Characterization of Uterine Heterogeneous Nuclear Ribonucleic Acid and the Effect of Oestradiol-17 β on its Synthesis

By SHAHARUDDIN AZIZ and JOHN T. KNOWLER

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

(Received 13 October 1977)

An early response to the administration of oestradiol-17 β to immature rats is a dramatic stimulation in the synthesis of uterine hnRNA (heterogeneous nuclear RNA). High-molecular-weight fractions of the hnRNA were purified and subfractionated on poly(U)-Sephadex into fractions that differed in their poly(A) content and their size profile on polyacrylamide gels. Oestrogen treatment of the rats stimulated the synthesis of all three fractions of high-molecular-weight hnRNA, but the kinetics of synthesis, degree of stimulation and size distribution of the newly synthesized RNA differed in each fraction.

Steroid hormones are bound specifically by receptor proteins in the cytoplasm of their target cells and are then transported to the nucleus, where they activate the transcription of RNA. In the uterus, this event causes the induction of a cycle of growth and development that prepares the tissue for implantation of the blastocyst and pregnancy. The initial stimulation of RNA synthesis, which precedes and is a prerequisite for the uterine growth, occurs in two phases. The earliest response, detected within 30 min of oestradiol-17 β administration to immature rats, is a stimulation in the rate of synthesis of uterine hnRNA (Knowler & Smellie, 1973). Subsequently, at 1–2 h after hormone treatment the synthesis of tRNA and rRNA is stimulated to a peak at 10–12 times control values 2–4 h after hormone treatment (Knowler & Smellie, 1971, 1973). Studies with various inhibitors (Knowler & Smellie, 1971; Borthwick & Smellie, 1975) and of polyribosome profiles (Merryweather & Knowler, 1977) have led us to propose that the hnRNA made in response to oestrogen contains mRNA sequences and that the maturation and translation of these mRNA species might be a necessary prerequisite for the stimulation of ribosome synthesis and the subsequent uterine hypertrophy. The conclusions of other workers using different hormone-responsive systems support the above concepts. Thus Tata & Baker (1975) demonstrated the oestrogen-induced synthesis of a large poly(A)-containing hnRNA in male *Xenopus* liver. Oestrogen-induced specific mRNA species have been demonstrated in *Xenopus* liver (Berridge *et al.*, 1976; Berridge & Lane, 1976; Wahli *et al.*, 1976) and in fowl oviduct (O'Malley *et al.*, 1975; Means *et al.*, 1975; Woo & O'Malley, 1975). Schmid & Sekeris

(1973) have suggested that hnRNA is involved in the synthesis of liver rRNA, and Nicolette & Babler (1974) have demonstrated that protein synthesis is a prerequisite of oestrogen-stimulated rRNA synthesis. In the present study we have isolated a high-molecular-weight fraction of uterine hnRNA and have initiated a study of its structure and the effect of oestrogen on its synthesis.

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 , T₁ ribonuclease and pancreatic ribonuclease A were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. NNN'-Tetramethylethylenediamine and ethylene diacrylate were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Poly(U)-Sephadex was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. PPO (2,5-diphenyloxazole) (scintillation grade) was purchased from International Enzymes, Windsor, Berks., U.K. Bis-MSB [*p*-bis(*O*-methylstyryl)benzene] was purchased from Eastman Kodak, Kirby, Lancs., U.K. [2-³H]Adenosine (30 Ci/mmol), [8-³H]guanosine (10 Ci/mmol) and [5-³H]uridine (5 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Scintillation fluid

Toluene/PPO scintillator was prepared by dissolving PPO at 0.5% (w/v) in AnalaR toluene. Triton/toluene scintillator was prepared by dissolving PPO at 0.5% (w/v) and bis-MSB at 0.05% (w/v) in a solution containing 35% (v/v) Triton X-100 and 65% (v/v) AnalaR toluene.

Abbreviation used: hnRNA, heterogeneous nuclear RNA.

Experimental animals

Rats derived from the Wistar strain and bred at the University of Glasgow were used. In all experiments the rats were 18–21 days old and limited to a weight of 25–30 g.

The preparation and administration of oestradiol-17 β and the techniques of radioactive-precursor injection or incorporation during incubation *in vitro* have been previously described (Knowler & Smellie, 1973).

Extraction of uterine RNA

For the preparation of total uterine RNA 8–12 uteri from oestrogen-treated rats that had received intravenous injections of radioactive precursor or that had been incubated with precursor *in vitro* were used, as described previously (Aziz & Knowler, 1977).

Preparation of uterine high-molecular-weight hnRNA

To obtain the high-molecular-weight hnRNA, total uterine RNA was fractionated on a linear 15–30% sucrose gradients in LETS buffer, pH 7.4 (0.01 M-Tris/HCl, 0.1 M-LiCl, 1 mM-EDTA and 0.2% sodium dodecyl sulphate), as described by Knowler & Smellie (1973). RNA fractions sedimenting above 45S were pooled, NaCl was added to 0.15 M and the RNA was precipitated overnight at –20°C with 95% (v/v) ethanol in the presence of 60 μ g of yeast RNA. High-molecular-weight hnRNA samples from two separate experiments were pooled for subsequent characterization.

Sucrose density gradients in denaturing conditions (Ross, 1976)

High-molecular-weight hnRNA was pelleted and dried in a gentle stream of N₂. The RNA was then resuspended in 0.2 ml of 98% (v/v) formamide containing 0.002 M-EDTA and 0.01 M-Tris/HCl (pH 7.5), heated in a 90°C water bath for 1 min and layered over 8–20% (w/v) sucrose gradients containing 98% deionized formamide, 0.002 M-EDTA and 0.01 M-Tris/HCl (pH 7.5) in polyallomer tubes for the SW56 rotor and centrifuged in the Beckman model L2 65B ultracentrifuge for 21 h at 30°C and 32000 rev./min. A parallel gradient containing ³²P-labelled HeLa-cell nucleolar RNA was centrifuged under similar conditions to provide the 45S and 32S markers. Fractions (approx. 0.2 ml) were collected under gravity by puncturing the polyallomer tubes and acid-insoluble material was prepared as described by Knowler & Smellie (1973). Radioactivity was counted in 10 ml of toluene/PPO scintillation fluid.

Characterization of hnRNA into species containing and lacking poly(A)

Poly(U)–Sephacryl affinity chromatography was

used by essentially the method of Molloy *et al.* (1974) as described elsewhere (Aziz & Knowler, 1977). Three main fractions were obtained, the unbound fraction, the fraction that bound but was eluted with 15% formamide and the fraction that bound but was eluted with 90% formamide.

Preparation of polyadenylate core

For this six to eight uteri from oestrogen-treated rats were removed, dissected free of adipose and connective tissues and incubated *in vitro* as described before (Knowler & Smellie, 1973) in Eagle's medium (Busby *et al.*, 1964) containing 125 μ Ci of [2-³H]-adenosine/ml for 1 h. High-molecular-weight hnRNA was prepared as described (Aziz & Knowler, 1977) and fractionated on poly(U)–Sephacryl into the three fractions as described above. The fractionated RNA from two separate experiments was pooled and precipitated in 95% ethanol overnight at –20°C in the presence of 0.15 M-NaCl. The RNA from each fraction was pelleted and dissolved in 1 ml of sterile water and reprecipitated in ethanol. The RNA was subsequently pelleted, freeze-dried and dissolved in digestion buffer (0.3 M-NaCl, 0.005 M-MgCl₂ and 0.01 M-Tris/HCl, pH 7.4) containing 0.6 μ g of pure T₁ ribonuclease (Sigma)/ml and 50 μ g of pancreatic ribonuclease A/ml (Dubroff & Nemer, 1975). Digestion was at 37°C for 90 min and was terminated by the addition of an equal volume of digestion-buffer-saturated phenol and 0.5% (w/v) sodium dodecyl sulphate. The polyadenylate core was extracted twice at room temperature (20°C) and precipitated with 2 vol. of 95% ethanol/0.15 M-NaCl in the presence of 40 μ g of yeast tRNA.

Gel electrophoresis of RNA and polyadenylate core

RNA on poly(U)–Sephacryl after fractionation was electrophoretically resolved on 2.7% polyacrylamide gels as described by Knowler & Smellie (1971). The polyadenylate core was resolved on 10% polyacrylamide gels (Loening, 1967). Radioactivity in gel slices was counted in Triton/toluene scintillation fluid.

Results and Discussion

Fig. 1 shows the radioactivity profile of total ³H-labelled uterine RNA after sucrose-density-gradient centrifugation. A parallel gradient of ³²P-labelled HeLa-cell nucleolar RNA provided the 45S and 32S markers. The gradient system separated the high-molecular-weight RNA from the rRNA and their precursors. Fractions containing heavy RNA and the rRNA species retained their characteristic size profile when re-examined on 2.7% polyacrylamide gels (results not shown). Fractions containing RNA of a size greater than 45S were pooled for the preparation of high-molecular-weight hnRNA. In a

Fig. 1. Separation of high-molecular-weight uterine RNA on sucrose density gradients

Immature rats (18–21 days old) received 50 μ Ci of each of [5- 3 H]uridine and [8- 3 H]guanosine by intravenous injection 30 min before death. Purified RNA was fractionated on 15–30% (w/v) sucrose density gradients as described in the Materials and Methods section; the gradients were pumped through the flow cell of a Gilford 240 recording spectrophotometer and the A_{260} was continuously monitored. Fractions (0.4 ml) were collected and assayed for acid-insoluble radioactivity. 32 P-labelled HeLa-cell nucleolar RNA was centrifuged in a parallel gradient to provide the 45S and 32S markers. $\square$, 32 P-labelled HeLa-cell nucleolar RNA; $\bullet$, 3 H-labelled uterine RNA; —, A_{260} of uterine RNA.

Fig. 2. Sedimentation of high-molecular-weight uterine hnRNA under denaturing conditions

RNA was purified from immature rats (18–21 days old) that had received radioactive RNA precursors as described in Fig. 1. The high-molecular-weight hnRNA was then isolated, denatured at 90°C in 98% formamide and fractionated on denaturing sucrose gradients at 30°C as described in the Materials and Methods section. Fractions (approx. 0.2 ml) of the gradients were collected dropwise, and acid-insoluble material was prepared from each fraction. 32 P-labelled HeLa-cell nucleolar RNA was centrifuged in a parallel gradient to provide 45S and 32S markers. Fractions are numbered from the bottom. $\bullet$, 3 H-labelled uterine high-molecular-weight RNA; $\square$, 32 P-labelled HeLa-cell nucleolar RNA.

previous investigation this high-molecular-weight RNA was characterized as hnRNA by its base composition, nuclear location, kinetics of decay and synthesis and by its lack of methylation (Knowler & Smellie, 1971, 1973).

Fig. 2 illustrates an experiment designed to elucidate the extent to which the apparent high molecular weight of the hnRNA fraction was an artifact of aggregation or secondary structure. The RNA was denatured in 98% formamide at 90°C and centrifuged in a sucrose gradient containing 98% formamide at 30°C. When the profile of the uterine hnRNA in Fig. 2 was compared with that of purified HeLa-cell 45S and 32S rRNA precursors, it was seen that the greatest part still sedimented more rapidly than 45S RNA, but a proportion appeared in the 45S–35S region. Only a small part sedimented more slowly than 35S RNA. The apparent decrease in sedimentation rate of this otherwise very large hnRNA could be attributed to the denaturation of duplex regions of hnRNA regions, as observed by Fedoroff *et al.* (1977) in their investigation of the physical basis of hnRNA complex-formation.

One of the most persuasive indications of a precursor-product relationship between hnRNA and

Fig. 3. Preparation of nuclease-resistant polyadenylate core of uterine hnRNA

The uteri of six immature rats (18–21 days old) were incubated for 1 h in 4 ml of Eagle's medium containing 125 μ Ci/ml of [3 H]adenosine. High-molecular-weight hnRNA was prepared from the uteri and was then fractionated on poly(U)–Sephacel and digested with a mixture of T_1 and pancreatic ribonucleases as described in the Materials and Methods section. The nuclease-resistant material was subsequently resolved on 10% polyacrylamide gels for 3 h at 5 mA/gel, with the carrier 4S RNA as a marker. The Figure illustrates the ribonuclease-resistant core of the hnRNA eluted with 90% formamide. The other fractions did not have detectable peaks of ribonuclease-resistant material. —, A_{260} ; ----, radioactivity per 1 mm slice (d.p.m.).

Fig. 4. Effects of oestradiol-17 β on the synthesis of total uterine high-molecular-weight hnRNA and poly(U)–Sephacel-fractionated hnRNA

High-molecular-weight hnRNA was prepared from groups of immature rats that had received 100 μ Ci of [3 H]uridine intravenously 30 min before death and 1 μ g of oestradiol-17 β or carrier at various times before death. In some experiments the RNA was further fractionated on poly(U)–Sephacel as described in the Materials and Methods section. Total high-molecular-weight hnRNA or the fractions obtained by poly(U)–Sephacel chromatography were acid-precipitated and prepared for determination of acid-insoluble radioactivity as described in the Materials and Methods section. Each point represents the mean values of two separate experiments and eight animals. $\circ$, Total high-molecular-weight hnRNA; Δ , high-molecular-weight hnRNA not bound by poly(U)–Sephacel; $\square$, high-molecular-weight hnRNA eluted from poly(U)–Sephacel with buffered 15% formamide; $\bullet$, high-molecular-weight hnRNA eluted from poly(U)–Sephacel with buffered 90% formamide.

mRNA is the evidence demonstrating the presence of poly(A) in both types of RNA (Edmonds *et al.*, 1971; Lee *et al.*, 1971; Adesnik *et al.*, 1972; Jelinek *et al.*, 1973). This poly(A) region, located at the 3'-OH terminus (Mendecki *et al.*, 1972; Molloy *et al.*, 1972), was about 200 nucleotides long (Mendecki *et al.*, 1972; Sheiness & Darnell, 1973). However, recent experiments showed that not all hnRNA contained such long stretches of poly(A), there being a heterogeneous distribution of lengths, with some species having very short poly(A) tails and others apparently

Fig. 5. Oestrogen effect on the electrophoretic profile of uterine high-molecular-weight hnRNA fractions

High-molecular-weight hnRNA was prepared from groups of immature rats that had each received $100\mu\text{Ci}$ of $[5\text{-}^3\text{H}]\text{uridine}$ intravenously 30 min before death and $1\mu\text{g}$ of oestradiol- 17β or carrier only intraperitoneally 2 h before death. The hnRNA was prepared and fractionated as described in the Materials and Methods section. RNA in the various fractions was precipitated in the presence of unlabelled uterine rRNA as carrier and subsequently resolved on 2.7% polyacrylamide gels for 5 h at 5 mA/gel. The positions of the 45 S and 32 S RNA were identified by resolving ^{32}P -labelled HeLa-cell nucleolar RNA on a parallel gel, and the carrier RNA provided 28 S and 18 S markers. —, A_{260} ; ----, radioactivity per slice (d.p.m.). (a) Unbound fraction (control); (b) fraction eluted with buffered 15% formamide (control); (c) fraction eluted with buffered 90% formamide (control); (d) unbound fraction (2 h oestradiol treatment); (e) fraction eluted with buffered 15% formamide (2 h oestradiol treatment); (f) fraction eluted with buffered 90% formamide (2 h oestradiol treatment).

lacking poly(A) at the 3'-OH terminus (Molloy *et al.*, 1974; Nakazato & Edmonds, 1974; Brawerman, 1976).

These findings were utilized by Dubroff & Nemer (1975) to fractionate sea-urchin hnRNA of differing poly(A) content by its variable affinity for poly(U)-impregnated filters and oligo(dT)-cellulose. We similarly fractionated uterine hnRNA on poly(U)-Sephadex and collected three fractions: one not bound on poly(U)-Sephadex, one bound but eluted by 15% formamide and one bound and eluted by 90% formamide. After a 30 min pulse with radioactive uridine, 42–47% of radioactivity was found in the high-molecular-weight hnRNA fraction. When a sample of high-molecular-weight hnRNA was applied to a column of poly(U)-Sephadex, approx. 13–25% of the total radioactivity bound and could be eluted with various concentrations of formamide. Fig. 3 illustrates the electrophoretic profile of the ribonuclease-resistant poly(A) tail of the uterine hnRNA eluted by 90% formamide. The radioactivity profile was heterodisperse and indicated the presence of poly(A) tails with a size range of 80–150 nucleotides relative to a 4S marker. Molloy *et al.* (1974) showed that not all poly(A)-containing hnRNA binds to poly(U)-Sephadex; therefore we could not rule out the presence of poly(A)-containing hnRNA in our unbound fraction. In our experiments we could not detect significant peaks corresponding to poly(A) tails in either the unbound fraction or in the fraction eluted with 15% formamide (results not shown). However, short tails of 15 bases or less, which might have been expected in the latter fraction (Dubroff & Nemer, 1975), would not have been detected in our gels.

Fig. 4 shows the effects of oestradiol-17 β on the synthesis of the various fractions of high-molecular-weight hnRNA. The incorporation of radioactive precursors into total hnRNA was stimulated by nearly 2-fold as early as 30 min after oestradiol treatment, thus confirming previous observations (Knowler & Smellie, 1973; Knowler, 1976). By 2 h of hormone treatment the stimulated synthesis of total hnRNA increased to 10 times unstimulated values. When the hnRNA was fractionated on poly(U)-Sephadex, it was observed that the increase in hnRNA synthesis at 30 min was reflected in the increase in the poly(A)-containing hnRNA, whereas the other fractions eluted showed marginal increases. At 2 h, however, the synthesis of poly(A)-containing hnRNA was least stimulated, whereas there was a 10-fold increase in the rate of synthesis of the unbound hnRNA fraction. It is possible that the poly(A)-containing hnRNA was being turned over rapidly or that there was rapid processing of the hnRNA into its possible putative messengers. At all time intervals the hnRNA fractions eluted with 15% formamide showed responses to oestradiol-17 β

that were roughly intermediate between those for the other two hnRNA fractions.

Fig. 5 illustrates radioactivity profiles of the three hnRNA fractions prepared from untreated and oestrogen-treated immature rats and electrophoresed in 2.7% polyacrylamide gels in the presence of unlabelled uterine rRNA as carrier. Parallel gels contained ³²P-labelled HeLa-cell nucleolar RNA. Each fraction exhibits a profile containing a number of peaks of radioactive precursor incorporation, and the profiles differ between fractions and between preparations derived from untreated and oestrogen-treated animals. The fraction not retained by poly(U)-Sephadex showed the greatest increase in incorporation of radioactivity in response to 2 h of oestradiol treatment, thus confirming the kinetic experiment (Fig. 4). This fraction also contained the greatest concentration of RNA in high-molecular-weight species. All three fractions of hnRNA, however, showed substantial radioactivity in species of lower molecular weight than originally isolated. In the poly(U)-Sephadex-bound fraction this change in molecular weight can be attributed to the denaturing effects of formamide and parallels the observation in Fig. 2. The fact that lower-molecular-weight species are also detected in the unbound fraction implies that some degradation during the column chromatography cannot be ruled out.

The results presented show that the uterine hnRNA can be fractionated into three types of molecules differing in their affinity for poly(U)-Sephadex. These affinities are retained if the fractions are re-applied to a column. Perhaps the most interesting fraction is that observed to contain poly(A) (Fig. 3). This fraction responds most rapidly to oestradiol treatment of immature rats, its synthesis being stimulated almost 2-fold within 30 min of hormone administration. Work with ribosomes in our laboratory has shown that within 30–60 min of oestradiol administration to immature rats ribosomes aggregate into polyribosomes (Merryweather & Knowler, 1977), and it is tempting to speculate that they are doing so because of the availability of mRNA derived from this hnRNA fraction. It is hoped that these concepts can be tested by hybridization experiments.

We thank Dr. Keith Vass for gifts of ³²P-labelled nucleolar RNA and Professor R. M. S. Smellie for critical reading of the manuscript. S. A. is in receipt of a staff training scholarship from the National University, Kuala Lumpur, Malaysia.

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