The Oestrogen-Stimulated Synthesis of Heterogeneous Nuclear Ribonucleic Acid in the Uterus of Immature Rats

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An early response to the administration of oestradiol-17β to immature rats is the synthesis of uterine RNA of very high-molecular-weight. This RNA is shown to be heterogeneous nuclear RNA. Increased precursor incorporation into the heterogeneous rRNA is not confined to entities of precise molecular weight but appears to involve much of the size range of the species. These findings are discussed with respect to the mode of action of oestradiol.

The precise interrelationship between the biochemical effects of oestrogens on the uterus and their physiological action on this tissue is as yet unclear. We have proposed that the primary action of the steroid could be to stimulate the synthesis of specific mRNA molecules (Knowler & Smellie, 1971). The translation of such messages might then result in the synthesis of a small number of proteins, which in turn could promote the synthesis of new ribosomes and tRNA and the subsequent hypertrophy of the tissue.

Studies on oestrogen action have produced a considerable body of evidence which can be marshalled in support of such an argument. First, oestrogen is found bound to the chromatin of target tissue within 2 min of its administration (Teng & Hamilton, 1968) and results in increases in RNA polymerase II activity (Glasser et al., 1972) and in chromatin template capacity (Church & McCarthy, 1970). This is followed by the early synthesis of new RNA species (Church & McCarthy, 1970) and the production of a small number of new protein species (Notides & Gorski, 1966; Barker, 1971) which appear to be dependent on RNA synthesis for their elaboration (De Angelo & Gorski, 1970) and probably include an activator of Mg+ ion-stimulated RNA polymerase (Barry & Gorski, 1971). Increased activity of this enzyme is associated with stimulated production of rRNA (Hamilton et al., 1965; Knowler & Smellie, 1971) and at the same time, or possibly earlier, synthesis of tRNA is also increased (Knowler & Smellie, 1971). These large increases in RNA synthesis are followed by protein synthesis and uterine hypertrophy (Aizawa & Mueller, 1961).

We have followed the oestrogen-stimulated synthesis of RNA in the uterus of the immature rat by separating the purified RNA on polyacrylamide gels (Knowler & Smellie, 1971). This technique allowed the increased synthesis of rRNA to be followed from its 45S precursor and also revealed the earlier synthesis of an RNA species which, by its location in the first few slices of polyacrylamide gels, appeared to have a very high molecular weight. This species was further found to be rapidly labelled: it was unmethylated and, whilst very sensitive to ribonuclease, was unaffected by Pronase. The present paper describes the further examination of this RNA and its identification as heterogeneous nuclear RNA (heterogeneous rRNA), a species for which a messenger precursor function has been proposed (Darnell et al., 1971).

Materials and Methods

The reagents, experimental animals, injection techniques, preparation of total uterine RNA and electrophoresis of RNA on polyacrylamide gels were the same as previously described (Knowler & Smellie, 1971).

Incubations in vitro

Uteri were removed from rats, dissected free of connective tissue and incubated in 5-ml conical flasks under O2+CO2 (95:5) at 37°C in a shaking waterbath. Incubation was in 2–4 ml of Eagle’s medium but precise conditions varied with each experiment and are described in the legends to the Figures.

Preparation of RNA from subcellular particles

The 12 uteri, which had been removed from treated animals or from incubations in vitro, were rapidly frozen in a bath of solid CO2–methanol and broken up with a footed glass-rod. They were homogenized in 2 ml of 1 mM-MgCl2 in an Ultra-Turrax homogenizer run at 40 V for 20 s. The homogenization and all subsequent steps were carried out at 0–4°C. The homogenate was filtered through two thicknesses of muslin, the homogenizer and muslin were washed
with 2ml of 0.1m-citric acid in 1mm-MgCl₂, and the washings were added to the homogenate. The crude nuclei were collected by sedimentation at 300g for 5min and the pellet was resuspended in 1mm-MgCl₂ containing 0.05m-citric acid and 1% (w/v) Triton X-100. The mixture was made 0.25M with respect to sucrose and underlaid with 2ml of 1mm-MgCl₂ containing 0.32m-sucrose, 0.05m-citric acid and 1% (w/v) Triton X-100. The clean nuclei were collected by sedimentation at 800g for 5min and their purity was checked by phase contrast and electron microscopy. The preparations were optically pure and the nuclei were devoid of outer membranes; however, the nuclear RNA exhibited distinct 28S and 18S RNA peaks. In this respect they differed from nuclear RNA from HeLa cells which contains very little 18S RNA (Penman, 1966). This may reflect some otherwise undetected impurities in the preparations, but nuclear RNA from other mammalian sources also differs from HeLa cells in containing readily detectable amounts of the 18S RNA species (AB & Malt, 1970; Higashi et al., 1966; Steele et al., 1965).

Nuclear RNA was prepared in the same manner as whole uterine RNA except that it was only necessary to perform one potassium acetate–ethanol precipitation to remove DNA oligonucleotides remaining after deoxyribonuclease digestion.

Nucleoli from HeLa cells were prepared from S₃ HeLa cells which were the generous gift of Dr. B. E. H. Maden of this department. They were grown in spinner culture to a concentration of 7 x 10⁶ cells/ml and were labelled for 2.5h with [2-¹⁴C]uridine present in the incubation medium at 0.1μCi/ml. Nucleoli were isolated by the method of Penman et al. (1966) and were suspended in 0.05M-sodium acetate buffer, pH5.2, containing 1% (w/v) sodium dodecyl sulphate and 1mg of bentonite/ml. RNA was extracted at 52°C with 88% (w/v) phenol in 0.05M-sodium acetate buffer, pH5.2, and precipitated with 2 volumes of ethanol in the presence of 0.15M-NaCl.

RNA analysis on sucrose density gradients

The method used was a modification of that described by Girard et al. (1965). RNA (80μg in 0.1ml of LETS buffer) [0.01M-Tris–HCl, pH7.4, 0.1m-LiCl, 1mm-EDTA and 0.2% (w/v) sodium dodecyl sulphate] was layered on to a 13ml, 15–30% linear sucrose density gradient in LETS buffer. Sedimentation was for 16h at 31800g and at 20°C in the SW40 rotor of a Beckman model L2 65B ultracentrifuge. Gradients were collected by using a peristaltic pump, through the flow cell of a Gilford 240 recording spectrophotometer and the E₂₆₀ was continuously monitored. Fractions (10s, about 0.35ml) were collected into an equal volume of ice-cold 10% (w/v) trichloroacetic acid and a further 5ml of cold 5% (w/v) trichloroacetic acid was added together with one drop of 2% (w/v) bovine serum albumin. After being left at 0–4°C for 15min the acid-insoluble material was collected on 2.5cm diameter cellulose acetate membranes with a 0.45μm pore size (Sartorius Membranefilter G.m.b.H., 34 Gottingen, West Germany). The filters were washed with 5ml of cold 5% (w/v) trichloroacetic acid, dried and radioactivity was measured in 10ml of toluene scintillator with efficiencies of 27–30% for ³H and 80–82% for ¹⁴C (Knowler & Smellie, 1971).

Denaturation of RNA

RNA was denatured with dimethyl sulphoxide as described by Katz & Penman (1966) except that dextran sulphate was omitted from the incubation as it markedly affected the subsequent separation of the RNA on polyacrylamide gels.

Base composition analysis

³²P-labelled RNA was separated on polyacryl-

amid gels and the gel slices were dried onto 2.5cm diameter Whatman No. 1 filter paper discs which were glued to planchettes. The radioactivity was measured in a Nuclear Chicago, low background, gas-flow counter. Selected gel slices were cut from the filter-paper discs and RNA was extracted by the method of Marcaud et al. (1971). The extracts were diluted tenfold with dilute saline, so as to decrease the NaCl concentration to 0.15M, and RNA was precipitated by the addition of two volumes of ethanol and storage at −20°C overnight. The hydrolysis, subsequent separation of the hydrolysate and analysis of base composition was essentially as described by Sebring & Salzman (1964).

Electron microscopy

The quality of nuclear preparations was kindly investigated with the assistance of the personnel of the Electron Microscopy Unit of the Physiology Department of this University. Nuclear pellets in polyallomer
tubes were fixed in 2% glutaraldehyde in 1mM-MgCl₂ containing 0.32M-sucrose and 0.05M-citric acid. They were post-fixed in 1% OsO₄ in the same buffer and embedded in Araldite. Sections 50nm thick were cut on a LKB III ultramicrotome, stained with lead and uranium ions and examined in an A.E.I. EM6B electron microscope.

Results and Discussion

Fig. 1 shows the distribution of radioactivity in high-molecular-weight RNA species of rat uterus 15min after the administration of radioactive precursors. When the purified RNA is separated on polyacrylamide gels, incorporation into control animals is seen to be low and largely confined to RNA species of very high molecular weight. At 30min after the administration of oestradiol there is a marked stimulation in the incorporation of precursors into RNA species remaining in the first few slices of the gel. This is again evident 1h after the administration of hormone when the synthesis of ribosomal precursor RNA is also slightly stimulated and at 2h when the synthesis of rRNA is greatly increased. At these later times the labelling of RNA was enhanced across the length of the gel with the ribosomal and pre-ribosomal peaks being superimposed on a background of heterogeneous radioactivity.

It appeared likely that the RNA made as an early response to the treatment of immature rats with oestradiol-17β was in fact heterogeneous nuclear RNA (heterogeneous nRNA). Two of the characteristics of this species have already been demonstrated in the uterine material, namely the high molecular weight and the absence of methylation. Heterogeneous nRNA has also been shown to be located in the cell nucleus, to have a very rapid turnover and a low G+C content. It remained to be seen whether the rapidly labelled uterine RNA also showed these properties and to confirm that the high molecular weight was not an artifact of aggregation. Difficulty was experienced in achieving these ends with the system in vivo. Thus, it proved impractical to label uterine RNA with ³²P in vivo to high enough specific radioactivities for base-ratio determination. Similarly, rate-of-decay studies did not give clear-cut results when radioactive precursors and inhibitors were given to the intact animal. However, when uteri were labelled in vitro by the methods of Gorski & Nicolette (1963) it was found that, although the effects of oestrogen were then slight and could be wholly attributed to increases in precursor uptake, the pattern of RNA labelling was identical with that in vivo (Fig. 2). The nature of the very high-molecular-weight RNA was therefore investigated after labelling in vitro as described in the Materials and Methods section.

Fig. 2 shows the electrophoresis patterns obtained using RNA from uteri labelled in vitro with ³²P and the base composition of the RNA extracted from selected gel slices. The very high-molecular-weight RNA confined to the first few slices of the gel has the high U and low G+C composition characteristic of heterogeneous nuclear RNA. In fact, the RNA extracted from slices 1 and 2 have similarly striking base compositions to those reported by Soeiro et al. (1966) for HeLa-cell heterogeneous nRNA with sedimentation coefficients of >65S and approximately 65S respectively. The base composition of the ribosomal RNA peaks also gives a result which might have been predicted from their known base ratios (Willems et al., 1968) bearing in mind the fact that radioactivity in these species is superimposed on a background of heterogeneous radioactivity probably due to RNA of low G+C content. If, for example, one postulates that in slice 9 radioactivity is roughly divided between 32S RNA of 70% G+C and a DNA-like RNA with a G+C content of 44%, then one would expect a G+C value of approximately 57% in the mixture.

Fig. 3 shows the decay of uterine RNA. Quite drastic measures were required to stop the incorporation of radioactive precursor into RNA rapidly and, after incorporation of labelled ribonucleosides, it was necessary to transfer the uterus to systems containing high concentrations of unlabelled nucleosides and actinomycin D. By using the conditions described in the legend to Fig. 3, RNA synthesis was stopped almost immediately. After short labelling-periods only the very high-molecular-weight RNA species were markedly labelled and Fig. 3 (a, b, c and d) shows that this species decayed very rapidly, the breakdown in the first four slices of the gel amounting to 43% of the total during the first 10min of decay and to 90% during 1h of decay. Fig. 3 (e and f) compares the profile of RNA labelled in vitro for 1h with that from a similar preparation that had been allowed to decay for a further 1h. Again, after 1h of decay, the radioactivity in the first four slices of the gel was decreased to 10%. Further, the radioactivity in all RNA components larger than 28S was drastically decreased, presumably through both decay and ribosomal maturation. When allowance was made for the decay of heterogeneous RNA associated with the ribosomal peaks, it was clear that the 28S and 18S RNA had not undergone marked degradation. It was concluded that the very high-molecular-weight RNA had a much faster turnover than rRNA.

Fig. 4 shows the distribution of precursor incorporation into high-molecular-weight RNA of uterine nuclei. In initial experiments, where nuclei were prepared as described in the Materials and Methods section but without the presence of citric acid, the very high-molecular-weight RNA was not present. Such nuclei appeared structurally sound when observed by phase-contrast microscopy, but
Rats (18–21-day-old, 25–30g) received 1 μg of oestradiol-17β, or carrier, by intraperitoneal injection at various times before death and 125μCi of each of [5-3H]uridine and [8-3H]guanosine by intravenous injection 15min before death. Purified RNA was separated for 5h on 2.7% polyacrylamide gels. (a) Control; (b) 30min after oestradiol-17β administration; (c) 1h after oestradiol-17β administration; (d) 2h after oestradiol-17β administration; ———, E260; ———, radioactivity/slice (d.p.m.). For reasons of clarity the E260 trace is omitted for the first few slices of the gels. The position and identity of 45S and 32S RNA has been determined by longer labelling-periods, methylation and co-fractionation with 14C-labelled HeLa-cell nucleolar RNA as previously described (Knowler & Smellie, 1971).
Fig. 2. Analysis of the base composition of uterine RNA

The uteri of four 18–21-day-old rats, weighing 25–30 g, were incubated for 1 h in 2 ml of Eagle's medium with a phosphate content 10% of the normal concentration, and containing 1 mCi/ml of $^{32}$P-Pi. After washing the incubated uteri thoroughly, the total RNA was purified and separated on 2.7% polyacrylamide gels for 5 h. The $E_{260}$ and the radioactivity were determined throughout the gel and the RNA was extracted from selected slices. This was precipitated in the presence of unlabelled RNA, hydrolysed and its base composition analysed. 

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when examined in the electron microscope they were found to be damaged and to have lost some of their nucleoplasmic content. Citric acid decreases nuclear fragility (Dounce, 1955) and Higashi et al. (1966) have reported that, in Walker carcinoma tissue, citric acid permitted the isolation of nuclei which
Fig. 3. **Decay of uterine RNA**

The uteri from 18–21-day-old rats, weighing 25–30g, were incubated in groups of four in 2ml of Eagle’s medium containing 3μCi/ml of each of [5-3H]uridine and [8-3H]guanosine. After being washed in 2×20ml of pre-warmed Eagle’s media, the uteri were then transferred to a further 2ml of Eagle’s media which contained 100μg/ml of each of uridine, guanosine and actinomycin D. After various lengths of time RNA was isolated and separated on 2.7% polyacrylamide gels for 5h. (a) Incubation for 15min, no decay; (b) 15 min of incubation, 10min of decay; (c) 15min of incubation, 30min of decay; (d) 15min of incubation, 1h of decay; (e) 1h of incubation, no decay; (f) 1h of incubation, 1h of decay. ---, $E_{260}$; -----, radioactivity/slice (d.p.m.).
retained high-molecular-weight RNA even though structural damage was still apparent. Our observations are in agreement with these findings in that citric acid did not appear to decrease structural damage but did permit the preparation of uterine nuclei which retained the very high-molecular-weight RNA (Fig. 4).

In addition to being unmethylated and located in the cell nuclei, therefore, the very high-molecular-weight RNA of rat uterus exhibits a rapid turnover and a base composition low in G+C, high in U. On this basis it seemed to satisfy most of the criteria for heterogeneous nuclear RNA. It was still possible, however, that the high-molecular-weight RNA was a product of aggregation, though this seemed unlikely since at the temperatures used and the concentrations obtained (never more than 0.1 mg/ml) aggregation should not occur during the extraction procedure (Wagner et al., 1967). Further, an aggregate of rRNA would be methylated and have a decay time at least as long as rRNA. Nevertheless, it was felt important to exclude this possibility. Aggregated rRNA molecules would be expected to have double-stranded regions and treatment with 85\% (v/v) dimethyl sulfoxide achieves strand separation of double-stranded RNA without degrading the single strands released (Katz & Penman, 1966). Fig. 5 shows the results of the dimethyl sulfoxide treatment of uterine RNA purified from immature rats 2h after oestrogen treatment and 15 min after administration of radioactive ribonucleoside. Clearly denaturation of the secondary structure of RNA has no effect on the amount of radioactivity present in the first few slices of the gel.

The heterogeneous nRNA which was synthesized in increased amounts as an early result of oestrogen action was located in the first few slices of polyacrylamide gels. This was not an entirely desirable situation but even prolonged electrophoresis failed to produce significantly greater migration. Other separation procedures were tested to extend the polyacrylamide gel results and to investigate the degree
Fig. 6. Separation of rapidly labelled uterine RNA on sucrose density gradients

The 18-21-day-old rats, weighing 25-30 g, received 1 μg of oestradiol-17β, or carrier, by intraperitoneal injection at various times before death and 125 μCi of each of [5-3H]uridine and [8-3H]guanosine by intravenous injection 15 min before death. Purified RNA was layered on to 15-30% sucrose density gradients and sedimented for 16 h at 31 800 g and at 20°C. The fractions are numbered from the bottom of the gradients; (a) control; (b) 30 min after oestradiol-17β administration; (c) 1 h after oestradiol-17β administration; (d) 2 h after oestradiol-17β administration. ---, $E_{260}$; ---, radioactivity/fraction (d.p.m.). The position of 45S and 32S RNA was determined by co-sedimentation with purified 14C-labelled nucleolar RNA from HeLa cells.

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Fig. 7. Separation of rapidly labelled RNA on agarose gels

The 18-21-day-old rats, weighing 25-30 g, received 1 μg of oestradiol-17β, or carrier, by intraperitoneal injection at various times before death and 125 μCi of each of [5-3H]uridine and [8-3H]guanosine by intravenous injection 15 min before death. Purified RNA was separated for 2.5 h on 1% agarose gels; (a) control; (b) 30 min after oestradiol-17β administration; (c) 1 h after administration of oestradiol-17β; (d) 2 h after oestradiol-17β administration. ---, $E_{260}$; ---, radioactivity/slice (d.p.m.). The position of 45S and 32S RNA was determined by co-fractionation with purified 14C-labelled nucleolar RNA from HeLa cells.
of heterogeneity of these very high-molecular-weight RNA species. Fig. 6 shows the separation of the RNA in sucrose density gradients which were run under conditions in which heavier RNA species occupied most of the gradients. Experiments in which RNA, fractionated in sucrose density gradients, was re-separated on polyacrylamide gels showed that two thirds of such gradients were occupied by RNA confined to the first five slices of the gels, while species with sedimentation coefficients of 4S and less were collected in the least-dense third of the gradient. The RNA separated in Fig. 6 was from the same preparations as illustrated in Fig. 1 and it is seen that the RNA synthesized in stimulated amounts 30 min after oestrogen administration is localized in fractions 0–3 of the gradient but is most noticeable in fractions 20–30. In rats exposed to hormone for longer periods, increased incorporation of precursor is noticeable across the gradient but becomes increasingly obvious in lower-molecular-weight species.

Fig. 7 shows the separation of the same RNA preparations on agarose gels. Here again the earliest response to oestradiol treatment is observed 30 min after administration in RNA species heavier than 45S and with longer periods of treatment, the synthesis of pre-rRNA and rRNA becomes most prominent. The separations of Figs. 6 and 7 reveal that the localization of the very high-molecular-weight RNA near the origins of polyacrylamide gels, whilst fortuitous in revealing the stimulated-synthesis in a striking manner, is in fact an artifact of the system. Sucrose density gradient and agarose gels confirm the increased synthesis but reveal that the increased incorporation of precursor is not into defined entities within heterogeneous mRNA but was of a heterogeneous nature.

In view of the evidence which has been accumulating suggesting that heterogeneous mRNA is a precursor of cytoplasmic mRNA (for a review see Darnell et al., 1971) the present evidence supports our contention that the primary step in oestrogenic control of uterine development could be the synthesis of mRNA. Recent studies have also shown that oestrogen controls the synthesis of specific mRNA species in the hen oviduct (Rosenfeld et al., 1972). The finding that the newly synthesized heterogeneous mRNA was not a defined entity but was spread over the wide range of molecular weights by which this species could be explained in a number of ways. Oestrogen could conceivably promote the synthesis of a large number of entities of various molecular weights. These could be messenger precursors or serve some other function in the subsequent control of the hormone-initiated process. Alternatively, the initial product of oestrogen action might be a small number of high-molecular-weight molecules, which during maturation or degradation produce the observed spectrum of products. The further elucidation of these possibilities must await a better understanding of the nature of heterogeneous mRNA.

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References