Identification and Isolation of Protamine Messenger Ribonucleoprotein Particles from Rainbow Trout Testis†

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ABSTRACT: Treatment of rainbow trout testis polyribosomes with ethylenediaminetetraacetic acid released polyadenylated protamine messenger RNA in the form of a ribonucleoprotein (mRNP) particle. This mRNP particle which sedimented at 12–14 S could be identified by hybridization to [^{3}H]poly(U) and was partially purified by two successive sucrose gradient sedimentations. When RNA was extracted from the mRNP particle and used as a template in the wheat germ cell-free protein synthesizing system the sole product of translation was protamine. Of this RNA, 30% contained poly(A) sequences and was shown to comigrate with polyadenylated protamine messenger RNA during polyacrylamide gel electrophoresis. When the proteins of the mRNP particle were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the two prominent polypeptides with apparent molecular weights of 73 000 and 29 000 appeared reproducibly. Treatment of trout testis polyribosomes with puromycin in the presence of 0.5 M KCl released a smaller (8–10S) mRNP particle which was similarly shown to contain protamine messenger RNA. Trout testis postribosomal supernatant fraction possessed 16–18S mRNP particles containing polyadenylated RNA which cosedimented with protamine messenger RNA when the particles were dissociated with sodium dodecyl sulfate.

In eukaryotic cells, mRNA appears to be associated with specific proteins to form cytoplasmic messenger ribonucleoprotein (mRNP)[1] particles. Such particles have been observed for a population of mRNAs in extracts of L cells (Perry and Kelly, 1968), silk gland cells of *Bombyx mori* (Kafatos, 1968), kidney (Irwin et al., 1975), liver (Henshaw and Lowenstein, 1970), HeLa cells (Spohr et al., 1970), and chick embryo brain (Bryan and Hayashi, 1973). This observation has been extended to specific mRNAs such as globin mRNA in rabbit reticulocytes (Lebleu et al., 1971; Blobel, 1972; Jacobs-Lorena and Baglioni, 1972), in duck erythroblasts (Morel et al., 1971, 1973), histone mRNA in unfertilized sea urchin eggs (Gross et al., 1973), crystallin mRNAs in calf lens (Chen et al., 1976), and actin mRNA in embryonic chick muscle (Bag and Sarkar, 1975). The observation also holds for mRNAs occurring in the postribosomal supernatant fraction of the cytoplasm (Gander et al., 1973; Bag and Sarkar, 1975; Jacobs-Lorena and Baglioni, 1972; Gross et al., 1973), as well as for those particles released from polysomes by EDTA dissociation or puromycin–0.5 M KCl treatment (Lebleu et al., 1971; Blobel, 1971a, 1972).

The proteins found in the mRNP particles are of interest because of the role they might play in regulating the transport and storage (Spohr et al., 1970) or translation (Schochetman and Perry, 1972; Hellerman and Shafritz, 1975) of eukaryotic mRNAs. The properties of mRNP particles from a variety of tissues and the molecular weights of the associated proteins have been reviewed (Irwin et al., 1975).

Previous investigations have demonstrated that protamine mRNA can be readily isolated from the polysomal and postribosomal supernatant fractions of rainbow trout testis (Gedamu and Dixon, 1976a,b) and translated in cell-free systems derived from Krebs II ascites cells and wheat germ.

In this study, we have examined the form in which protamine mRNA occurs in the polysomes and postribosomal supernatant fractions of trout testis. Evidence is presented that protamine mRNA occurs in these fractions in the form of mRNP particles and not as free mRNA. Several properties of this mRNA, such as its small size (6 S), nuclease resistance (Dixon et al., 1976), and ease of characterization (Gedamu and Dixon, 1976b), facilitate the study of its mRNP particles. In addition, protamine mRNA is the predominant messenger RNA present in maturing trout testis, which allows the mRNP particle to be readily identified and isolated in good yield. Preliminary evidence is available (Iatrou and Dixon, 1977) that protamine mRNA sequences are present in the cell at an early stage of spermatogenesis, whereas its translational product, protamine, does not appear until considerably later, at the spermatid stage of development. The expression of the protamine mRNA in trout testis is thus a promising system to study the storage and/or control of a eukaryotic mRNA.

Materials and Methods

Oligo(dT)-cellulose (T_3) was purchased from Collaborative Research, Inc. RNase A was from Worthington Biochemical Corp.; puromycin hydrochloride was from Calbiochem; poly(A) was from P-L Biochemicals Inc. Human γ-globulin, sperm whale myoglobin, bovine serum albumin, and ribonuclease-free sucrose were obtained from Schwartz/Mann. [^{14}C]Arginine of specific activity 336 mCi/mmol came from New England Nuclear. [^{3}H]Poly(U) of specific activity 54.2 μCi/μmol P_i came from Miles Laboratories, Inc. Rainbow trout testis were collected from Dantrout, Brande, Denmark, in early October and after freezing on dry ice were stored at −80 °C. Fresh testes were obtainable throughout the year by the method of Schmidt et al. (1965). Protamine messenger RNA was prepared as described by Gedamu and Dixon (1976b). Non-idet P40 was a gift from Shell Research Ltd., Sittingbourne, Kent, U.K.

Fractionation of Trout Testis. Trout testis was fractionated

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[1] Abbreviations used: mRNP, messenger ribonucleoprotein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate.

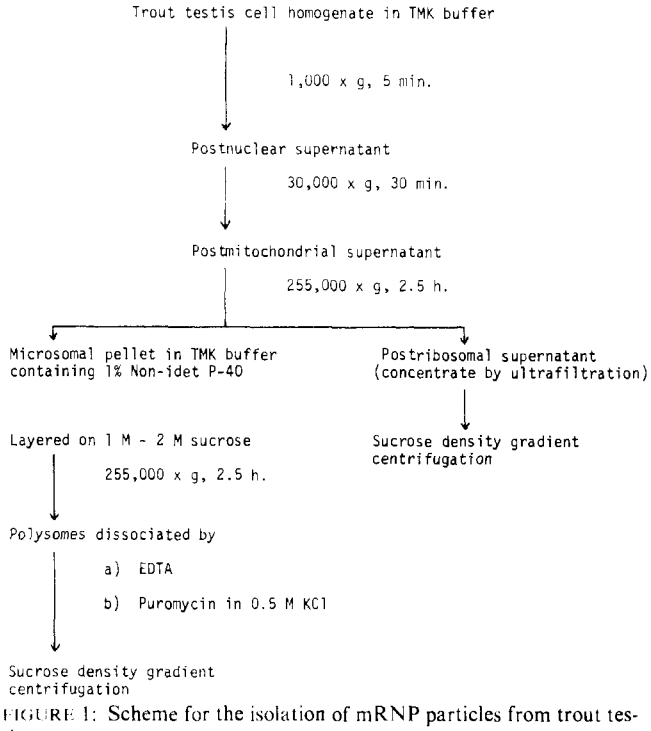

FIGURE 1: Scheme for the isolation of mRNP particles from trout testis.

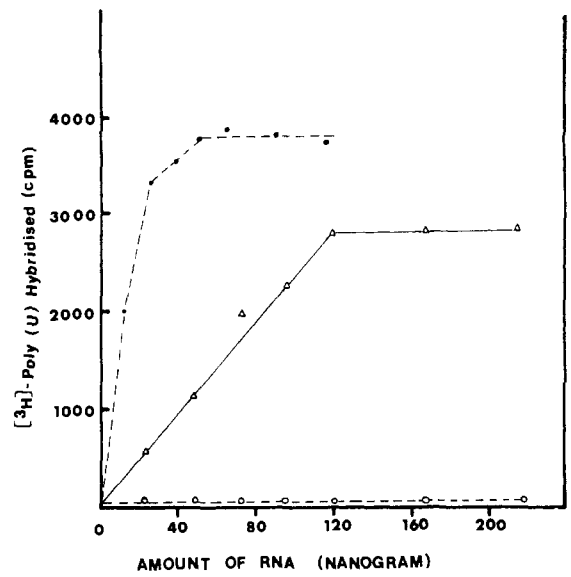

FIGURE 2: Assay for poly(A) tracts by hybridization with [^{3}H]poly(U). The assay was performed according to the method of Jeffery and Brawerman (1974), except that the final volume was decreased to 200 μL. Each assay contained 0.16 μg of [^{3}H]poly(U) of specific activity 54.2 μCi/μmol of P_i. Synthetic poly(A) (●- - -●), purified protamine mRNA (△—△), and wheat germ tRNA (○- - -○) were assayed at increasing concentrations. The weight of RNA per sample was estimated from absorbance readings, assuming 1 A_{260} unit is equivalent to 40 μg of RNA.

for the isolation of mRNP particles according to the scheme shown in Figure 1. All procedures were carried out at 0–4 °C unless otherwise indicated.

Frozen trout testis (200 g) was thawed in TMK buffer (400 mL) containing 40 mM Tris-HCl (pH 7.5), 25 mM KCl, and 5 mM magnesium acetate. After scissor-mincing the testes, the resultant suspension was homogenized by a motor-driven Teflon pestle in a glass homogenizer (Tri-R Instruments). The homogenate was filtered through two layers of cheesecloth and centrifuged at 1000*g* for 5 min to remove nuclear material, and at 30 000*g* to collect the postmitochondrial supernatant. This supernatant was further separated into microsomal pellets and postribosomal supernatant by centrifugation in a Beckman 60 Ti rotor at 255 000*g* for 2.5 h. The microsomal pellets were resuspended by homogenization in TMK buffer (120 mL) containing 1% Non-idet P-40. This suspension was layered on a double cushion of 1 M sucrose in TMK buffer (5 mL) over 2 M sucrose in TMK (8 mL) and was centrifuged at 255 000*g* for 2.5 h as above. The polysomal pellets obtained from this step were washed gently with TMK buffer.

Release of mRNP Particles from Polyribosomes. Trout testis polysomes were dissociated to release mRNP particles by one of two methods. In the first method polysomes from 100 g of trout testis were suspended in buffer (12 mL) containing 10 mM Tris-HCl (pH 7.5), 30 mM KCl, and 30 mM EDTA (pH 7.5). The preparation was then centrifuged at 12 000*g* for 10 min to pellet material which was not in suspension. In the second method, polysomes from 200 g of trout testis were first salt-washed by suspension in 24 mL of 0.5 M KCl containing 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and 1 mM dithiothreitol followed by sedimentation of the polysomes at 255 000*g* for 2.5 h through a 12-mL cushion of 30% sucrose made up in the same buffer. The salt-washed polysomes were resuspended in this buffer (20 mL) containing 1 mM puromycin hydrochloride (final pH 7.0). The suspension was incubated at 37 °C for 10 min before being centrifuged at 12 000*g* for 10 min to remove insoluble material.

Isolation of mRNP Particles. Dissociated polysomes were fractionated by sucrose gradient sedimentation. Aliquots (4 mL) of EDTA-dissociated polysomal suspensions were layered onto linear 15–40% sucrose gradients (34 mL) which contained 10 mM Tris-HCl (pH 7.5), 30 mM KCl, and 1 mM EDTA. The gradients were centrifuged for 48 h in a Beckman SW 27 rotor at 25 000 rpm. After centrifugation the gradients were emptied from the bottom of the tubes and collected into fractions or regions on the basis of the absorbance profile read at 260 nm. These fractions were analyzed for protamine mRNP particles. Small aliquots (1 mL) of the dissociated polysomal suspensions were fractionated on linear 15–35% sucrose gradients (11.4 mL) containing 10 mM Tris-HCl (pH 7.5), 30 mM KCl, and 1 mM EDTA. These smaller gradients were centrifuged for 24 h in a Beckman SW 41 rotor at 39 000 rpm and were analyzed as detailed above. Total RNA from trout testis, containing 28S, 18S, and 4S RNAs was used as a reference throughout these sedimentations. Postribosomal supernatant was fractionated in the same manner either after a fivefold concentration by ultrafiltration through an Amicon PM 10 membrane or without prior concentration. Gradient fractions which were rich in protamine mRNP particles were combined and dialyzed against 10 mM Tris-HCl (pH 7.5) to remove sucrose. The dialyzed material was concentrated by ultrafiltration and then reapplied to sucrose density gradients as described above. Puromycin-dissociated polysomal suspensions were fractionated on 11.4- or 34-mL gradients as described above, except that the gradients were prepared in 50 mM Tris-HCl (pH 7.5), 500 mM KCl, and 2 mM $MgCl_2$.

Poly(U) Hybridization Assay. Poly(A)-containing material, including protamine mRNP particles, was detected in the gradients by a poly(U) hybridization assay based on that described by Jeffery and Brawerman (1974). The [^{3}H]poly(U) used in the assay had a specific activity of 54 μCi/μmol of P_i and was present in excess over the hybridizable poly(A) in the sample. The stoichiometry of the assay (Figure 2) performed in a volume of 200 μL was assessed by hybridizing [^{3}H]-

poly(U) against increasing amounts of pure protamine messenger RNA and synthetic poly(A). Blank assays were performed on wheat germ tRNA and gave no precipitable counts above background (no sample added).

Extraction and Characterization of RNA. Regions from the sucrose density gradients, made 0.2 M in ammonium acetate, were precipitated overnight at −40 °C by the addition of 2 volumes of 95% ethanol. The precipitated material was recovered by centrifugation at 30 000*g* for 30 min and was then dissolved at 22 °C in 100 mM Tris-HCl (pH 9.0), 100 mM NaCl, 1 mM EDTA containing 1% sodium dodecyl sulfate. This solution was twice extracted with equal volumes of phenol–chloroform–isoamyl alcohol (50:50:1, v/v) as described by Gedamu and Dixon (1976b) for the preparation of protamine mRNA from trout testis polysomes. The RNA in the aqueous phase was ethanol precipitated as above. The resultant pellet was washed gently with 65% ethanol, dried in vacuo, and resuspended in water to measure absorbance at 260 nm. The extracted RNA was translated in a wheat germ cell-free system as described by Gedamu and Dixon (1976b). The activity of this RNA in directing [^{14}C]arginine incorporation into hot trichloroacetic acid–tungstate precipitable material was measured relative to the activity of highly purified protamine messenger RNA. The isolation of the cell-free products and their characterization by starch gel electrophoresis and polyacrylamide slab gel electrophoresis of the extracted RNA in 98% formamide were performed as described by Gedamu and Dixon (1976a,b). The extracted RNA was chromatographed on oligo(dT)-cellulose columns (3 mL bed volume) according to the procedure of Aviv and Leder (1972), as modified by Gedamu and Dixon (1976b). The poly(A)-containing RNA fraction was eluted from the column with water.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Proteins were analyzed by cylindrical polyacrylamide gel electrophoresis in a discontinuous system at pH 8.9 as described by Gabriel (1971) with the modification that sodium dodecyl sulfate was included in all solutions at a concentration of 0.1%. The acrylamide concentration in the separating gel (6.5 cm) was 10%. Samples were dissolved in Tris-glycine running buffer containing 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 15% glycerol and were heated to 100 °C for 2 min before being applied to the gel. Electrophoresis was performed at 2 mA per tube until the bromophenol blue tracking dye reached the bottom of the gel. The gels were stained overnight in 0.25% Coomassie brilliant blue, 10% acetic acid, and 45% methanol and were destained in 10% acetic acid and 45% methanol at 60 °C.

Results

The poly(U) hybridization assay of Jeffery and Brawerman allows the detection of small quantities of poly(A)-containing material. When the assay was performed on polyadenylated protamine mRNA (Figure 2), there was a linear relationship between the amount of mRNA added and the extent of [^{3}H]poly(U) hybridization up to a saturating value of 120 ng of protamine mRNA. As little as 10 ng of protamine mRNA was readily detectable. Synthetic poly(A) saturated the system at lower values (30–50 ng) and wheat germ tRNA did not hybridize at all.

Using this assay sucrose density gradient separations of trout testis postribosomal supernatant and EDTA-dissociated polysomes were analyzed for poly(A)-containing material (Figure 3). These two cell fractions from fresh trout testis each contained material which hybridized to labeled poly(U). In

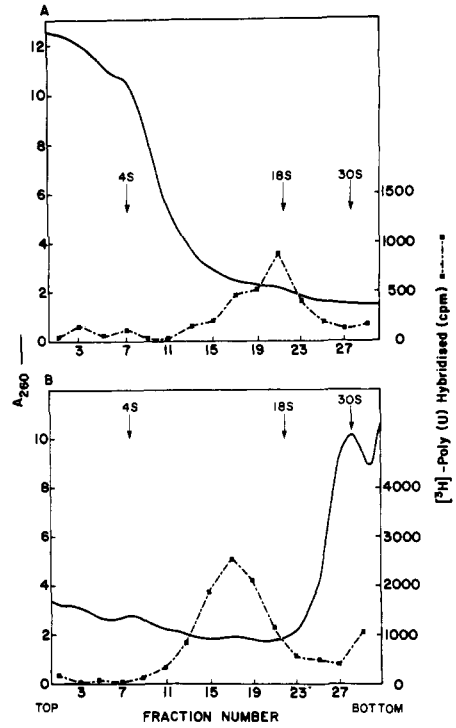

FIGURE 3: Comparison of polysomal and postribosomal protamine mRNP particles from trout testis on sucrose gradients. Polysomal and postribosomal supernatant fractions were prepared from fresh trout testis (17 g) as described in Materials and Methods. One-eighth (4 mL) of the postribosomal supernatant fraction (A) and all (80 A_{260}) of the EDTA-dissociated polysomal fraction (B) were fractionated on 34-mL 15–35% linear sucrose density gradients (see Materials and Methods). Gradients were centrifuged at 25 000 rpm for 48 h in a SW 27 rotor. Fractions (1.2 mL) were collected and read at 260 nm (—), and every second fraction was assayed for poly(A) content by [^{3}H]poly(U) hybridization (■- - -■). The amounts of fractions assayed were: (A) 100 μL and (B) 25 μL. A blank of 100 cpm, representing [^{3}H]poly(U), incompletely degraded by pancreatic RNase in the absence of added mRNP, was subtracted from each value.

the postribosomal supernatant, this hybridizable material showed a broad distribution, centered, with reference to 4S and ribosomal RNA markers, around 16–18 S, while the bulk of the material which absorbed at 260 nm sedimented in the 4S region. The EDTA-dissociated polysomes contained poly(U) hybridizable material which had a lower sedimentation value of 12–14 S but again showed a broad distribution. This profile also showed a prominent peak of 260-nm-absorbing material at 30 S corresponding to the small ribosomal subunit dissociated by EDTA.

Gedamu and Dixon (1976b) have shown that protamine mRNA, in the absence of protein, sediments on sucrose density gradients with a peak at 6 S and a shoulder on the low molecular weight side. Such profiles, obtained by scanning the gradient at 260 nm, were very similar to the one shown in Figure 4A, which was obtained by assaying fractions for poly(U) hybridization.

From the evidence of Figure 3 neither the postribosomal supernatant nor the EDTA-dissociated polysomes contained free protamine mRNA sedimenting at 6 S. However, when an aliquot of postribosomal supernatant was treated with 1% sodium dodecyl sulfate and then sedimented through a sucrose density gradient, there was a shift of the poly(A)-containing material from 16–18 S toward the top of the gradient (Figure 4B). The new profile showed a major peak similar to protamine mRNA in its shape and sedimentation velocity, together with a smaller peak on the high molecular weight side. This higher

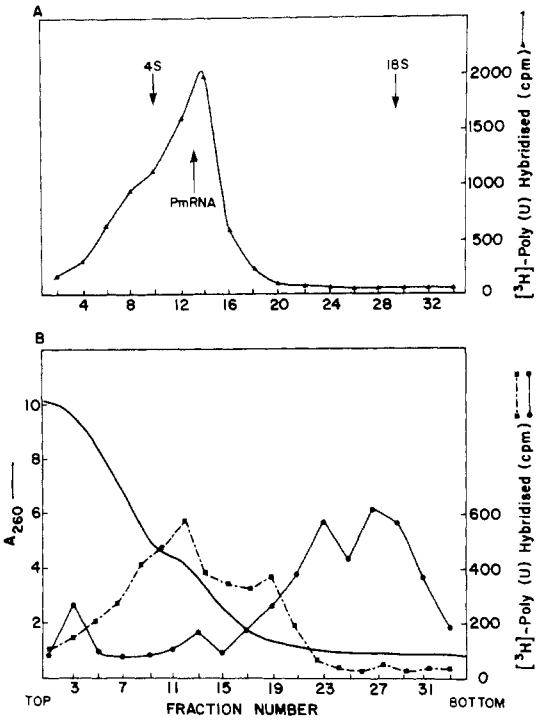

FIGURE 4: Comparison of the size difference of protamine mRNP particles from postribosomal supernatant fraction of trout testis with protamine mRNA. The following samples were applied on an 11-mL 15–35% linear sucrose density gradient and centrifuged at 39 000 rpm for 24 h in a SW 41 rotor. (A) PolyA(+) protamine mRNA marker (5 μg) purified as described by Gedamu and Dixon (1976b). (B) Postribosomal supernatant fraction (1 mL) without sodium dodecyl sulfate treatment (●- - -●) and incubated with 1% sodium dodecyl sulfate at 37 °C for 3 min to release proteins from mRNP (■- · · · · ·■). Fractions (0.36 mL) were collected, optical density at 260 nm (—) was read, and aliquots were assayed for poly(A) content by the [^{3}H]poly(U) hybridization technique. Twenty-five microliters (A) and 100 μL (B) from every second fraction were used. The arrows indicate positions of trout testis RNA markers.

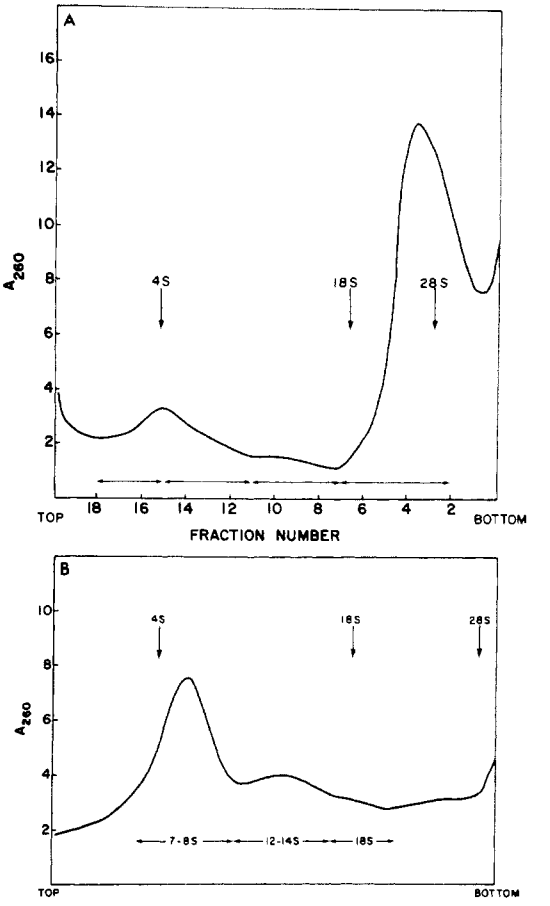

FIGURE 5: Purification of protamine mRNP particles from EDTA-dissociated trout testis polysomes on sucrose density gradients. In profile A, polysomes from frozen trout testis (33 g) were EDTA-dissociated (see Materials and Methods) and then fractionated on a linear 15–40% sucrose gradient as described for the isolation of mRNP particles in Materials and Methods. Fractions (2 mL) were collected at the end of the run and were later combined for RNA extraction and further purification as indicated by the arrows. Fractions 8–11 inclusive from three such gradients were combined, dialyzed, and concentrated by ultrafiltration to a volume of 4 mL (see Materials and Methods). This sample was then applied to a 34-mL linear 15–35% sucrose gradient and centrifuged in a Beckman SW 27 rotor for 48 h at 25 000 rpm as shown in profile B. The regions indicated were combined for RNA extraction. The solid line in each profile indicates absorbance at 260 nm. The positions of RNA markers sedimented under identical conditions are shown by arrows.

molecular weight RNA could have arisen from mRNPs other than that for protamine.

More direct evidence for the existence of protamine mRNA in an mRNP particle was obtained after extracting RNA from regions of the sucrose density gradient separation of EDTA-dissociated polysomes (Figure 5A). RNA extracts from four regions of the gradient were tested in the wheat germ cell-free system for the incorporation of [^{14}C]arginine into trichloroacetic acid-tungstate precipitable material. The specific activities of the four fractions in terms of cpm incorporated per μg of RNA are shown in Table I and compared with that of pure protamine mRNA. The most active fraction came from tubes 8–11 inclusive in Figure 5A. This 12–14S region is equivalent to the region of poly(A)-containing material shown in Figure 3B. The nature of the messenger RNA activity in this region was investigated by analyzing the translation products extracted with 0.2 M sulfuric acid, which also contained some free arginine (Gedamu and Dixon, 1976a,b), on starch gel electrophoresis. In the absence of RNA, one peak of radioactivity (thick line) was observed which migrated in the same region as free arginine (Figure 6D). In the presence of the RNA, there were two peaks of radioactivity. The faster moving peak comigrated with free arginine and the slower with carrier trout testis protamine (Figure 6D, dotted line). Free arginine did not appear as a band in the autoradiograph of the starch gel because the prior staining and destaining of the gel allowed the amino acid to diffuse away (Figure 6A, B). We have observed that there is slight proteolytic activity in the wheat germ extract and, in Figure 6, the presence of a shoulder of [^{14}C]-arginine-labeled material migrating slightly faster than the major protamine peak was observed on the autoradiograph and after quantitating the labeled gel slices. In this gel system, fragments of labeled protamine would be expected to migrate in this region. On the basis of the cell-free assay, the RNA in tubes 8–11 (Figure 5A) contained 25% protamine mRNA. The purification of this polysomal protamine mRNP particle was taken a stage further in order to investigate the nature of the proteins associated with the mRNA in the 12–14S particle. The most active regions (tubes 8–11 in Figure 5A) from several sucrose density gradients were combined, dialyzed against 10 mM Tris-HCl to remove sucrose, concentrated by ultrafiltration, and then fractionated on a second, 15–35% sucrose density gradient. The profile of absorbance at 260 nm in this second gradient showed a major peak at 7–8 S comprising approximately 80% of the total A_{260} units (Figure 7). A smaller, broader peak was seen in the 12–14S region. When fractions through this gradient were assayed for poly(A) content, the peak of poly(U)-hybridizable material corre-

TABLE I: Stimulation of [^{14}C]Arginine Incorporation by RNA Fractions Isolated from mRNP Particles (Figure 5A) in a Wheat Germ Cell-Free System.[a]

Source of RNA	RNA used (μg)	[^{14}C]Arginine Incorp (cpm/40 μL reaction)	Sp Act. (cpm/μg)
Minus RNA		1465	
Total RNA from pooled tubes 2–7	31.0	1856	59.9
Total RNA from pooled tubes 8–11	6.2	12494	2015.2
Total RNA from pooled tubes 12–15	25.2	10136	402.2
Total RNA from pooled tubes 16–18	27.0	2650	98.2
Protamine mRNA	0.90	7177	7974.4

[a] RNA fractions were extracted from the regions shown in Figure 5A (see Materials and Methods) and assays performed in a 50-μL reaction mixture as described by Gedamu and Dixon (1976b). Purified protamine mRNA was used as a standard. Concentration curves were carried out in each case and the specific activity was calculated from the linear range. Tubes 2–7 correspond to 18–30S material, tubes 8–11 to 11–18S material, tubes 12–15 to 4–11S material, and tubes 16–18 contain material sedimenting at less than 4 S.

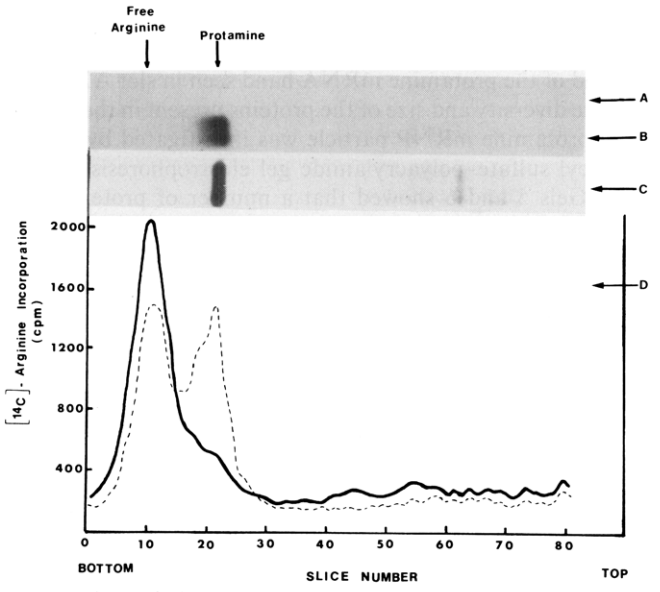

FIGURE 6: Analysis by starch gel electrophoresis of the polypeptides synthesized in the wheat germ cell-free system in the presence of protamine mRNA isolated from the 12–14S region (Figure 5B). Reaction mixtures (250 μL) containing 125 μL of preincubated wheat germ cell-free extract were incubated as described previously (Gedamu and Dixon, 1976b), with 50 μg of RNA extracted from the 12–14S protamine mRNP particle. Trichloroacetic acid–tungstate precipitable radioactivity was estimated in 20-μL aliquots and acid-soluble peptides were extracted from the remainder (Gedamu and Dixon, 1976a). Dried samples were resuspended in 100 μL of 0.1 N HCl and 50 μL of this solution was applied to a starch gel. Electrophoresis was performed and the gel was bisected horizontally (for details, see Gedamu and Dixon, 1976a). The lower slab was stained, destained, and then photographed. The gel was then dried under suction onto a filter paper and was exposed for approximately 30 days to a Kodirex x-ray film. The upper slab was cut into 2-mm slices and radioactivity determined. (A) Indicates autoradiograph of the gel with no RNA added. (B) Shows autoradiograph of the product synthesized in the presence of RNA. (C) Photograph of the destained gel corresponding to that autoradiographed in B. The major, stained band was due to protamine added as a carrier during the extraction of the labeled translation product(s). (D) Radioactivity in gel slices: (—) no RNA added; (- - -) in the presence of RNA.

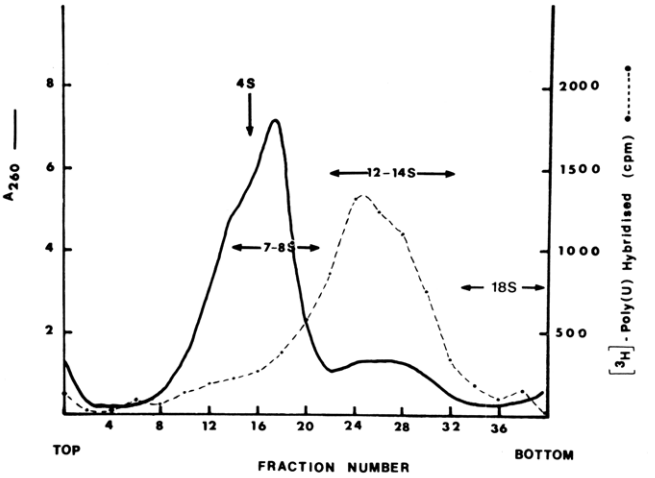

FIGURE 7: Association of poly(A) tracts with protamine mRNP particles. An analytical sample (1 mL) similar to that applied to the gradient in Figure 5B was fractionated on an 11.4-mL linear 15–35% sucrose gradient (see Materials and Methods). Fractions (0.3 mL) were collected after monitoring the absorbance at 260 nm (—) and were assayed for poly(A) content by [^{3}H]poly(U) hybridization (- - -).

TABLE II: Stimulation of [^{14}C]Arginine Incorporation by RNA Fractions Isolated from mRNP Particles (Figure 5B) in the Wheat Germ Cell-Free System.[a]

Source of RNA	RNA added (μg)	[^{14}C]Arginine Incorp (cpm/40 μL reaction)	Sp Act. (cpm/μg)
Total RNA from 7–8S region	4.16	670	161.1
Total RNA from 12–14S region	1.25	2894	2315.2
Total RNA from 18S region	3.9	504	129.2
Protamine mRNA	0.9	4832	5368.9

[a] RNA fractions were extracted from the regions indicated in Figure 5B and assayed in the wheat germ cell-free system (50 μL) as described previously in the legend to Table I (Gedamu and Dixon, 1976b). Incorporation due to endogenous protein synthesis (which averaged 2898 cpm) was subtracted.

sponded to the smaller 12–14S A_{260} peak. Fractions from this gradient were combined into three regions as shown in Figure 7. These regions, 7–8 S, 12–14 S, and 18 S corresponded to the regions combined in Figure 5B. RNA was extracted from aliquots of each region and assayed in the wheat germ cell-free system (Table II). Nearly all of the protamine mRNA activity was found in the region centered around 12–14 S. Analysis of the [^{14}C]arginine-labeled product by starch gel electrophoresis (Figure 6) confirmed that it was authentic protamine. The RNA from the 12–14S mRNP particle was 43% as active as pure protamine mRNA.

This RNA was further characterized by passing 3.6 A_{260} units through an oligo(dT)-cellulose column of bed volume 3 mL. Figure 8 shows that 70% of the RNA was unabsorbed in 0.5 M KCl while 30% was bound to the oligo(dT)-cellulose and remained so after the KCl concentration was decreased to 0.1 M. The adsorbed RNA was eluted with distilled water. This fraction and the pass-through fraction in 0.5 M KCl were each desalted by passage through Sephadex G-25 and were then assayed in the wheat germ cell-free system. Table III shows that the fraction of RNA which bound to oligo (dT)-cellulose

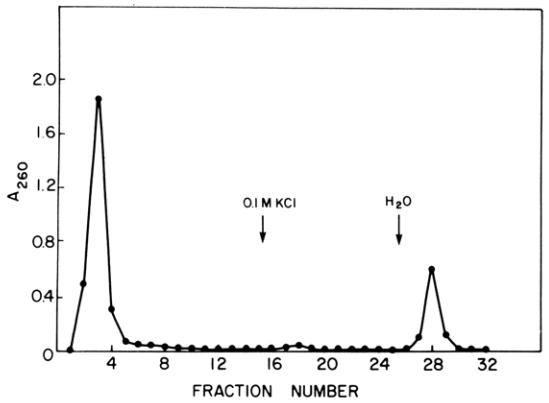

FIGURE 8: Oligo(dT)-cellulose chromatography of RNA extracted from the polysome mRNP particles. An aliquot (3.6 A_{260} units) of the RNA extracted from the 12–14S region shown in Figure 5B was fractionated on an oligo(dT)-cellulose column of bed volume 3 mL. The column was equilibrated with 0.5 M KCl in 10 mM Tris-HCl (pH 7.5) before application of the sample (270 μL) in the same solution. The column was then washed with a further 15 mL of this solution before elution with 0.1 M KCl in 10 mM Tris-HCl (pH 7.5) (10 mL) and finally with water. Fractions (1 mL) were collected at a flow rate of 0.5 mL/min and their absorbance at 260 nm was recorded.

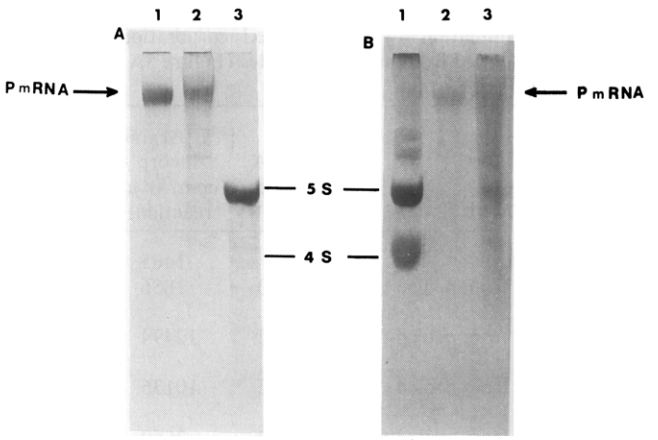

FIGURE 9: Formamide gel electrophoresis of RNA. The following RNA samples were analyzed in a 10% slab polyacrylamide gel containing 98% formamide as described previously (Gedamu and Dixon, 1976b). (A) Slot 1, poly A(+) protamine mRNA purified by oligo(dT)-cellulose chromatography and sucrose gradient centrifugation (10 μg); slot 2, RNA extracted from the 12–14S region (Figure 5B) of the EDTA-dissociated polysomes (18 μg); slot 3, RNA extracted from the 7–8S region (Figure 5B) of the EDTA-dissociated polysomes (20 μg). (B) Slot 1, RNA extracted from samples in tubes 10–15 (Figure 11) of the puromycin dissociated polysomes (44 μg); slot 2, RNA (9 μg) extracted from the 12–14S region which was bound to oligo(dT)-cellulose in high salt (Figure 8); slot 3, unbound RNA fraction (27 μg) from oligo(dT)-cellulose of Figure 8.

TABLE III: Comparison of the Activity of RNA Isolated from the 12–14S Region (Figure 5B) after Oligo(dT)-Cellulose Chromatography with Purified Protamine mRNA in a Wheat Germ Cell-Free System.[a]

Source of RNA	Amount of RNA added (μg)	[^{14}C]Arginine Incorp (cpm/40 μL reaction)	Sp Act. (cpm/μg)
Bound fraction	1.5	9914	6609.3
Unbound fraction	6.0	4271	711.8
Poly A$^{(+)}$ protamine mRNA	0.9	6399	7110

[a] The RNA extracted from the 12–14S region of the sucrose gradient (Figure 5B) was chromatographed on oligo(DT)-cellulose. The bound (containing poly(A)) and the unbound (devoid of poly(A)) fractions were assayed in the wheat germ system. [^{14}C]Arginine incorporated into acid precipitable counts (2715 cpm) due to the endogenous RNA was subtracted.

was almost as active as the pure protamine mRNA standard. The pass-through fraction was only 10% active in relation to the standard.

The RNA extracted from the polysomal 12–14S mRNP particle was also characterized by polyacrylamide gel electrophoresis run under denaturing conditions (Figure 9). Pure protamine mRNA was applied in slot A1. RNA extracted from the 12–14S region shown in Figure 5B appears alongside in slot A2. Protamine mRNA is clearly a major component in this RNA extract. Slot A3 contained RNA extracted from the 7–8S region in Figure 5B. The major RNA species in this region was 5S RNA. Slot B1 contained RNA extracted from the puromycin–0.5 M KCl-released mRNP particle (Figure 11). The major species in this fraction was also 5S RNA with substantial contamination of 4S RNA. Protamine mRNA appeared as a minor band. Slots B2 and B3 contained RNA from the 12–14S region in Figure 5B after fractionation on oligo(dT)-cellulose. The polyadenylated RNA from this region which was eluted from the oligo(dT)-cellulose column by distilled water (Figure 8) appears in slot B2 to be almost entirely protamine mRNA. This observation is supported by the translation data in Table III. The RNA which passed through the oligo(dT)-cellulose column in 0.5 M KCl (slot B3) was devoid of the protamine mRNA band seen in slot A2.

The diversity and size of the proteins present in the polysomal protamine mRNP particle was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 10). Gels 5 and 6 showed that a number of proteins were present in the region of the 12–14S mRNP particle after the second sucrose gradient. Two predominant polypeptides which were consistently observed in this fraction had molecular weights of 73 000 and 29 000. For comparison the 7–8S region of Figure 5B also contained many minor protein species (gel 2) but the major polypeptide in this fraction had a molecular weight of 34 000.

Treatment of trout testis polysomes with puromycin in 0.5 M KCl released poly(A)-containing material which sedimented at 8–10 S (Figure 11); a region of the gradient where there was very little material absorbing at 260 nm. RNA extracted from these regions was translated in the wheat germ cell-free assay. The region most active in [^{14}C]arginine incorporation comprised fractions 10–15 inclusive (Table IV) which corresponded to the region of the poly(U) hybridization peak.

Discussion

Protamine mRNA is found in both the polysomes and the postribosomal supernatant of trout testis (Gedamu and Dixon, 1976a,b). The form in which polyadenylated protamine mRNA exists in these two fractions was examined initially using the poly(U) hybridization assay of Jeffery and Brawerman (1974). This analysis showed that free protamine mRNA did not occur in these fractions since poly(A)-containing material was absent from the 6S region after sucrose gradient sedimentation. Instead, as with other eukaryotic mRNAs studied (Irwin et al., 1975), the mRNA was present in the form of mRNP particles. Hellerman and Shafritz (1975) have used the poly(U) hybridization assay to locate globin mRNP particles released from reticulocyte polysomes on su-

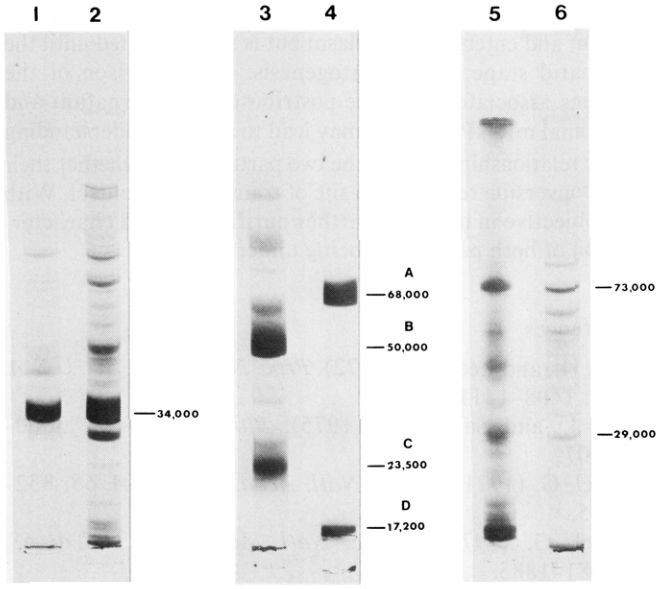

FIGURE 10: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of mRNP proteins. Gels were electrophoresed, stained, and destained as described in Materials and Methods. Molecular weight markers were applied to gels 3 and 4. Human γ-globulin (20 μg) gave heavy- and light-chain markers (50 000 daltons and 23 500 daltons respectively) on gel 3. Bovine serum albumin (68 000 daltons) (15 μg) and myoglobin (17 200 daltons) (15 μg) were the markers applied to gel 4. Samples 5 and 6 were of EDTA-dissociated polysomal mRNP particles from two separate preparations corresponding to the 12–14S region in Figure 5B. Sample 2 was an aliquot of the 7–8S region in Figure 5B. The major component of this region, a complex between 5S ribosomal RNA and a 34 000 dalton protein, was further purified by gel filtration and run as sample 1.

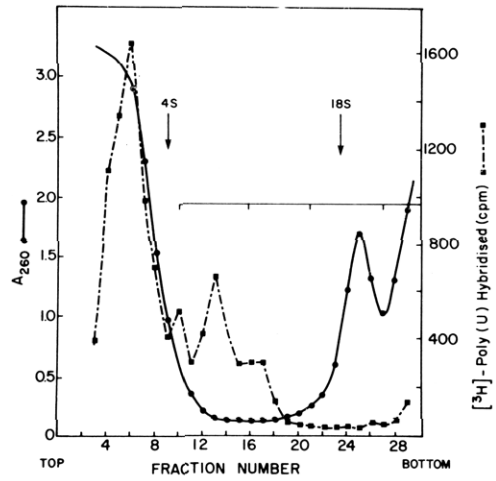

FIGURE 11: Fractionation of protamine mRNP particles from puromycin-dissociated trout testis polysomes on a sucrose density gradient. Polysomes from frozen trout testis (33 g) which had been salt washed and dissociated by puromycin (see Materials and Methods) were fractionated on a linear 15–40% sucrose gradient made up in 50 mM Tris-HCl (pH 7.5), 500 mM KCl, and 2 mM $MgCl_2$ as described in Materials and Methods for the isolation of mRNP particles. Fractions (1.3 mL) were collected, read at 260 nm (●—●), and assayed for poly(A) content by [^{3}H]poly(U) hybridization (■- - -■). The sedimentation positions of 4S and 18S RNAs are shown by arrows. Fractions between bars were combined for RNA extraction.

TABLE IV: Activity in a Wheat Germ Cell-Free System of RNA Fractions Isolated from Regions after Puromycin Treatment of Polysomes.[a]

Source of RNA	Amount of RNA Added (μg)	[^{14}C]Arginine Incorp (cpm/assay)	Sp Act. (cpm/μg)
RNA from pooled tubes 10–15	2.20	1755	797.7
	5.50	4342	790.4
RNA from pooled tubes 16–21	2.52	906	359.5
	4.20	1006	239.5
RNA from pooled tubes 22–27	4.10	207	50.5
	12.30	704	57.2
RNA from pooled tubes 28–30	8.50	476	56.0
	17.00	1132	66.6
Protamine mRNA	0.90	6399	7110

[a] RNA fractions were extracted from regions on a sucrose gradient (Figure 11) after treating polysomes with 1 mM puromycin hydrochloride (see Materials and Methods). Assays were performed in a wheat germ cell-free system for supporting [^{14}C]arginine incorporation into acid-precipitable counts. Incorporation due to endogenous protein synthesis (2715 cpm) was subtracted. Tubes 10–15 correspond to 5–11S material, tubes 16–21 to 11–16S material, tubes 22–27 to 16–22S material, and tubes 28–30 contain material sedimenting at greater than 22 S.

crose gradients. Rabbit globin mRNA has been reported to have a poly(A) tract 149 residues long (Soreq et al., 1974), while protamine mRNA isolated by oligo(dT)-cellulose chromatography has a poly(A) tract of mean length 18 residues[2] (Davies et al., 1977). This is sufficiently long to hybridize to poly(U) and allows the detection of very small amounts (10 ng) of protamine mRNA. The ability of the assay to detect protamine mRNP particles also suggests that, if a poly(A)-binding protein exists in these particles, as has been reported for other mRNP particles such as globin (Blobel, 1973), mouse sarcoma (Kwan and Brawerman, 1972), and lens crystallin (Chen et al., 1976), then it does not significantly hinder hybridization to poly(U).

Three distinct protamine mRNP particles of different sizes were identified in testis extracts. EDTA dissociation of polysomes released a 12–14S particle, whereas dissociation with puromycin in 0.5 M KCl gave rise to an 8–10S particle. The discrepancy in size between the two polysomal particles released by these two techniques is consistent with observations made about other mRNP particles. Lebleu et al. (1971) observed a 15S globin mRNP particle after EDTA dissociation of rabbit reticulocyte polysomes and this finding was confirmed by Hellerman and Shafritz (1975). The puromycin–0.5 M KCl method of Blobel (1972) released an 11–12S mRNP particle from the same source, which was also confirmed by Hellerman and Shafritz (1975). The smaller size of the protamine and globin mRNP particles prepared by the latter technique may reflect the effect of the higher salt concentration in dissociating extraneous and loosely bound proteins from the mRNA (Perry and Kelley, 1968).

In contrast, the protamine mRNP particle present in the postribosomal supernatant at 16–18 S is even bigger than the EDTA-dissociated particle. This finding is also consistent with the observation that the rabbit globin mRNP particle from reticulocyte postribosomal supernatant (20 S) is larger than the 15S EDTA-dissociated mRNP particle (Jacobs-Lorena and Baglioni, 1972). A similar size difference was observed in

[2] The length of the poly(A) tract in protamine mRNA is heterogeneous and more recent determinations indicate three size classes of 60, 80, and 110 residues (Iatrou, K., Gedamu, L., and Dixon, G. H., manuscript in preparation). The initial lower estimate (18 residues) was caused by traces of nuclease activity in the commercial alkaline phosphatase preparation used prior to the labeling procedure.

globin mRNP particles from duck erythroblasts (Morel et al., 1973; Gander et al., 1973).

The poly(U) hybridization assay for poly(A) tracts is so sensitive that the position of mRNP particles in a gradient can be determined while leaving the bulk of the sample for subsequent purification or analysis of RNA and proteins. Purification of the EDTA-dissociated protamine mRNP particle can be judged on the basis of the template activity of the extracted RNA relative to the activity of pure protamine mRNA. On this basis the protamine mRNP particle was 25% pure after the first sucrose gradient and 43% pure after the second sucrose gradient. This assumes that the protamine mRNA after extraction is fully active, rather than being proportionally less active but more homogeneous. This assumption is borne out by the evidence from polyacrylamide gels, where, although protamine mRNA was the major RNA band, there were other RNA species present. Furthermore, oligo(dT)-cellulose chromatography of RNA extracted from the 12–14S particle yielded polyadenylated protamine mRNA with a template activity equivalent to that of the protamine mRNA standard. The remaining 70% of RNA, which was not bound to oligo(dT)-cellulose also had some template activity. This activity in the pass-through fraction was not due to having exceeded the capacity of the column since the unbound material could be repassed through the regenerated column without it binding more of the RNA. This activity was probably due to the presence of a nonpolyadenylated edition of protamine mRNA which has been observed previously in trout testis (Gedamu and Dixon, 1976b; Gedamu et al., 1977).

Although the 12–14S mRNP particle was not completely homogeneous, protamine mRNA was clearly its major RNA component. Sodium dodecyl sulfate gel electrophoresis showed a number of proteins to be present in the 12–14S region some of which may not be associated with protamine mRNA in a definite complex. The two predominant polypeptides which were consistently seen had molecular weights of 73 000 and 29 000. The neighboring 7–8S region contained smaller amounts of the 73 000 polypeptide which were removed from the main 7–8S material by an additional gel filtration step, suggesting that this polypeptide was indeed associated with the 12–14S region. A 73 000 or 78 000 polypeptide has been observed in many mRNP particles, both homogeneous (Blobel, 1972; Morel et al., 1973; Hellerman and Shafritz, 1975) and heterogeneous (Blobel, 1973; Bryan and Hayashi, 1973; Lindberg and Sundquist, 1974). There is evidence (Blobel, 1973; Kwan and Brawerman, 1972; Jeffery and Brawerman, 1974) that this may be a poly(A)-binding protein.

The 7–8S region consisted mainly of a ribosomal RNP complex between 5S ribosomal RNA and the 34 000 dalton protein designated L3 (unpublished observations). Similar complexes have been seen in other systems following EDTA treatment of eukaryotic polysomes (Lebleu et al., 1971; Blobel, 1971b). In comparison to the discrete 7–8S complex, the 12–14S mRNP particle appeared as a broad peak on sucrose gradients, indicating a heterogeneity in the size of the particle. Some of this heterogeneity may lie with the protamine mRNA whose poly(A) tract is known to be of variable length. Furthermore, there is evidence of different subcomponents within the protamine mRNA. However, the variation in particle size is likely to be due to differences in protein populations bound to the mRNA.

The 16–18S particle of the postribosomal supernatant may be a storage form of protamine mRNA. During the development of trout testis, evidence from hybridization studies with protamine cDNA (Iatrou and Dixon, 1977) suggests that protamine mRNA is synthesized at an early stage of development and enters the cytoplasm but is not translated until the spermatid stage of spermatogenesis. A comparison of the proteins associated with the postribosomal supernatant and polysomal mRNP particles may lead to a better understanding of the relationship between the two particles, and whether their interconversion represents a site of translational control. With this objective in mind the further purification and characterization of both particles is being undertaken.

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Evidence for a Precursor–Product Relationship in the Biosynthesis of Ribosomal Protein S20†

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ABSTRACT: The kinetics of labeling ribosomal protein S20 of *Escherichia coli* strains H882 and H882 *groE*$_{44}$ have been examined using partial reconstitution as a means of binding this and some other 30S subunit proteins selectively to 16S RNA from crude extracts prepared by acetic acid extraction of pulse-labeled whole cells. The rate of labeling of S20 during short pulses at 44 °C is less than 20% of that observed at 28 °C. S20 can be recovered from the cells labeled at the higher temperature if they are chased at 28 °C, but not at 44 °C, in the presence of excess sulfate prior to their extraction. These observations suggest that S20 is derived from a precursor whose processing is blocked at 44 °C. Among the proteins extracted from cells labeled at 44 °C capable of binding to 16S RNA is a novel polypeptide, p2, which is not normally present on the 30S subunit. The kinetics of its appearance at 44 °C, and its chasing at 28 °C, suggest a precursor–product relationship with S20. p2 contains a tryptic peptide with the chromatographic properties of the peptide Ser-Met-Met-Arg at position 25–28 in S20. A second methionine-containing peptide at positions 49–59 of S20 is missing from p2. In addition, the apparent molecular weight of p2 (8600) is less than that of S20 (9500). p2 may represent the product of degradation of a precursor to S20, yet retains the ability to bind to 16S RNA. It is much less likely that p2 is a bona fide precursor which is converted into S20 by fusion to some other polypeptide.

It is now well established that ribosomal proteins of the 30S subunit of *Escherichia coli* can self-assemble in the presence of 16S RNA to produce a physically and enzymatically complete 30S subunit in vitro (Nomura, 1973; Nomura and Held, 1974). It is not clear, however, that the same pathway is followed in vivo. In fact, several lines of reasoning argue against such a supposition. First, the conditions under which ribosomal assembly must occur in vivo are somewhat different than those which are optimal in vitro. In particular, ribosomal reconstitution requires a rather high concentration of KCl in the buffers used (Traub and Nomura, 1969), heating to 42 °C (Traub and Nomura, 1969), and mature 16S RNA rather than precursor RNA (Wireman and Sypherd, 1974). Secondly, there are both genetic (Bryant and Sypherd, 1974) and physiological (Mangiarotti et al., 1975) experiments which implicate the participation of nonribosomal morphogenetic factors in ribosomal biogenesis. Third, the slow kinetics of labeling of S18 in 30S subunits in vivo are best rationalized by the existence of a precursor to this protein (Dennis, 1974). Similar precursor–product relationships are quite common in other assembling systems. Proteolytic cleavages, for example, frequently intervene during the assembly of bacteriophages and viruses from their macromolecular precursors (Eiserling and Dickson, 1972; Hershko and Fry, 1975). The additional information present in the putative precursors to some ribosomal proteins might alleviate some of the kinetic or ionic barriers to ribosomal assembly enumerated above.

In the present work, I have examined a temperature-sensitive strain of *E. coli* K12 (Georgopoulos and Eisen, 1974) which is deficient in the processing of some viral precursor polypeptides during infection by T4 or λ bacteriophages in the expectation that this would facilitate the identification of putative precursors to ribosomal proteins. I have found that nonpermissive temperatures and sulfate deprivation block the appearance of ribosomal protein S20 reversibly. Simultaneously, a novel polypeptide not normally associated with the 30S subunit accumulates in extracts of both the mutant and its parent. This protein is capable of binding to 16S RNA under conditions of partial reconstitution, possesses a methionine-containing tryptic peptide chromatographically indistinguishable from one in S20, and behaves kinetically as if it were a precursor to S20, or directly derived from such a precursor.

Materials and Methods

Strains. Strain H882 *groE*$_{44}$ (F$^-$, thi$^-$, *proA*$_{35}$, thr$^-$, leu$^-$, arg$^-$, str^R, ton, tsx, *groE*$_{44}$), and its groE$^+$ parent, H882, are *E. coli* K12 strains and were obtained from C. P. Georgopoulos (Georgopoulos and Eisen, 1974; Georgopoulos et al., 1972). These strains were tested periodically for their growth requirements and for their ability to plate phage T4D. MRE 600 (Cammack and Wade, 1965) was used for the preparation of ribosomal components on a large scale.

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