarly triggered by the binding of specific metabolites of testosterone in the prostate gland (Mangan & Mainwaring, 1972). When administered together with testosterone *in vivo*, the relatively high dose of androsterone promoted a higher rate of incorporation of [3 H]uridine into poly(A)-rich RNA than was obtained with the lower dose of testosterone alone. However, Bruchovsky (1971) has shown that androsterone may be partially converted into 5 α -

dihydrotestosterone, the presumed active androgen in the prostate gland. Although the optimum conditions for the labelling of prostate RNA *in vitro* were initially established in studies on prostate glands taken from non-castrated animals (see Fig. 4), a similar rate but lower extent of labelling was found in prostate glands derived from castrated animals. It was assumed that the time-course of labelling RNA in the control (androgen-independent) tissues was similar if not identical to that in the prostate gland. If differences exist, they are unlikely to invalidate the interpretation of the results presented in Table 3, notably with respect to the tissue specificity of the androgenic stimulation of poly(A)-rich RNA.

Throughout the investigations described above on the isolation of prostate mRNA from polyribosomes, extensive use was made of sucrose-density-gradient ultracentrifugation to resolve the polyribosomal RNA components after labelling with [3 H]uridine *in vivo*. Despite our subsequent adoption of procedures for labelling prostate RNA with [3 H]uridine *in vitro* (see Fig. 4) and the resolution of the labelled poly(A)-rich or mRNA by oligo(dT)-cellulose chromatography (see Table 3), there is little doubt that the prostate mRNA was labelled and analysed with a similar authenticity by both experimental approaches. When prostate glands from androgen-treated animals were labelled with [3 H]uridine *in vitro* and the total RNA fraction was analysed in sucrose density gradients (Fig. 5), a conspicuous increase in the labelling of the heterogeneous 6–15S prostate RNA fraction was observed in comparison with similar RNA preparations derived from castrated animal controls. This finding closely follows the experimental evidence presented in Table 3 where an increase in the amount and labelling of prostate mRNA after androgenic stimulation was quantitatively monitored by oligo-

Fig. 5. Analysis of prostate RNA labelled *in vitro* in sucrose density gradients

Prostate glands from either castrated animals or castrated animals after 8 h of testosterone phenylpropionate treatment *in vivo* (2.5 mg/animal) were incubated with [3 H]uridine *in vitro* and the total RNA fraction prepared as described in the Materials and Methods section. Samples equivalent to 10 E_{254} units were analysed in sucrose gradients with the direction of centrifugation from left to right. In both figures, E_{254} , —; 3 H, ●. (a) RNA from castrated controls; (b) RNA from androgen-treated animals. Fractions (0.25 ml) were collected.

(dT)-cellulose chromatography. As reported below, the RNA retained by oligo(dT)-cellulose chromatography is capable of stimulating the mRNA-depleted ascites-tumour S-30 preparations and thus fulfils a further characteristic property of mRNA in addition to a heterogeneous sedimentation coefficient.

One contentious aspect of tacitly correlating changes in RNA synthesis with rates of incorporation of radioactive precursors should be broached, namely the extent to which such changes reflect alterations in metabolic precursor pools. Even refined demonstrations of tissue and steroid specificity in the rate of labelling do not counter this stricture satisfactorily. Three lines of defence may be cited in the present instance. First, as judged by spectrophotometric analysis, the amount of poly(A)-rich RNA increases rapidly after hormonal stimulation relative to other classes of RNA. Secondly, by using the more time-consuming approach of labelling prostate polyribosomes with 5'-fluoro-[^{14}C]orotic acid *in vivo*, an increased rate of labelling of the poly(A)-rich 6-15S polyribosomal RNA component was similarly demonstrated after hormonal stimulation (P. A.

Wilce, unpublished work). Criticisms of the use of this precursor are less severe, since it has no strict intracellular counterpart. Thirdly, and most important, the demonstration that new proteins may be synthesized in the presence of the poly(A)-rich RNA fraction after androgenic stimulation strongly upholds the viewpoint that certain poly(A)-rich (mRNA) species are fabricated *de novo* during the hormonal response.

Changes in the synthesis of prostate proteins after androgenic stimulation. At timed intervals after the administration of testosterone to castrated animals *in vivo*, the poly(A)-rich RNA fraction was isolated from the prostate gland and spleen by oligo(dT)-cellulose chromatography of total RNA fraction extracted by the modified Rosenfeld *et al.* (1972) procedure. Samples of the poly(A)-rich RNA were sub-

Fig. 6. Pattern of protein synthesis in the prostate gland after hormonal stimulation

At 72h after castration, animals were injected with either oil (control animals) or 2.5mg of testosterone phenylpropionate. Poly(A)-rich RNA was isolated after various times of androgenic stimulation and 4 μg of mRNA was added to an ascites-cell S-30 system. Either [^3H]methionine (9.1 Ci/mmol) or [^{35}S]methionine (diluted to 15.0 Ci/mmol with non-radioactive L-methionine) was used as tracer. Unless otherwise stated, poly(A)-rich RNA from the castrated controls was incubated with [^3H]methionine and that from androgen-treated animals with [^{35}S]methionine. The labelled S-30 preparations were then mixed, dissociated in sodium dodecyl sulphate and fractionated in 7% (w/v) polyacrylamide gels. The doubly labelled gels were sliced and counted for radioactivity when the tracking dye, Bromophenol Blue, approached the anodic end of the gels. Proteins identified *in vitro* as a consequence of the androgenic stimulation of poly(A)-rich RNA *in vivo* are characterized by their high $^{35}\text{S}/^3\text{H}$ ratio. No attempt was made to remove free radioactive methionine before analysis. As a consequence the protein profiles are recorded only from slice no. 10, in the regions not containing free methionine (slices were 1.5mm thick). For simplicity in presentation, only one representative profile is illustrated in the upper panel; remaining experiments are presented in the simpler form of the $^{35}\text{S}/^3\text{H}$ ratio across the gels. The range of this ratio given in Fig. 5(d) is understood to apply to all experiments. The anode is to the left throughout. Each gel is described in terms of the source of mRNA and the androgenic status of the animals under comparison. Upper panel: prostate; castrated ([^3H]methionine) (○); 1h of testosterone treatment ([^{35}S]methionine) (●). Gel (a) prostate; castrated, 1h of testosterone treatment; (b) prostate; castrated, 2h of testosterone treatment; (c) prostate; castrated, 8h of testosterone treatment; (d) prostate; castrated, 16h of testosterone treatment; (e) spleen; castrated, 8h of testosterone treatment; (f) prostate; both 8h of testosterone treatment, but separate incubation with each radioisotope; (g) none, mRNA-free incubation of S-30 system with both radioisotopes.

sequently added to the ascites-tumour S-30 preparation with the objective of investigating the proteins made during the early phase of androgenic stimulation. These results are presented in Fig. 6. To ensure that even small androgen-induced changes in the pattern of protein synthesis would be detected, the experiments were based on the double-labelling technique. Accordingly, the poly(A)-rich RNA fraction from castrated (control) animals was initially incubated with [^3H]methionine, whereas the mRNA from testosterone-stimulated animals was added to S-30 preparations supplemented with [^{35}S]methionine. On completion of the incubation, the preparations were mixed and the appearance of androgen-induced proteins was monitored by changes in the $^{35}\text{S}/^3\text{H}$ ratio of labelled proteins separated in polyacrylamide gels. Even within the first hour of androgenic stimulation, fairly extensive changes in the pattern of protein synthesis were detected. Such changes became even more marked at longer periods of hormonal stimulation and then possibly declined. The authenticity of the androgen-mediated changes in protein synthesis in the prostate gland was verified by the failure to detect significant changes in the $^{35}\text{S}/^3\text{H}$ ratio of proteins made in control incubations. These included studies with mRNA isolated from an androgen-independent tissue, spleen; experiments with S-30 preparations in the absence of added mRNA; and analyses of proteins synthesized in the presence of mRNA extracted from animals of identical androgenic status, but incubated separately with either labelled amino acid, before mixing and analysis. These findings confirm that new species of poly(A)-rich mRNA are synthesized during the early part of the androgenic response.

Electrophoretic analysis of poly(A)-rich RNA. Despite the proven sensitivity of the electrophoretic method for the analysis of complex mixtures of RNA (Dingman & Peacock, 1968), insufficient amounts of prostate poly(A)-rich RNA were isolated during the present investigation to assess accurately the biochemical complexity of the poly(A)-rich fraction. On the evidence of the gels presented in Fig. 6, the fraction is heterogeneous and contrasts sharply, for example, with the poly(A)-rich fraction of rabbit reticulocytes, which essentially contains only globin mRNA. In the latter case, this poly(A)-rich mRNA could be detected by the staining of similar polyacrylamide-agarose gels (Aviv & Leder, 1972).

Discussion

The prostate mRNA fraction has many properties in common with similar preparations from other eukaryotic sources. This similarity is particularly evident in terms of size (Laycock & Hunt, 1969; Lockhard & Lingrel, 1969), selective labelling with 5'-fluoro-orotic acid (Wilkinson *et al.*, 1971) and the presence of polyribonucleotide sequences enriched with respect to poly(A) (Darnell *et al.*, 1971; Lee *et al.*,

1971; Aviv & Leder, 1972; Rosenfeld *et al.*, 1972). On the evidence of its wide distribution in sucrose density gradients and the complexity of the pattern of proteins synthesized *in vitro*, however, the prostate mRNA fraction appears to be conspicuously heterogeneous. This is not surprising in the light of the fact that the entire growth and morphology of the gland is under androgenic control. Such a complex process of regulation must require a conspicuously wide spectrum of regulatory proteins and thus a comprehensive arsenal of mRNA molecules.

Like all other major classes of prostate RNA, the synthesis of the poly(A)-rich RNA fraction is ultimately controlled by the selective binding of 5 α -dihydrotestosterone-androgen receptor complex within the nuclear chromatin. This is particularly evident from the antagonism of the testosterone-induced stimulation of poly(A)-rich RNA synthesis by the anti-androgen 6 α -bromo-17 β -hydroxy-17 α -methyl-4-oxa-5 α -androstane-3-one. This synthetic steroid is a competitive inhibitor for the 5 α -dihydrotestosterone-binding sites on the receptor protein and it also ablates the androgenic stimulation of nuclear RNA polymerase (Mangan & Mainwaring, 1972). The apparent stimulation of poly(A)-rich RNA synthesis by androsterone can probably be explained by its partial conversion into 5 α -dihydrotestosterone (Bruchovsky, 1971); such steroids related to 5 β -androstane exert no influence on the binding of steroids to the androgen receptor (Mangan & Mainwaring, 1972). The molecular basis for the connexion between the nuclear binding of 5 α -dihydrotestosterone and the stimulation of poly(A)-rich RNA synthesis, like all classes of prostate RNA, remains essentially unknown. However, new insights into the androgenic regulation of genetic transcription are now forthcoming (Davies & Griffiths, 1973), particularly with respect to the integral part played by the receptor complex in the control of RNA synthesis.

Since there is at least a superficial similarity between the mechanism of action of androgens and oestrogens at the molecular level, a comparison of the proteins made during the early phases of the hormonal response is of more than passing interest. The oestrogen-mediated response in rat uterus is characterized by the early induction of possibly a single protein, generally termed simply the oestrogen-induced protein (Notides & Gorski, 1966; Katzenellenbogen & Gorski, 1972). This contrasts with the several protein peaks identified here in the first hour of the androgenic stimulation of the prostate gland. This difference may almost certainly be attributed to the methods used in the two investigations. The results from rat uterus were obtained solely from experiments conducted in whole uterine tissue whereas the present investigation depended exclusively on the use of cell-free systems. It remains a distinct possibility that the regulation of protein synthesis is controlled

by steroid hormones at many stages of the translational process and that part of the regulatory machinery may be absent in protein-synthesizing systems derived from seemingly steroid-insensitive cells.

A very severe limitation will be imposed on future work on the mRNA fraction of the prostate gland until a specific marker for the androgenic response can be identified. Certain enzymes have been identified as sensitive markers of the androgenic response (Butler & Schade, 1958; Geller *et al.*, 1969) and it is essential that antibodies be raised against purified forms of these prostatic enzymes for resolving the complex mixture of proteins synthesized *in vitro* in the presence of the poly(A)-rich mRNA fraction. Only by such immunological means may future advances be ensured.

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