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Purification and properties of rabbit uterus preuteroglobin mRNA

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ABSTRACT

The mRNA for preuteroglobin, a precursor of the hormonally induced protein uteroglobin, has been partially purified from the endometrium of progesterone treated rabbits. The purification procedure starts with total endometrial polysomes and involves treatment with proteinase K and dodecylsulfate, chromatography on oligo(dT)-cellulose, sucrose density gradient centrifugation, electrophoresis in polyacrylamide gels containing dodecylsulfate, and a second absorption to oligo(dT)-cellulose. The final mRNA preparation codes exclusively for preuteroglobin in a wheat germ cell-free system and migrates as a single band in polyacrylamide gels containing 99% formamide. The average length of the poly(A) segment is 60 nucleotides and the translation of the preuteroglobin mRNA is inhibited by $m^7G(5')ppp(5')A$, indicating that it contains a "capped" 5'-terminus. Comparison with known standards yields a molecular weight of 200,000 (600 nucleotides) for preuteroglobin mRNA, approximately twice as many nucleotides as required for encoding the 90 aminoacids of its cell-free product.

INTRODUCTION

Progesterone administration causes an accumulation of the mRNA for preuteroglobin (PUmRNA) in the endometrium of the rabbit, followed by increased secretion of uteroglobin in the uterine lumen (1,2,3). In order to understand the molecular mechanisms underlying the hormonal regulation of uteroglobin biosynthesis it is necessary to carry out a precise titration of the PUmRNA during induction, and to study its intracellular distribution. These experiments involve hybridization of cellular RNA to labelled DNA complementary to the mRNA which can be synthesized *in vitro* using the purified mRNA as template. In this paper we describe a procedure for the preparation of PUmRNA which is biologically over 90 % pure, and homogeneous in polyacrylamide gels containing 99 % formamide.

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MATERIALS AND METHODS

L-(^{35}S)Methionine (595 Ci/mmol) and L-(^{3}H)leucine (60 Ci/mmol) were obtained from the Radiochemical Center, Amersham. Polyuridylic acid, sodium salt urydylate-5,6-(^{3}H) (500 mCi/mmol) was obtained from New England Nuclear Inc. The steroids were a gift of Schering AG, Berlin. Oligo(dT)-cellulose, type T-2, was purchased from Collaborative Research Inc., Waltham, Mass. Ribosomal ribonucleic acids, 5s, 16s and 23s, from Escherichia coli, ribonuclease A from bovine pancreas, ribonuclease T1 from Aspergillus oryzae, proteinase K, adenylyl-$(3'\rightarrow5')_5$-adenosine, and adenylyl-$(3'\rightarrow5')_9$-adenosine were obtained from Boehringer, Mannheim. t-RNA was prepared from rabbit liver or rabbit reticulocytes by the procedure of Rogg et al.(4). Ribonuclease-free sucrose was obtained from Serva, Heidelberg. m^7G(5')ppp(5')A was purchased from P.L. Biochemical Inc., Milwaukee. All other chemicals were analytical grade from Merck, Darmstadt. Acrylamide and bis-acrylamide, obtained from Serva, Heidelberg, were recrystallized from chloroform.

Preparation of endometrial poly(A)-containing RNA. All solutions were either autoclaved or treated with diethylpyrocarbonate (1). Total polysomes were isolated from the endometrium of rabbits treated sequentially with estradiol and progesterone by a previously published procedure (1). The purified polysomes were resuspended in tris-SDS buffer (10 mM tris-HCl, pH 7.5, containing 0.5 % dodecylsulfate) to a concentration of 20 A_{260} units/ml, and incubated for 30 min at 37° with 100 µg/ml proteinase K. At the end of the incubation 0.125 volumes of 4M NaCl in tris-SDS buffer were added, and the mixture was heated at 60° for 10 min. After cooling down to room temperature, the sample was applied to a column of oligo(dT)-cellulose equilibrated with tris-SDS buffer containing 0.5 M NaCl. One gram of oligo(dT)-cellulose was used for every 600-800 A_{260} units polysomes and the flow rate was 20 ml/h. The column was extensively washed first with the starting buffer and then with the same buffer but without dodecylsulfate, before eluting the poly(A)-containing RNA with sterile water. The RNA was then precipitated overnight at -20° after the addition of 0.1 volume of 2M LiCl and 2 volumes of ethanol. The RNA contained in the

material which did not bind to oligo(dT)-cellulose was extracted with phenol/chloroform (5) and precipitated in a similar way.

Sucrose density gradient centrifugation. Linear 5%-20% sucrose gradients in 10 mM tris-HCl buffer, pH 7.5, containing 1 mM $EDTANa_2$ were prepared in 12 ml polyallomer tubes. The RNA sample was heated at 60° for 5 min, chilled, and applied in 0.2 ml aliquots per gradient at a concentration of 40 A_{260} units/ml in sterile water. The gradients were centrifuged at 40,000 rpm and 0° for 22 h in the Beckman SW41 rotor, and collected into 0.4 ml fractions. Fractions from 3-4 gradients were pooled and 5 µl aliquots taken for assaying protein synthesis in the wheat germ cell-free system (see below). The fractions containing PUmRNA activity were pooled and precipitated overnight at -20° after addition of 0.1 volumes of 3M NaOAc and 2 volumes of ethanol.

Electrophoresis in polyacrylamide gels containing dodecylsulfate. 4% polyacrylamide gels containing 0.2% dodecylsulfate were prepared according to Loening (6) in plastic tubes of 6 mm inner diameter. The gels were preelectrophoresed at room temperature, 2mA/tube for 1-2h, and the RNA sample was applied dissolved in 20 µl electrode buffer A (18 mM tris, 15 mM NaH_2PO_4, 0.5 mM $EDTANa_2$ and 0.2% dodecylsulfate, adjusted to pH 7.6 with HCl) containing 10% glycerol. Before application the samples were heated at 60° for 10 min. Electrophoresis was carried out at room temperature and 2mA/gel until the dye front reached to the end of the tube (around 8 cm). The gels were soaked for 2 h in 200 ml of electrode buffer and scanned at 260 nm. The absorbance peak was cut and placed into a small plastic tube of 6 mm inner diameter closed by dialysis membrane. The RNA was electrophoretically eluted at 2mA/tube for 1 h and incubated for 30 min at 20° with 0.1 g oligo(dT)-cellulose, after raising the NaCl concentration to 0.5 M. The mixture was then applied to a small column and extensively washed with electrode buffer containing 0.5 M NaCl, and with 10 mM tris-HCl, pH 7.5 also containing 0.5 M NaCl. The bound mRNA was finally eluted with 0.5 ml sterile water and precipitated at -20° overnight by addition of 0.1 volume of 3M NaOAc and 2 volumes of ethanol.

The precipitate was washed with 1 ml 3M NaOAc, pH 5.0, followed by 70%, 90% and absolute ethanol. The RNA pellet was dried, resuspended in sterile water, and stored frozen at -85°C.

Cell-free protein synthesis and product analysis. The wheat germ system was prepared according to Roberts and Paterson (7). Preincubation was carried out at 35° for 30 min. The composition of the assay has been published (1). Standard incubations were carried out at 25° for 30 min, and the incorporation of radioactivity was determined in filter paper discs according to the method of Bollum (8).

Immunoprecipitation of the cell-free products was carried out by a previously described double antibody technique (1), with the only difference that a monospecific goat antiserum against uteroglobin was used as a source of the immunoglobulin fraction, and rabbit immunoglobulin prepared against goat immunoglobulin was used as second antibody. The goat antiserum was prepared as follows. Purified uteroglobin (1 mg) was treated at 90° for 5 min with 2% dodecylsulfate and 2% 2-mercaptoethanol before application to a 10% polyacrylamide slab gel (0.3 x 10 x 10 cm) containing 0.1% dodecylsulfate (9). After electrophoresis at 50 mA for 7 h the uteroglobin band was cut and homogenized in 1 volume of complete Freund's adjuvant, and the paste subcutaneously injected. Each goat received 4 injections (0.5 mg uteroglobin) with monthly intervals, and the animals were bleeded 3 weeks after the last injection. The immunoglobulin fraction was prepared from serum by repeated ammonium sulfate fractionation (10). The antiserum was monospecific as judged by the same criteria used with guinea pig antiuteroglobin (11,12).

Antibodies against goat immunoglobulin were prepared in rabbits by conventional techniques, and the immunoglobulin fraction isolated by ammonium sulfate precipitation (10). An immunoprecipitation curve showed that 100 µg of rabbit immunoglobulin were sufficient to quantitatively precipitate 40 µg of goat immunoglobulin.

The cell-free radioactive products were analyzed in 12.5% polyacrylamide gels containing 8M urea and 1% sodium dodecylsulfate (13) using either round gels (0.6 x 14 cm) or slab

gels (0.15 x 10 x 10 cm). When slab gels were used the concentration of bis-acrylamide was reduced by 50% in order to facilitate drying of the gels, before submitting then the fluorography according to Laskey and Mills (14). The round gels were sliced and processed for radioactivity determination as previously described (1).

Polyacrylamide gel electrophoresis in formamide and hybridization with (^{3}H)poly(U). 4% polyacrylamide gels were prepared in purified formamide as described by Pinder *et al.* (15). The samples to be applied to the gels were lyophilized, resuspended in formamide and heated at 60° for 10 min. Electrophoresis was carried out at 2 mA/tube (0.6 x 10 cm). The gels were then immersed for 2 h at room temperature in Loenings electrophorese buffer (6), scanned at 270 nm in order to identify the markers, and sliced into 2 mm fractions. The RNA was extracted by mincing the slices and shaking overnight at room temperature in 1 ml of 20 mM tris-HCl, pH 7.6, containing 0.5 M NaOAc and 1% dodecylsulfate. The extraction was repeated once, and the dodecylsulfate in the combined extracts was precipitated by addition of concentrated KCl to a final concentration of 0.2M and chilling on ice. After centrifugation, the supernatant was extracted first with one volume of *n*-butanol and then with one volume of chloroform, and the aqueous phase was precipitated overnight at -20° with two volumes of ethanol after addition of 20 µg tRNA as carrier. The precipitate was washed with 3M NaOAc, pH 5.0, and with 80% ethanol, dried, and resuspended in sterile water.

Aliquots of each fractions (0.1 volumes) were lyophilized and used for hybridization with (^{3}H)-labelled poly(U) (16). The dried RNA samples were resuspended in 0.2 ml of 0.3M NaCl, 0.03M trisodium citrate, pH 6.8, and incubated at 35° for 20 min with 1 µl (50,000 cpm) (^{3}H)-labelled poly(U). At the end of the incubation 20 µg of ribonuclease A were added in 10 µl buffer, and incubation continued at 0° for 20 min. After addition of 100 µg of bovine serum albumine as carrier the ribonuclease resistant material was precipitated at 0° for 15 min with 1 ml of 10% trichloracetic acid. The precipitate was recovered in GF/C Whatman glass fiber paper discs, washed, and counted in

5 ml of a liquid scintillation mixture (5 g PPO, 0.5 g POPOP in 1 l toluol).

Determination of the length of the poly(A) segment. The RNA sample to be analyzed (0.5 µg) was treated at 35° for 15 min with ribonuclease A (20 µg/ml) and ribonuclease T1 (2µg/ml) (17), chilled and lyophilized. The ribonuclease resistant material was resuspended in formamide and applied to 10% polyacrylamide gels prepared in formamide (15).
Rabbit reticulocyte 5s RNA and tRNA as well as poly(A) segments of defined length were used as standards. After the run, the gels were cut into 2mm slices, and extracted overnight at room temperature as described above. The extracted material was precipitated after addition of tRNA (20 µg), washed and used for hybridization with (^{3}H)-labelled poly(U), as described in the preceeding section.

RESULTS

Purification procedure. Treatment of endometrial polysomes with dodecylsulfate and proteinase K followed by column chromatography on oligo(dT)-cellulose results in the retention in the column of 2-3% of the total RNA. The bound RNA can be eluted with water and contains most of the PUmRNA activity. The material that does not bind to oligo(dT)-cellulose, exhibits virtually no PUmRNA activity in a wheat germ cell-free system. If, however, RNA is extracted from the same polysomes by the phenol:chloroform procedure (5) and applied to an oligo(dT)-cellulose column, a considerable proportion of PUmRNA does not bind to the column (1,18), indicating that treatment with dodecylsulfate and proteinase K is more efficient in preserving the ability of PUmRNA to interact with oligo(dT).

Figure 1 shows the sedimentation behaviour of the poly(A)-containing RNA on linear sucrose gradients. The mRNA activity assayed in a wheat germ system shows a peak of activity in the 9s region, coinciding with the position of rabbit globin mRNA run in a parallel gradient (Fig. 1, inset). Between 85-90% of the radioactive cell-free products encoded by this 9s RNA are preuteroglobin, when (^{35}S)-methionine is used as label (Fig. 2). If, however, (^{3}H)leucine is used as labelled amino acid

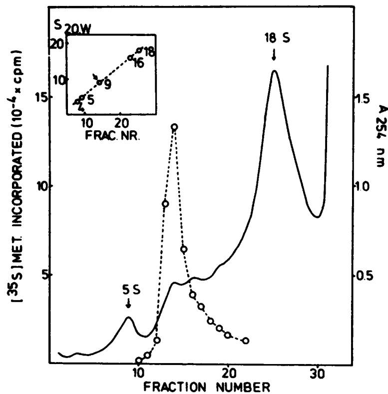

Fig. 1. Sucrose density gradient centrifugation of poly(A)-containing RNA from endometrial polysomes. Poly(A)-containing RNA from endometrial polysomes was applied to 5-20% linear sucrose gradients (8 A260 units/gradient) and centrifuged at 0° and 40,000 rpm for 22 h. The figure represents the optical density profile at 254 nm (solid line) and the mRNA activity in 5 µl aliquots, as determined in a wheat germ system using (35S)methionine as label (o---o). The inset shows the sedimentation position of the mRNA peak (arrow) compared with known standards (rabbit reticulocyte 18s rRNA, 9s globin mRNA, 5s rRNA and tRNA, and E. coli 16s rRNA) run in a parallel gradient.

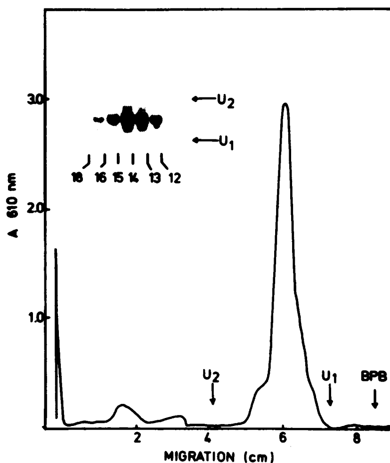

Fig. 2. Cell-free products of sucrose gradient fractions. The wheat germ cell-free products obtained with aliquots of the sucrose gradient fractions shown in figure 1 were analyzed in polyacrylamide slab gels containing dodecylsulfate and urea. The inset shows a fluorogram of the slab gel, to which the products of fractions 12 (slot 1), 13 (slot 2), 14 (slot 3), 15 (slot 4), 17 (slot 5), 19 (slot 6) and 22 (slot 7) were applied. The arrows indicate the position of oxydized (U2) and reduced (U1) uteroglobin. The fluorogram corresponding to fraction 14 (slot 3) was scanned at 610 nm and the absorbance is depicted as a function of electrophoretic migration.

only 70% of the radioactive cell-free products encoded by endometrial 9s RNA could be identified as preuteroglobin, reflecting the high methionine content of uteroglobin (12).

Chromatography on oligo(dT)-cellulose does not select for a particular size class of PUmRNA. Analysis of total polysomal RNA on sucrose gradients shows that the peak of PUmRNA activity sediments at 9s both in the presence of 0.5% dodecylsulfate or in gradients containing 50% formamide (Fig. 3a & b). In both cases, a shoulder of PUmRNA activity is detected around 12s, and activity is also observed sedimenting between the 18s and

28s peak. This finding suggests that PUmRNA tends to form heavy aggregates under conditions of sucrose gradient centrifugation. Optimal conditions to prevent aggregation involve heating the RNA at 60° for 10 min and rapidly cooling the sample before application to sucrose gradient containing only 10 mM tris and 1 mM $EDTANa_2$ (Fig. 1).

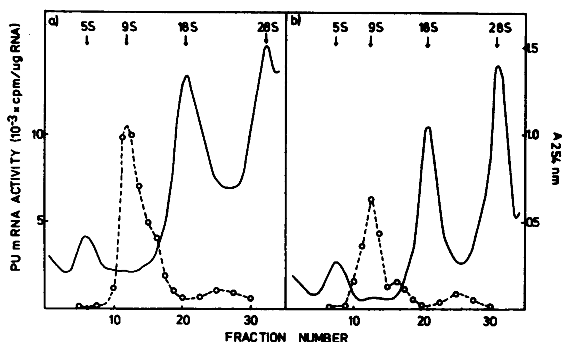

Fig. 3. Fractionation of total polysomal RNA on sucrose gradients. a) One ml of total polysomal RNA (70 A260 units) was applied to a linear sucrose gradient (15-30% sucrose in 10 mM tris/ HCl, pH 7.5 containing 0.1 M NaCl, 0.5% Na dodecylsulfate and 1 mM EDTA) and centrifuged at 25° and 27,000 rpm in the Beckman SW27 rotor. Fractions (30 drops) were collected and the absorbance at 254 nm recorded (solid line). The RNA was precipitated from each fraction and 1μg RNA was used in the wheat germ system for assaying the PUmRNA activity. The values (o---o) represent the radioactivity immunoprecipitated with goat anti-uteroglobin using a previously described double antibody technique (1).

b) Polysomal RNA was treated with 90% formamide, and centrifuged through linear sucrose gradients (5-20% sucrose in 50% formamide), prepared as described by Anderson et al. (19). 25 A260 units were applied to each 35 ml gradient, and centrifugation was carried out at 4° and 27,000 rpm for 40 h in the Beckman SW27 rotor. Fractions were collected and assayed for PUmRNA activity as described in a).

The position of known RNA standards (5s, 18s and 28s ribosomal RNA, as well as 9s globin mRNA from rabbit reticulocytes) are indicated.

The fractions of the gradient containing the peak of PUmRNA activity were pooled, and the RNA was precipitated by ethanol and electrophoresed in 4% polyacrylamide gels containing 0.2% dodecylsulfate (6). The absorbance profile of such a gel at 260 nm shows a main band in the 9s region and some minor bands corresponding to RNAs of lower molecular weight (Fig. 4a). If the pooled fractions from sucrose gradients are bound to oligo (dT)-cellulose and eluted with H_2O before application to polyacrylamide gels, these minor bands disappear (Fig. 4b), indicating that they represent RNA not containing poly(A). This

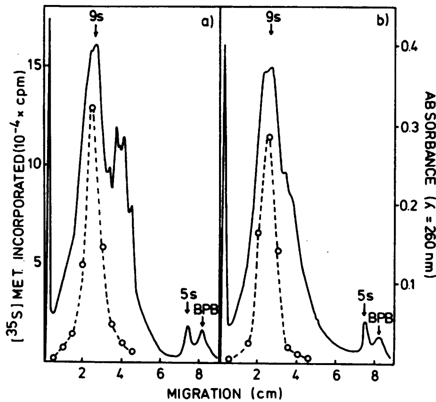

Fig. 4. Polyacrylamide gel electrophoresis of partially purified PUmRNA. The 9s RNA obtained from sucrose gradients was divided into two halves. One half (a) was precipitated with ethanol, washed, and resuspended in water. The other half (b) was brought to 0.5 M NaCl and 0.5% dodecylsulfate and applied to a column containing 0.1 g oligo(dT)-cellulose. After washing the column with 10 mM tris-HCl, pH 7.5, 0.5 M NaCl, the bound RNA was eluted with 0.5 ml water, precipitated with ethanol, washed, and resuspended in water. Aliquots (15 µg) of both RNA fractions were electrophoresed in 4% polyacrylamide gels containing 0.2% dodecylsulfate (6). The gels were scanned at 260 nm and cut into 5 mm slices. The RNA was eluted electrophoretically, bound to oligo(dT)-cellulose, and precipitated with ethanol. The precipitates were washed with 3 M NaOAc, and the RNA (0.1 µg) was translated in the wheat germ system using (35S)methionine as labelled amino acid. The full line represents the absorbance at 260 nm, and the broken line the radioactivity incorporated into protein in each 25 µl assay. The position of globin mRNA (9s) is indicated.

suggestion is confirmed by translation of the different bands in a cell-free system. A single peak of mRNA activity is detected coincident with the main band of absorbance (Fig. 4).

Criteria of purity. The biological purity of the final mRNA preparation was determined by the analysis of its cell-free products immunologically and electrophoretically. The use of (^{35}S)methionine as labelled amino acid leads to an overestimation of the degree of purification due to the relatively high methionine content of uteroglobin (12). Therefore, (^{3}H)-leucine was used as label in the wheat germ system. As can be seen in Figure 5, the only detectable product comigrates with preuteroglobin and is completely removed by incubation with anti-uteroglobin immunoglobulin.

The physical purity of the final mRNA preparation was determined by polyacrylamid gel electrophoresis. Both, in gels containing dodecylsulfate or formamide, a single absorbance band was detected after electrophoresis. In addition, this band contains over 95% of the poly(A)-RNA as demonstrated by hybridi-

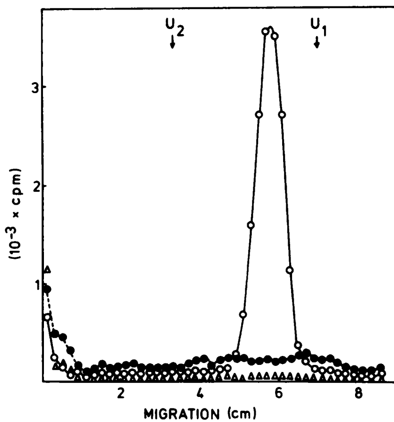

Fig. 5. Gel electrophoresis of the PUmRNA cell-free products labelled with tritiated leucine. Purified PUmRNA (0.4 µg) was translated in a 50 µl wheat germ assay using (3H)leucine as labelled amino acid. The reaction mixture was centrifuged at 105,000 x g for 60 min and the supernatant was divided into two equal halves. One half (o——o) was processed for electrophoresis by alkaline hydrolysis and precipitation with trichloracetic acid (1), and the other half (△△△) was submitted to an immunoprecipitation with goat antiuteroglobin immunoglobuline (5 µg, 4° C, 48 h) and rabbit antigoat immunoglobuline (100 µg, 4°, 24 h), before processing the supernatant for electrophoresis in 12.5% acrylamide gels (1,13). The gels were stained, destained and processed for determination of radioactivity as previously described (1). Native uteroglobin (U2) as well as, reduced and carboxymethylated uteroglobin (U1) were added as internal markers and their positions are indicated by arrows. As a control, the products of a wheat germ assay without added mRNA were also analyzed in similar gels (●---●).

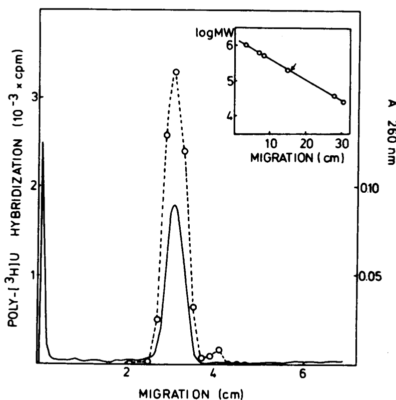

Fig. 6. Electrophoresis of purified PUmRNA on 4% polyacrylamide gels containing formamide. Purified PUmRNA (2 µg) was electrophoresed in 4% polyacrylamide gels, prepared in formamide. The gels were scanned at 270 nm (solid line, right ordinate) and cut into 2 mm slices. The RNA was extracted from the slices and hybridized to (3H)poly-(U). The left ordinate represents the radioactivity in the ribonuclease resistant hybrids (o---o). The inset shows a plot of the electrophoretic mobility versus the logarithm of the molecular weight of RNA standards (26s and 16s rRNA from E. coli, 18s rRNA, globin mRNA and 5s RNA from rabbit reticulocytes, and 4s tRNA from calf liver) run in a parallel gel. The position of the PUmRNA is indicated by an arrow.

zation with (^{3}H)-labelled poly(U) (Fig. 6).

Characterization of the PUmRNA. The electrophoretic mobility of PUmRNA in gels containing 99% formamide can be used to estimate its molecular weight. A comparison with appropriate standards, including purified rabbit globin mRNA yields a value close to 200,000, corresponding to 600 nucleotides (Fig.6,

inset). This value is in good agreement with the sedimentation coefficient of 9s observed in sucrose gradients with or without formamide (Fig. 1 and 2).

The length of the poly(A)segment in PUmRNA and rabbit globin mRNA was determined after digestion with ribonucleases A and T1 by electrophoresis of the resistant material in 10% polyacrylamide gels containing 99% formamide, and hybridization to (^{3}H)labelled poly(U). In agreement with previously published data (17,20) the length of the poly(A) segment of rabbit globin mRNA was found to vary between 30 and 90 nucleotides with a peak at 50 nucleotides (Fig. 7). The results obtained with PUmRNA indicate a length of the poly(A) segment between 40-120 nucleotides, with a peak at 63 nucleotides (Fig. 7).

The presence of a methylated "cap" structure at the 5'end of PUmRNA was investigated by determining the influence of the "cap" analogue m^{7}G(5')ppp(5')A on the translation of PUmRNA in the wheat germ system. m^{7}G(5')ppp(5')A inhibits the synthesis of preuteroglobin at concentrations which have been shown to compete with capped mRNAs for ribosomal binding (21,22,23). GTP has no effect on the cell-free translation of PUmRNA and does not affect the inhibition caused by the "cap" analogue (Fig. 8).

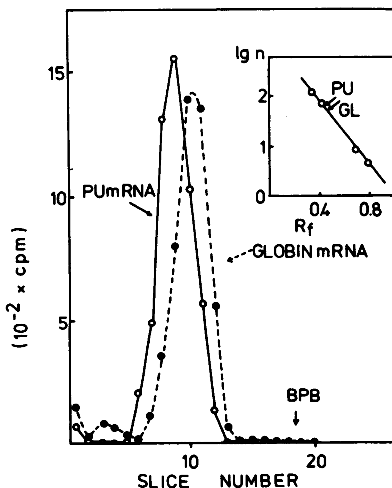

Fig. 7. Determination of the length of the poly(A) segment in PUmRNA. Aliquots (1 µg) of purified PUmRNA (o——o) and rabbit globin mRNA (•---•) were digested with ribonucleases A and T1 and the digests were electrophoresed in 10% polyacrylamide gels made in formamide. The gels were sliced into 4 mm pieces and the RNA was extracted and precipitated with ethanol. The figure represents the ribonuclease resistant cpm after hybridization to (3H)poly(U). The inset shows a plot of the logarithm of the nucleotide number of known standards (5s RNA and tRNA from rabbit reticulocytes, poly(A)9 and poly(A)5, versus their relative electrophoretic migration (Rf). The positions of the peaks obtained with PUmRNA and rabbit globin mRNA are indicated by arrows.

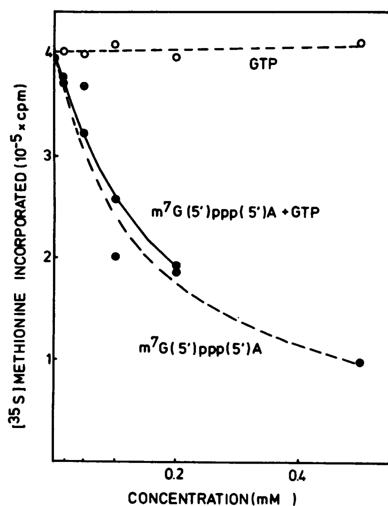

Fig. 8. Influence of GTP and $m^7G(5')ppp(5')A$ on the translation of PUmRNA. Purified PUmRNA (0.2 µg) was translated in the wheat germ system using (35S)methionine as labelled aminoacid. GTP (o---o), the"cap" analogue $m^7G(5')ppp(5')A$ (●---●), or both (●——●), were added to the assay mixtures up to the final concentrations indicated in the abscisa. The ordinate represents the radioactivity incorporated into preuteroglobin in each 25 µl assay.

DISCUSSION

The first question in our attempts to purity the PUmRNA concerned the choice of starting material. Although the use of whole endometrial tissue would probably give better yields, we decided to start with purified polysomes since our main objective was to obtain highly purified PUmRNA. Preliminary experiments had shown that preuteroglobin was synthesized predominantly in the disome-trisome fraction of membrane-bound polysomes (24), but the fractionation procedure is time consuming and poor yields were observed in pilot experiments. We, therefore, decided to start with total endometrial polysomes.

Another important question concerned the purification strategy. Elegant work has been presented by several groups on the isolation of specific mRNAs by immunological methods (as an example see Ref. 25). Unfortunately, this procedure was not successful in our hands. Very little polysomal binding of purified goat (^{125}I)-immunoglobulin against uteroglobin was observed, indicating that the antigenic determinants of the nascent preuteroglobin chains were not available for antibody binding (24).

A remarkable finding of the purification procedure concerns

the influence of the RNA extraction procedure on the subsequent binding of PUmRNA to oligo(dT)-cellulose. After extraction of polysomes with phenol:chloroform at low pH a considerable proportion of the PUmRNA looses its ability to bind to oligo(dT)-cellulose (1). Similar results have been reported by Bullock et al. (18), who employed a pH 9 buffer-phenol mixture (26) for extracting total endometrial tissue. By means of hybridization with (^{3}H)-poly(U) these authors could not detect poly(A) in the flow-through fraction of the oligo(dT)-cellulose column, suggesting that phenol extraction reduces the size of the poly(A) fragment on PUmRNA. The PUmRNA found in the flow-through of oligo(dT)-cellulose after phenolization sediments in sucrose gradients in the 9s region as does the poly(A)-containing PUmRNA.

In respect to the biological purity of PUmRNA it is important to use as radioactive amino acid one which is not unusually frequent in uteroglobin. This is the case for methionine and, therefore, the use of (^{35}S)methionine gives the wrong impression that PUmRNA is over 85% pure after sucrose gradient centrifugation (figure 2).

Purified PUmRNA exhibits a molecular weight of 200,000 in polyacrylamide gels made in formamide, corresponding to a length of some 600 nucleotides. As preuteroglobin is only 90 aminoacids long (1) and the average length of the poly(A)segment in PUmRNA is 60 nucleotides, about 50% of the nucleotides of PUmRNA represent untranslated sequences. Similar findings were originally reported for the globin mRNA (27), and have since been found for most of the eukaryotic mRNAs. Although our evidence in this respect is indirect and based on inhibition of translation by "cap" analogues, PUmRNA seems to contain a modified nucleotide at the 5'-end, a structure also found in the majority of the eukaryotic mRNAs studied till now.

After completion of this manuscript a paper by Atger and Milgrom (28) was published which confirms some of our previous findings (1), and is in basic agreement with the data present here.

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