The Kinetics of the Incorporation of Newly Synthesized Ribonucleic Acid and Protein into the Ribosomes of the Uterus of the Oestrogen-Stimulated Immature Rat

Michael J. MERRYWEATHER and John T. KNOWLER

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

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The kinetics of the synthesis of the components of polyribosomes was investigated in the uterus of the immature rat responding to the administration of oestradiol-17 β . The hormone brings about a rapid stimulation of the association of newly synthesized mRNA with uterine ribosomes, which is maximal 2–4 h after oestradiol administration and causes the aggregation of pre-existing ribosomes into polyribosomes. Despite the striking stimulation of rRNA synthesis 2–4 h after hormone treatment [Knowler & Smellie (1971) *Biochem. J.* **125**, 605–614], the accumulation of new rRNA into ribosomes does not reach a peak until 12 h after administration. At this time, the incorporation of new ribosomal protein is also maximal. A second peak of incorporation of newly synthesized mRNA into polyribosomes follows the peak of ribosome synthesis and coincides with the oestrogen-activated synthesis of DNA.

Oestrogen-induced hypertrophy of the uterus is characterized by a sequential stimulation of the synthesis of macromolecules (Billing *et al.*, 1969), the formation of new ribosomes and the mobilization of the tissue's protein-synthesizing machinery (Tata, 1967).

The synthesis of new rRNA is maximal 2–4 h after a single administration of oestradiol-17 β to immature rats (Knowler & Smellie, 1971) and is preceded by and dependent on the prior stimulation of hnRNA synthesis (Knowler & Smellie, 1973; Aziz & Knowler, 1978). Studies with various inhibitors (Knowler & Smellie, 1971; Borthwick & Smellie, 1975), work on the accumulation of hnRNA extractable as hnRNP particles (Knowler, 1976), and experiments showing a similarity in the sequence of certain hnRNA and cytoplasmic mRNA species (S. Aziz & J. T. Knowler, unpublished work) lead us to suggest that mRNA sequences present in the hnRNA must be translated before rRNA synthesis is activated. The nature of the protein products of this early translation is largely unknown, but one possible product is ribosomal protein, which will be required, in combination with rRNA, in the formation of new ribosomes. In an attempt to understand better the importance of new and pre-existing ribosomes in oestrogen-induced growth, the present paper describes a study of the accumulation of

newly synthesized mRNA, rRNA and ribosomal protein in uterine polyribosomes.

Materials and Methods

Chemicals

All materials used were AnalaR grade supplied by BDH Chemicals, Poole, Dorset, U.K., except for the following. Trizma base, oestradiol-17 β , cycloheximide and puromycin were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Oligo(dT)–cellulose (type T₂; Collaborative Research) was obtained from Uniscience Ltd., 8 Jesus Lane, Cambridge CB5 8BA, U.K.; heparin at 5000 units/ml was purchased from Evans Medical Ltd., Speke, Liverpool, U.K., and Triton X-100 was supplied by Rohm and Haas (U.K.) Ltd., Croydon, Surrey, U.K. [5,6-³H]Uridine (50 Ci/mmol), L-[G-³H]tryptophan (11 Ci/mmol) and L-[4,5-³H]leucine (1 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. [5,6-³H]Uridine was diluted to 5 Ci/mmol before use and all radioisotopes were made 0.9% (w/v) with respect to NaCl before administration.

Experimental

Experimental animals, the preparation and administration of oestradiol-17 β , the administration of radioactive precursor and the measurement of radio-

Abbreviations used: hnRNA, heterogeneous nuclear RNA; hnRNP, heterogeneous ribonucleoprotein particle.

active-precursor incorporation into total uterine acid-insoluble material have been previously described (Knowler & Smellie, 1971).

Analysis of RNA on sucrose density gradients was as described by Knowler & Smellie (1973), except that sedimentation was at $77\,000g_{av.}$ for 16 h in buffered linear 10–25% (w/v) sucrose density gradients.

Polyribosome preparation

Polyribosomes were prepared by a modification of the method of Berridge *et al.* (1976). Uteri were collected in groups of 6–15 in thick-walled glass tubes immersed in a solid- CO_2 /methanol bath and were broken into small segments with a glass rod. While still frozen they were transferred to a sterile Teflon/glass homogenizer and homogenized in 5 ml of buffer 1 [200 mM-Tris/HCl (pH 8.5)/50 mM-KCl/15 mM- MgCl_2 , containing cycloheximide ($5\mu\text{g/ml}$) and 7 mM-2-mercaptoethanol]. All subsequent steps were at $0-4^\circ\text{C}$ and used sterile solutions and glassware. The homogenate, adjusted to 2% (w/v) with respect to Triton X-100, was centrifuged at $10\,000g$ for 10 min. The resulting supernatant was layered over a 1 ml cushion of buffer 2 [1 M-sucrose in 50 mM-Tris/HCl (pH 8.5)/50 mM-KCl/15 mM- MgCl_2 , containing cycloheximide ($5\mu\text{g/ml}$) and 7 mM-mercaptoethanol] and spun at $200\,000g_{av.}$ for 90 min. The polyribosomal pellet was washed once with buffer 3 [50 mM-Tris/HCl (pH 7.6)/250 mM-KCl/5 mM- MgCl_2 , containing heparin ($50\mu\text{g/ml}$) and cycloheximide ($5\mu\text{g/ml}$)] and either used in the preparation of ribosomal subunits or further purified in sucrose density gradients. For gradient fractionation, the pellet was suspended in buffer 3 and $0.5-2.0A_{260}$ units in 0.4 ml of buffer 3 were layered over linear 5 ml gradients of 15–45% (w/v) sucrose in buffer 3. Sedimentation was at $230\,000g_{av.}$ for 40 min and the polyribosome profiles were scanned with a gradient-scanning attachment for the Gilford 240 spectrophotometer.

Preparation of ribosomal subunits

The preparation of ribosomal subunits was derived from the method of Leader & Wool (1972). Ribosomal pellets were prepared from the uteri of immature rats which had each received $200\mu\text{Ci}$ of L-[G^3H]tryptophan or $100\mu\text{Ci}$ of L-[4,5- ^3H]leucine by intravenous injection 1 h before death. The pellets were suspended by gentle homogenization in 0.3 ml of a buffer containing 10 mM-Tris/HCl, pH 7.6, 80 mM-KCl and 5 mM- MgCl_2 . To this were sequentially added 0.15 ml of 1.25 M-KCl, $10\mu\text{l}$ of 1 M-2-mercaptoethanol and $5\mu\text{l}$ of 10 mM-puromycin and the suspension was incubated for 15 min at 37°C . It was then immediately layered over 5 ml gradients of 15–30% (w/v) sucrose in 10 mM-Tris/HCl (pH 7.6)/0.5 M-KCl/5 mM- MgCl_2 /20 mM-mercaptoethanol and

sedimented at $230\,000g_{av.}$ for 90 min at 28°C . The gradients were scanned by using a gradient-scanning attachment for the Gilford 240 spectrophotometer and the peaks corresponding to the 40S and 60S subunits (identified by protein and RNA analysis) were collected and dialysed against $2 \times 250\text{ ml}$ of 50 mM-Tris/HCl (pH 7.6)/80 mM-KCl/12.5 mM- MgCl_2 /10 mM-2-mercaptoethanol at 4°C for 12 h. The subunits were then precipitated with 2 vol. of ethanol at -20°C . The collected precipitates were dissolved in 2 ml of water and 0.1 ml samples were removed for analysis of protein by the method of Bramhall *et al.* (1969). The remainder was precipitated by 2 ml of 10% (w/v) trichloroacetic acid in the presence of 0.5 mg of bovine serum albumin added as a carrier. After standing at 0°C for 30 min, the precipitate was collected on 2.5 cm-diam. glass-fibre filters and the filters were washed with $3 \times 10\text{ ml}$ of 5% (w/v) trichloroacetic acid, $2 \times 5\text{ ml}$ of ethanol and $2 \times 5\text{ ml}$ of diethyl ether before drying and scintillation counting of radioactivity in 2,5-diphenyloxazole/toluene scintillation fluid (Knowler & Smellie, 1971).

Preparation of polyribosomal RNA

RNA was prepared from purified polyribosomes by a modification of the method of Berridge *et al.* (1976). The polyribosomal profiles, including the monoribosome peaks, were collected during the scanning of the gradient, pooled and precipitated with 2 vol. of ethanol at -20°C for 16 h. The precipitate was recovered by centrifugation for 15 min at $2500g_{av.}$, gently dried in a stream of pure N_2 and suspended in 0.25 ml of a buffer containing 50 mM-Tris/HCl, pH 7.6, 250 mM-KCl, 5 mM- MgCl_2 and heparin ($50\mu\text{g/ml}$). To the suspension was added 5 ml of 10 mM-Tris/HCl, pH 7.6, containing 10 mM-EDTA and 1% (w/v) sodium dodecyl sulphate followed by 2.5 ml of 88% (w/v) phenol in the same buffer. The mixture was shaken for 15 min at 20°C , after which 2.5 ml of chloroform was added and the shaking continued for a further 20 min. The phases were separated by centrifugation at $10\,000g_{max.}$ for 10 min at 20°C and the aqueous phase was re-extracted with 5 ml of phenol/chloroform (1:1, v/v) for 30 min. After centrifugation as above, the aqueous layer was made 0.15 M with respect to NaCl, and RNA precipitated by the addition of 2 vol. of ethanol.

Oligo(dT)-cellulose chromatography of polyribosomal RNA

Oligo(dT)-cellulose chromatography was based on the method of McGrath (1978), which was a modification of the method of Aviv & Leder (1972). For this 0.07 g of oligo(dT)-cellulose was suspended in sterile water and packed into a mini

column consisting of a sterilized Pasteur pipette plugged with glass wool. The columns were washed with 5 ml of 2% (w/v) sodium dodecyl sulphate and then equilibrated with loading buffer [10 mM-Tris/HCl (pH 7.5)/0.5 M-LiCl/1 mM-EDTA/0.1% sodium dodecyl sulphate]. RNA preparations in 1 ml of loading buffer were cycled three times through the columns, which were then washed with intermediate buffer [10 mM-Tris/HCl (pH 7.5)/0.1 M-LiCl/1 mM-EDTA/0.1% sodium dodecyl sulphate] until the A_{260} of the eluate was less than 0.01. The initial eluate and washings were combined and the RNA, which constituted the unbound fraction, was precipitated with 2 vol. of ethanol in the presence of carrier unlabelled uterine RNA. The bound RNA component was eluted with 2×1.5 ml of elution buffer [10 mM-Tris/HCl (pH 7.5)/1 mM-EDTA], made 0.15 M with respect to NaCl and precipitated with 2 vol. of ethanol in the presence of unlabelled carrier RNA. Fresh oligo(dT)-cellulose was used for each preparation and RNA recovery was monitored by its A_{260} at all stages of the chromatography. The precipitated RNA was dissolved in water, reprecipitated by 5% (w/v) trichloroacetic acid in the presence of 0.5 mg of bovine serum albumin and filtered on to 2.5 cm-diam. glass-fibre filters. The filters were washed with 3×10 ml of 5% (w/v) trichloroacetic acid, 2×5 ml of ethanol and 2×5 ml of ether before drying and scintillation counting for radioactivity in 2,5-diphenyloxazole/toluene scintillation fluid.

Results and Discussion

Fig. 1 shows the polyribosome profiles obtained from immature rat uteri at various times after oestrogen administration. The unstimulated immature rat uterus contained few polyribosomes and only showed peaks of monomers and dimers. Within 1 h of oestrogen treatment, the ribosomes were aggregating into polyribosomes. This was also seen in two out of three preparations from animals receiving oestrogen 30 min before death. The percentage of ribosomes in large polyribosomes was much increased 2–4 h after a single oestrogen administration, and was maximal after 8–12 h, but by 16 and 24 h the size and number of polyribosomes were falling.

Fig. 2 illustrates the incorporation of [5,6- 3 H]uridine into uterine polyribosomal RNA that has been fractionated into poly(A)-containing mRNA and species not bound by oligo(dT)-cellulose. The Figure is a composite of a series of experiments, each normally taking the form of a control (unstimulated preparation) and two hormone-treated preparations. Average preparations produced 200–300 μ g of unbound RNA, which was shown on polyacrylamide gels to consist of 28S, 18S and 4S

RNA. The recovery of bound RNA ranged between 2 and 6% of unbound RNA, depending on whether it was recovered from unstimulated or oestrogen-treated animals, and contained no detectable rRNA (results not shown). The data in Fig. 2 are expressed as specific radioactivity of RNA (c.p.m./ μ g) and plotted as a percentage of values for unstimulated controls. Control incorporation was 0.4 c.p.m./ μ g of unbound RNA and 12 c.p.m./ μ g of bound RNA.

The incorporation of radioactivity into the bound RNA, presumed to represent polyribosomal polyadenylated mRNA, is stimulated in excess of 20-fold by 2 and 4 h after oestrogen administration. Incorporation of precursor falls dramatically by 8–12 h, but shows a second rise at 16 and 24 h at the time when uterine DNA synthesis is activated by the hormone.

The stimulation of the incorporation of newly labelled RNA into the unbound fraction of polyribosomal RNA at 30 min and 1 h after hormone administration is presumed to represent mainly tRNA synthesis. The stimulated synthesis of 4S RNA is known to precede that of rRNA, which is only just noticeable in fractionated total uterine RNA 1 h after the administration of oestrogen (Billing *et al.*, 1969; Knowler & Smellie, 1971). The incorporation of newly labelled non-polyadenylated RNA into uterine polyribosomes reached a peak at 3.5 times the unstimulated incorporation, 2 h after the administration of oestrogen. By this time, the synthesis of uterine rRNA as well as that of tRNA is known to be strongly stimulated (Knowler & Smellie, 1971). This degree of stimulation is maintained for up to 8 h after the administration of oestrogen, but then exhibits a further dramatic increase such that, by 12 h after oestrogen treatment, precursor incorporation is more than 11-fold that of unstimulated animals. Thereafter incorporation rates decline, but 24 h after oestrogen administration are still more than twice that into the unbound fraction of control animals.

One problem in the design of the experiment in Fig. 2 was selecting the best length of time between the injection of radioactive precursor and the death of the animal. For the detection of rapid hormonal responses and of short-lived mRNA species, a short time period was desirable. Because of this, precursor was given 30 min before death. However, it was conceivable that 30 min would not be long enough to label all the unbound RNA species. tRNA and 18S RNA might be expected to be labelled to a greater extent than 28S RNA. Fig. 3 shows that, although 28S RNA was indeed labelled to a lower specific radioactivity than the other species, all three species showed considerable precursor incorporation. Nevertheless, a partial repeat of the experiment of Fig. 2 was conducted with a 1 h incorporation of precursor. Similar results were obtained.

Fig. 1. Polyribosomal profiles of the immature rat uterus responding to oestradiol-17 β

Polyribosomes were prepared and their profiles monitored as described in the Materials and Methods section. (a) Unstimulated control; (b) 1 h after oestradiol-17 β administration; (c) 4 h after oestradiol-17 β administration; (d) 8 h after oestradiol-17 β administration; (e) 12 h after oestradiol-17 β administration; (f) 16 h after oestradiol-17 β administration.

Fig. 4 shows the incorporation of L-[4,5- ^3H]leucine into uterine ribosomal protein. The preparations were made from polyribosomes that had been separated into 40S and 60S subunits to remove nascent protein, but were then pooled for analysis because their separation from each other was not reproducibly complete enough to consider them in isolation.

Synthesis of total uterine protein in Fig. 4 is expressed as c.p.m. of precursor incorporated into total uterine protein/ μg of uterine DNA and then plotted as a percentage of controls. The amount of

uterine DNA detectable by colorimetric assay does not change appreciably over 24 h of oestrogen treatment. Typical unstimulated control values obtained were 6.2 c.p.m./ μg of DNA.

Incorporation of precursor into ribosomal protein has been expressed as specific radioactivity, i.e. c.p.m. incorporated/ μg of ribosomal protein recovered. It is necessary to relate incorporation to recovery in this way because the oestrogen-induced imbibition of water by the uterus makes it easier to homogenize and leads to more efficient isolation of ribosomes. Analysis by this method does, however,

lead to some distortion of the results. Since the hormone stimulates ribosomal protein synthesis continually from 4 to 24 h after administration (Fig. 4), some of the increased recovery of ribosomal protein

Fig. 2. Effects of oestradiol-17 β on the incorporation of newly synthesized RNA into uterine polyribosomes. Groups of 12 18–21-day-old female rats each received 1 μ g of oestradiol-17 β , or carrier, at various times before death and 100 μ Ci of [5,6- 3 H]uridine 30 min before death. Polyribosomal RNA was prepared and fractionated into polyadenylated and non-polyadenylated species on oligo(dT)-cellulose as described in the Materials and Methods section. ●, Unbound polyribosomal RNA; ○, bound polyribosomal RNA.

Fig. 4. Comparison of the effects of oestradiol-17 β on the synthesis of uterine ribosomal protein and total protein. Groups of 12 immature rats were given 1 μ g of oestradiol-17 β , or carrier, at various times before death. Total protein synthesis (○) was estimated, as described in the Materials and Methods section, by measurement of incorporation into total uterine acid-insoluble material of [4,5- 3 H]leucine administered at 20 μ Ci/rat 1 h before death. Incorporation of newly synthesized ribosomal proteins into uterine polyribosomes (●) was estimated, as described in the Materials and Methods section, by measurement of incorporation into purified ribosomal subunits of [4,5- 3 H]leucine administered at 100 μ Ci/rat 1 h before death.

Fig. 3. Incorporation of precursor into uterine polyribosomal RNA of rats receiving [5,6- 3 H]uridine 30 min before death. For this experiment 25 immature female rats received 1 μ g of oestradiol-17 β 16 h before death and 200 μ Ci of [5,6- 3 H]uridine 30 min before death. RNA was prepared from the post-mitochondrial supernatant and 250 μ g in 0.25 ml of LETS [0.01 M-Tris/HCl (pH 7.4)/0.1 M-LiCl/1 mM-EDTA/0.2% (w/v) sodium dodecyl sulphate] buffer was subjected to analysis on sucrose density gradients as described in the Materials and Methods section. —, A₂₆₀; ----, radioactivity per fraction (c.p.m.).

in treated rats will be due to synthesis. This will have the effect of lowering the specific radioactivity for short periods of precursor incorporation and may partially contribute to the apparent decline in ribosomal protein synthesis seen at 16 and 24 h in Fig. 4. The results are plotted as a percentage of unstimulated controls, for which typical recoveries were 67 μ g of ribosomal protein containing 5.4 c.p.m. of incorporated precursor/ μ g.

The incorporation of newly synthesized proteins into ribosomes approximately mirrors that of rRNA. Thus the peak of stimulation, of just over 6-fold the incorporation into unstimulated animals, occurs 12 h after oestrogen administration. The time course does, however, differ from that of rRNA incorporation in that stimulation is near maximal 8 h after oestrogen administration. Clearly, the rapid aggregation of ribosomes into polyribosomes 2–4 h after oestrogen administration (Fig. 1) must involve pre-existing ribosomes linking to newly synthesized mRNA (Fig. 2). A repeat experiment employing L-[G-³H]tryptophan as the radioactive precursor gave similar results (results not shown).

The results of Figs. 2 and 4 were not as expected. When total uterine RNA was separated on polyacrylamide gels, the incorporation of precursor into rRNA was stimulated by up to 10-fold by 2–4 h after oestrogen administration (Knowler & Smellie, 1971). It was known, however, as shown in Fig. 4, that total protein synthesis was maximally stimulated considerably later in the hormone-induced growth. The above experiments were therefore conducted to investigate the kinetics of the incorporation of precursor into ribosomal protein, and it was considered likely that ribosomal protein synthesis would be stimulated earlier than the synthesis of total protein. Fig. 4 shows that this is not the case. Furthermore, Fig. 2 indicates that, despite a 10-fold stimulation of rRNA synthesis 2–4 h after oestrogen administration (Knowler & Smellie, 1971), incorporation of newly synthesized rRNA into polyribosomes is not stimulated to the same extent until new ribosomal protein incorporation is maximal at 12 h after oestradiol administration. The

implication is that between 2 and 12 h after hormone treatment a large pool of unincorporated rRNA accumulates in uterine cells. There are indications that this pool of rRNA is incorporated into ribosomes between 8 and 12 h after oestrogen administration, as at this time the incorporation of radioactive protein is near maximal, whereas that of rRNA is still only 3.5-fold that of unstimulated controls. This may reflect the association of the newly made protein with the pool of rRNA built up over the preceding 6 h. It is hoped that further studies on cytoplasmic pools of rRNA and ribosomal proteins and on the synthesis of ribosomal proteins *in vitro* on uterine polyribosomes may clarify these issues

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