Translational Efficiency of Cytoplasmic Nonpolysomal Messenger Ribonucleic Acid from Sea Urchin Embryos†

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ABSTRACT: Messenger ribonucleic acid (mRNA) has been isolated from polysomes and the free cytoplasmic nonpolysomal ribonucleoprotein particles (free RNPs or informosomes) of sea urchin embryos. These RNA populations were examined for their capacities to direct protein synthesis in both the rabbit reticulocyte and wheat germ cell-free amino acid incorporation systems, pretreated with micrococcal nuclease to reduce endogenous incorporation. At equivalent concentrations of each RNA the rate of protein synthesis was greater with the templates from polysomes, and the maximal rate (V_{max}) at saturating levels of RNA was about 2–3 times greater for polysomal mRNA compared with the free RNP mRNA. Experiments using mixtures of the two poly(A+) mRNA populations at nonsaturating levels show that the difference in amino acid incorporation is not due to the presence of inhibitors in the free RNP RNA. The ability of each class of RNA to form 80S initiation complexes was measured through ribosome-binding studies. The free RNP mRNA has a 20% lower capacity to bind ribosomes, suggesting that these nonpolysomal mRNA molecules are enriched for inefficient initiators of protein synthesis relative to the templates present in polysomes. However, a specific subset of mRNAs (the 9S histone mRNA) isolated from either the polysomes or free RNPs was found to be equivalent in its ability to form 80S initiation complexes. The difference in efficiency to be translated between the polysomal and nonpolysomal poly(A+) mRNA does not appear to lie in the structures of the 5′ termini since the translation of both classes of mRNA is inhibited to the same extent with the cap analogue m^7G^5p. Gel analysis of in vitro translation products reveals that the free RNPs and polysomes contain a similar highly complex array of messages. However, several major quantitative differences in the pattern of translation products exist. Differences in the inherent abilities of various messages to be translated are suggested as an important means of regulating the array of proteins synthesized in an embryo during development.

A significant fraction of the cytoplasmic messenger ribonucleic acid (mRNA) of most eucaryotic cells can be isolated in the form of ribonucleoprotein particles which are not actively engaged in protein synthesis (Samarina et al., 1973; Spirin, 1972; Bag & Sarkar, 1975; Jacobs-Lorena & Baglioni, 1972; Buckingham et al., 1976). In sea urchin embryos, such nonpolysomal mRNA particles (free RNPs or informosomes) constitute anywhere from 20 to 50% of the newly synthesized cytoplasmic mRNA population, depending on the developmental stage (Infante & Nemer, 1968; Dworkin & Infante, 1976). Both the free RNP and the polysome populations contain polyadenylated RNA [poly(A+) RNA], although the free RNPs are enriched for nonpolyadenylated nonhistone species [poly(A−) RNA] and the poly(A) tail shortens more slowly than for polysomal mRNA (Dworkin et al., 1977).

We have shown through half-life determinations and other studies that the newly synthesized (embryonic) free RNPs in sea urchins do not represent a pool of stable messages awaiting utilization later in development. Also, kinetic studies have shown that the free RNPs are not the normal intermediates on the way to polysome assembly, nor are they the result of polysome disassembly. Similar studies in Chinese hamster cells (Enger & Campbell, 1975) and murine myeloma cells (MacLeod, 1975) have yielded similar conclusions. Thus, it appears that newly synthesized mRNA, upon exit from the nucleus, is simultaneously distributed to both the free RNPs and polysomes, and it is the mechanism for regulating this distribution which has become of primary interest. A possible explanation is that the free RNPs represent a pool of mRNA which is in excess of what the translational system of a cell can effectively utilize (Dworkin & Infante, 1976). We have suggested, on the basis of the turnover kinetics of the mRNA and an analysis of the metabolism of the poly(A) tails of the free RNP RNA, that the free RNPs synthesized in embryos do form polysomes in vivo but that they initiate only infrequently (Dworkin et al., 1977). In this case it would be of interest to determine whether there is random utilization of a given portion of all cytoplasmic messengers or if certain messengers have an inherent selective advantage over others to be translated, possibly as a result of their nucleotide sequence or secondary structure.

We have compared the ability of RNA from polysomes and free RNPs to direct protein synthesis in a wheat germ and a rabbit reticulocyte cell-free system. Our results show that free RNP RNA of blastula stage embryos has a lower translational capacity in vitro than mRNA from polysomes. The results support a model suggesting that newly made embryonic free RNPs contain mRNA which is enriched with inefficient ("weak") templates.

Methods

Preparation of RNA. [^{3}H]Uridine-labeled free RNP and polysomal RNA were prepared from blastula stage *S. purpuratus* embryos as previously described (Dworkin & Infante, 1976; Dworkin et al., 1977). Essentially, postmitochondrial supernatants were sedimented through sucrose gradients, and the region lighter than the ribosome (free RNPs) was separated from the polysome pellet. Ethanol precipitated free RNPs, and polysome pellets were extracted with phenol–chloroform and separated into poly(A+) RNA and poly(A−) RNA by using oligo(dT)–cellulose (Type T-3, Collaborative Research). Columns contained about 0.2 g of oligo(dT)–cellulose in a 5-mL plastic syringe. The use of such a physically thin pad of oligo(dT)–cellulose substantially reduced nonspecific binding of RNA to the column. After the column

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was washed extensively with binding buffer (0.5 M KCl, 0.01 M Tris-HCl, pH 7.5), the RNA solution was passed over the column two times in the cold using a flow rate of ~0.2 mL/min. Additional binding buffer was added, and 8 mL was collected as the poly(A−) fraction. The column was washed with binding buffer until no absorbance could be detected, and the poly(A+) RNA was then quickly eluted at room temperature with 4 mL of elution buffer (0.01 M Tris, pH 7.5). A 1-mL amount of eluate was added to a cold cuvette and the A_{260} determined. This measurement was used to determine the approximate microgram quantity of poly(A+) RNA obtained. After ethanol precipitation at −20 °C (with 0.2 M KOAc, pH 5), the RNA was quantitatively recovered by ultracentrifugation in a Beckman SW41 rotor for 10 min at 35000 rpm. RNA was washed one time with 95% ethanol, dried by lyophilization, dissolved in sterile glass-distilled water at about 1 μg/μL, and stored at −70 °C. Such steps as performing the column chromatography in the presence of $NaDodSO_4$, preempting with tRNA, passing the RNA more than two times over the column, or using a larger column volume did little to increase the yield or integrity of the poly(A+) RNA finally obtained and often resulted in increased nonspecific binding of RNA and also less active mRNA in cell-free translation.

Reticulocyte Translation Assay. A rabbit reticulocyte lysate prepared as described (Dworkin et al., 1977) and stored at −70 °C was treated with 20 μg/mL micrococcal nuclease (Boehringer Mannheim) essentially as described by Pelham & Jackson (1976) using a 15-min treatment time at 20 °C. Nuclease was inactivated by chelating Ca^{2+} with 2 mM EGTA. Assays contained the following in a volume of between 25 and 100 μL: 12 mM Tris-HCl, pH 7.8, 0.85 mM ATP, 0.17 mM GTP, 10 mM creatine phosphate, 30 mg/mL creatine phosphokinase (Sigma), 1 mM dithiothreitol, 90 mM KCl, 1.2 mM $Mg(OAc)_2$, 15 mM hemin, 0.1 mM of each amino acid except leucine, 0.1 μCi/μL of [^{3}H]leucine with a specific activity of 60 Ci/mmol, and mRNA as indicated. Incubations were at 30 °C usually for 1 h. To assay incorporation, 1–5-μL aliquots were spotted in duplicate onto Whatman 3-MM filter disks, which were then incubated in ice-cold 10% Cl_3AcOH for 15 min, followed by several 15-min incubations in boiling and room temperature 5% Cl_3AcOH in the presence of the appropriate unlabeled amino acid. Filters were bleached for 30 min in a solution made up of 10% Cl_3AcOH, 30% H_2O_2, and 88% formic acid (2:1:1). After being washed in an ethanol–ether solution (1:3), the filters were dried and counted in Econoflour (New England Nuclear). The remaining reaction mixture was stored at −70 °C for further analysis.

Wheat Germ Assay. Wheat germ was obtained from General Mills and stored under vacuum at 4 °C. An S-23 extract was prepared according to Gallis et al. (1975), stored in small aliquots in liquid nitrogen, and treated with 10 μg/mL micrococcal nuclease for 5 min at 20 °C just before use. Incubations were performed essentially as described by Tse & Taylor (1977) and contained (in 10–100 μL) 1.75 A_{260} units of wheat germ S-23 (per 100 μL), 25 mM Hepes, pH 7.5, 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate, 0.15 μg/mL creatine phosphokinase, 40 μM spermine, 40 μM of 19 amino acids except leucine, 2 mM DTT, 100 mM KOAc, 2.5 mM $Mg(OAc)_2$, and 1 μCi/μL [^{3}H]leucine. Incubations were at 22.5 °C usually for 90 min. Incorporation was assayed as described for the reticulocyte system.

Ribosome-Binding Assay. The ability of mRNA to bind ribosomes was assayed as described previously (Dworkin et al., 1977) by adding [^{3}H]uridine-labeled RNA [specific activity for poly(A+) RNA = 5000 cpm/μg] to either the wheat germ or the reticulocyte cell-free systems under the conditions described above, except that all amino acids were present unlabeled, and cycloheximide was included at 3.2 μg/mL to inhibit polysome formation. The reaction volume was normally 100 μL, and limiting levels of RNA were used. With reticulocyte lysates the extent of RNA–ribosome binding reached a maximum in 2–3 min of incubation and did not change in the next 6 min; incubations were routinely for 4 min at 30 °C. Incubations with the wheat germ system were allowed to proceed for 10 min at 25 °C (Weber et al., 1979). The reaction was stopped by addition of 2 volumes of ice-cold buffer [0.05 M Tris, pH 7.5 (4 °C), 0.24 M NH_4Cl, 0.005 M $Mg(OAc)_2$, 0.25 M sucrose], and the mixture was centrifuged through a 5-mL 15–30% sucrose gradient (made up in the same buffer) at 39000 rpm for 140 min in a Spinco SW 50.1 rotor. Gradients were fractionated and assayed for Cl_3AcOH precipitable radioactivity as described (Dworkin et al., 1977).

Polyacrylamide Gel Electrophoresis of Translation Products. A 10–25-μL amount of the translation reaction mixture was treated with 60 μg/mL pancreatic RNase A for 15 min at 37 °C and then adjusted with 2% $NaDodSO_4$, 0.05 M Tris, pH 6.8, 1 mM β-mercaptoethanol, and 10% glycerol. Samples were then heated to 100 °C for 1 min and cooled to room temperature before application to gels. Samples were electrophoresed on 10–16% polyacrylamide gradient slab gels (Laemmli, 1970) (11 cm × 1.5 mm) with a 2-cm 5% acrylamide stacking region containing 0.2% $NaDodSO_4$. BSA, rabbit globin, and calf thymus histones were run as markers. Electrophoresis was performed at 120 V (constant voltage) for about 8 h. Gels were stained with 0.1% Coomassie blue in 50% Cl_3AcOH and destained in 30% methanol and 10% acetic acid. Gels were prepared for flourography, dried, and exposed to prefogged X-ray film for 24 h at −70 °C as described by Bonner & Laskey (1974) and Laskey & Mills (1975).

Results

Sedimentation Profiles of Free RNP and Polysomal RNA. The distribution of cytoplasmic RNA between polysomes and free RNPs is shown in Figure 1A. The proportion of RNA in the free RNPs is strongly related to the developmental stage of the embryo, such that in the first 8 h after fertilization about 50% of the RNA is in the free RNPs and by the mesenchyme blastula stage (25 h) only 15% of the mRNA is in free RNPs (Dworkin & Infante, 1976). Shown is a 14-h stage where approximately 35% of the labeled cytoplasmic non-4S RNA is in the free RNPs. The conditions given in Figure 1A are sufficient to achieve steady-state labeling of the cytoplasmic RNA; therefore, the distribution of labeled RNA represents the actual distribution of putative mRNA since in these early embryos there is virtually no detectable synthesis of rRNA. The regions of the free RNP and polysomes (the regions indicated as I and II, respectively, in Figure 1A) were isolated, and the poly(A+) RNA was prepared. Figure 1B shows the sedimentation patterns of these two RNA preparations. The profiles are both heterogeneous, and clearly they are extremely similar. This is also the case with the poly(A−) RNA constituents of the free RNPs and polysomes, with the exception that a predominant 9S histone RNA peak in the profile of polysomal poly(A−) RNA is not present in the free RNP RNA (Dworkin & Infante, 1976). Most of the studies described below utilize the 12–14-h embryonic RNA preparations shown in Figure 1.

Template Activity of Free RNP RNA. Comparison of

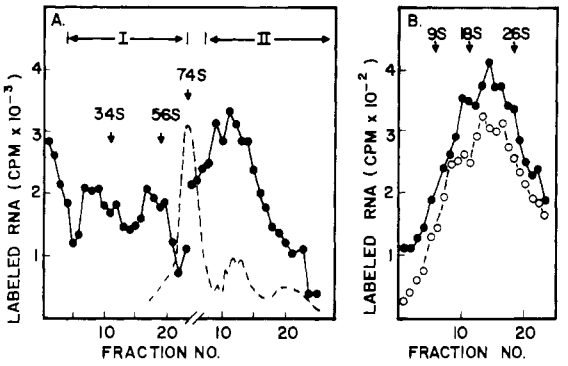

FIGURE 1: (A) Distribution of newly made RNA in the cytoplasm of sea urchin embryos. The 10-h embryos of *S. purpuratus* were labeled with [^{3}H]adenosine for 2 h, and a cytoplasmic extract was prepared (Dworkin et al., 1977). The extract was sedimented on 15–30% (w/w) sucrose gradients in TNM buffer [50 mM Tris-HCl, pH 7.8 (4 °C), 240 mM NH_4Cl, 5 mM $Mg(AcO)_2$, 250 mM sucrose] for either 27 (polysome gradient) or 150 min (free RNP gradient) at 49 000 rpm. The sedimentation profiles have been drawn together to show the total profile of cytoplasmic labeled RNA. The dashed line shows the A_{206} profile in the polysome gradient. (B) Sedimentation profiles of blastula RNA from free RNPs and polysomes. RNA from regions I and II of part A was precipitated in ethanol, extracted, and chromatographed on oligo(dT)–cellulose. The poly(A+) RNA was sedimented through 5-mL, 5–20% (w/v) sucrose gradients in 0.01 M sodium acetate, pH 5.0, and 0.0001 M EDTA in the Spinco SW 50.1 rotor at 49 000 rpm for 210 min (Dworkin & Infante, 1976). Fractions of 10 drops were collected and precipitated with 10% trichloroacetic acid in the presence of carrier BSA (40 μg). rRNA markers and 9S histone mRNA are indicated by arrows. Polysomal poly(A+) RNA (●—●); free RNP poly(A+) RNA (O---O).

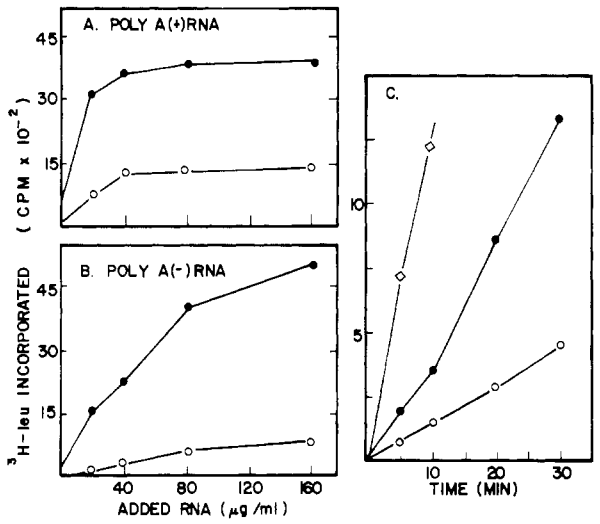

FIGURE 2: Characteristics of translation of free RNP and polysomal RNA in mRNA-dependent reticulocyte systems. Translation assays were performed by using the nuclease-treated reticulocyte system with polysomal RNA (●), free RNP RNA (O), or globin mRNA (for part C) (◊). In parts A and B, varying amounts of either poly(A+) RNA (A) or poly(A–) RNA (B) were added to 100-μL incubations and 5-μL aliquots were assayed for incorporation of [^{3}H]leucine into protein after 60 min at 30 °C. Amounts of RNA were extrapolated from A_{260} measurements. In part C, 100-μL incubations with 40 μg/mL poly(A+) RNA were performed and 5-μL aliquots were assayed at varying times for ^{3}H protein; initial rates are shown.

Translational Efficiency with Polysomal mRNA. In order to further demonstrate the existence of mRNA in the free RNPs and to compare its template activity with that of polysomal mRNA, we examined the response of the nuclease-treated reticulocyte lysate system to varying amounts of poly(A+) and poly(A–) free RNP and polysomal RNA (Figure 2). Both classes of poly(A+) RNA stimulate polypeptide synthesis and saturate the system at a concentration of about 40 μg/mL (Figure 2A). However, it is clear that a saturating level of free RNP RNA is only about 30–40% as efficient in amino acid incorporation as poly(A+) polysomal RNA. The reduced extent of amino acid incorporation for the free RNP RNA compared to the polysomal RNA is also observed for the nonpolyadenylated RNA species of the two classes of RNP (Figure 2B). In this system the poly(A–) RNA resulted in a more linear response over the same concentration range that yielded saturation for poly(A+) RNAs. This is very likely due to the fact that most (98%) of the poly(A–) fractions consist of ribosomal RNA, so that here we are testing mRNA concentrations which are far below saturation. It is thus surprising that, compared to the poly(A+) RNA, the polysomal poly(A–) RNA is as efficient as found. This is probably because most of the messages in this RNA fraction are templates for histones (Dworkin & Infante, 1976), which are particularly efficient (Stahl & Galliwitz, 1977; Gabrielli & Baglioni, 1977).

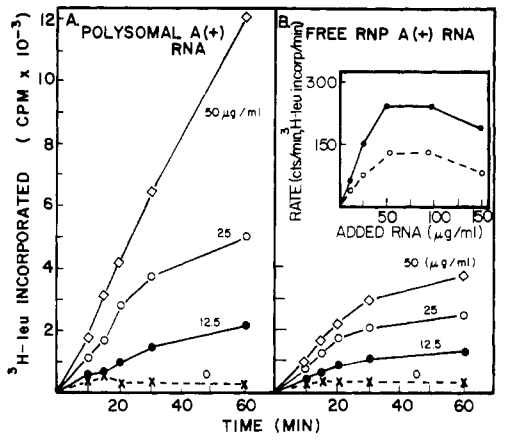

FIGURE 3: Dose–response and kinetics of cell-free protein synthesis with poly(A+) RNA in the wheat germ system. Varying amounts of poly(A+) RNA from polysomes (A) and free RNPs (B) were assayed in the wheat germ system, and the time course of incorporation at each RNA concentration was measured. Incubations (10 μL) were performed, and ^{3}H protein in 1-μL aliquots was measured at the indicated times. RNA concentrations: (X---X) 0 μg/mL; (●—●) 12.5 μg/mL; (O—O) 25 μg/mL; (◊—◊) 50 μg/mL. The kinetics of incorporation with RNA concentrations of 100 and 150 μg/mL are not shown for the purpose of clarity. (Insert) The rate of incorporation was calculated for each RNA concentration from the 0–30-min portions of the curves in parts A and B. (●—●) Polysomal RNA; (O---O) free RNP RNA.

Shown in Figure 2C are the early linear rates of incorporation for saturating concentrations of polysomal and free RNP poly(A+) RNA along with the rate with rabbit reticulocyte poly(A+) RNA, which is primarily globin mRNA. The rate of incorporation of [^{3}H]leucine with polysomal RNA is nearly three times greater than the rate of incorporation due to free RNP RNA. Therefore, the phenomenon observed in Figures 2A,B results from a reduced rate of polypeptide synthesis in the presence of free RNP RNA rather than reflecting a difference in the overall extent of translation.

Translation Efficiencies with Wheat Germ Extracts. In order to obtain confirmation that the findings given in Figure 2 are not due to conditions peculiar to the reticulocyte system (cf. Rose & Lodish, 1976; Weber et al., 1977), we performed similar translation studies in the wheat germ cell-free system (Figure 3). Also, in this case lower levels of RNA were tested and the rate of [^{3}H]leucine incorporation was determined for each level of RNA added (Figures 3A,B). It is clear that at all concentrations used the rate of synthesis directed by polysomal RNA is greater than that directed by the free RNP

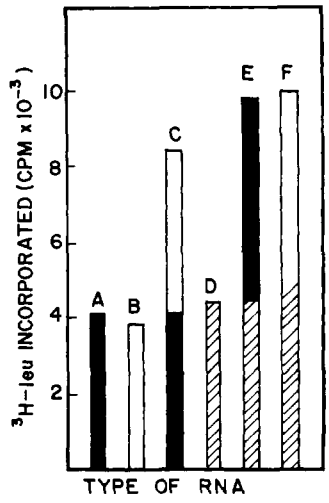

FIGURE 4: Translations of mixtures of nonsaturating levels of various poly(A+) mRNA. Wheat germ translation assays were performed with poly(A+) RNA added alone in some incubations and mixed with another preparation of mRNA in other incubations. (A) 12.5 μg/mL polysomal RNA alone; (B) 25 μg/mL free RNP RNA alone; (C) 12.5 μg/mL polysomal RNA plus 25 μg/mL free RNP RNA; (D) 3 μg/mL globin mRNA alone; (E) 3 μg/mL globin mRNA plus 12.5 μg/mL polysomal RNA; (F) 3 μg/mL globin plus 25 μg/mL free RNP RNA. Incorporation was measured in 2-μL aliquots taken after 60 min at 22.5 °C from 25-μL incubations. Background incorporation was subtracted in all cases.

RNA. This difference varies from two- to threefold for the poly(A+) RNA and is in the same range as observed in the rabbit reticulocyte system. The results with both cell-free systems are therefore strongly supportive of each other and indicate that the free RNPs contain higher concentrations of "poorer" messages.

It should be noted that the RNA assayed in the experiment shown here is the same sample of RNA which is displayed on sucrose gradients in Figure 1. Thus, although the polyadenylated free RNP and polysomal mRNAs are nearly identical in size, they differ significantly in their abilities to direct protein synthesis. The insert in Figure 3B shows that the rate of protein synthesis is linearly dependent on the concentration of each type of RNA added up to about 50 μg/mL, where the system becomes saturated, and the maximum rate (V_{max}) is obtained with both classes of messengers. Data obtained as in Figures 2 and 3 permit an ordering of efficiencies for the various RNAs tested as follows: globin mRNA > polysomal poly(A−) RNA ≥ polysomal poly(A+) RNA > free RNP poly(A+) RNA > free RNP poly(A−) RNA.

Absence of Inhibitors in Poly(A+) RNA Fractions. The observed differences in translation efficiency between free RNP and polysomal RNA might be attributed to the presence of contaminating inhibitors of protein synthesis in the free RNP fraction. This was tested by preparing mixtures of polysomal RNA, free RNP RNA, and rabbit globin mRNA and measuring the level of protein synthesis supported by the mixtures and by each RNA separately. For such an experiment it is essential that the RNA constituents are below saturating concentrations. Under this condition the level of protein synthesis supported by the mixture should equal the sum of the incorporations directed by both types of RNA examined separately, if there is no inhibitor present. In Figure 4 the concentrations of the three poly(A+) RNA classes were adjusted to yield similar levels of leucine incorporation (A, B, and D). When the concentration of each RNA was doubled, there resulted a doubling of the incorporation (not shown); hence, the system is limited by the amount of added RNA. The extent of polypeptide synthesis supported by the mixture of polysomal RNA and free RNP RNA (Figure 4C) was equal to the sum of the incorporations obtained with each RNA used separately. Additive levels of incorporation were also obtained with mixtures of globin mRNA and poly(A+) RNA from either the polysomes (Figure 4E) or the free RNPs (Figure 4F), indicating that no inhibitor is present in the free RNP RNA to account for the results in Figures 2 and 3. However, a similar study using poly(A−) RNA showed that, whereas the mixture of polysomal RNA and globin RNA yielded protein synthesis expected from the incorporation supported by each separately, the mixture of free RNP RNA and globin mRNA yielded lower than expected incorporation (data not shown). The latter result indicates that the poly(A−) RNA of the free RNP may contain some inhibitory element, which may account for the reduced incorporation shown in Figure 2B. The nature of this inhibitor and whether it is of physiological or artifactual origin are not known, and we have limited our examinations to the poly(A+) mRNA fractions. The result with the poly(A−) RNA fraction was not consistent since in some cases no inhibition was obtained.

Reduced Ribosome-Binding Ability of Free RNP RNA. We have compared the abilities of free RNP RNA and polysomal RNA to bind to ribosomes in vitro in an attempt to determine whether the differences observed in their efficiencies of translation may reflect differences in the RNAs to function at some initiation step. Radioactive poly(A+) RNAs of equal specific activities were incubated in either a standard or a nuclease-treated reticulocyte lysate, and then the reaction mixtures were sedimented on sucrose gradients to determine the extent of association of labeled RNA with ribosomes and polysomes. Figure 5 shows that the polysomal RNA binds to ribosomes to a greater extent than does the free RNP RNA. In the nonnuclease-treated lysate (Figures 5A,B) the difference is very slight as reported previously (Dworkin et al., 1977), and in this series of experiments 58–60 and 52–56% of the polysomal and free RNP RNA, respectively, become bound to ribosomes. This difference between the two RNA populations is much more evident with the nuclease-treated lysate, where a greater proportion of both radioactive RNA classes becomes associated with ribosomes and polysomes. In this system (Figures 5C,D) 76–78 and 60–62% of polysomal and nonpolysomal RNA, respectively, bind to ribosomes.

The improved binding of RNA to ribosomes after nuclease treatment of the reticulocyte lysate is very likely due to the removal of endogenous mRNA. Examination of the A_{260} profiles of the standard and the nuclease-treated reticulocyte lysates in sucrose gradients (Figures 5A,C; dashed curves) shows that the nuclease treatment effectively destroys the endogenous globin polysomes, thereby releasing ribosomes and the rest of the translational machinery for use in translating exogenously added RNA. Clearly the polysomal mRNA can make more effective use of this condition than the free RNP RNA.

The difference in the binding of the two RNA classes to ribosomes supports the incorporation data given above and indicates that the mRNA of free RNPs is a "weaker" initiator than polysomal RNA. This is further substantiated below (Figures 6C,D), where the binding of free RNP RNA to wheat germ ribosomes is only about 60% as efficient as that of polysomal RNA. However, the difference in ribosome binding between the two classes of mRNA in the reticulocyte system is much less (only about 20%) and is inadequate to account completely for the different extents of translation (i.e., two-

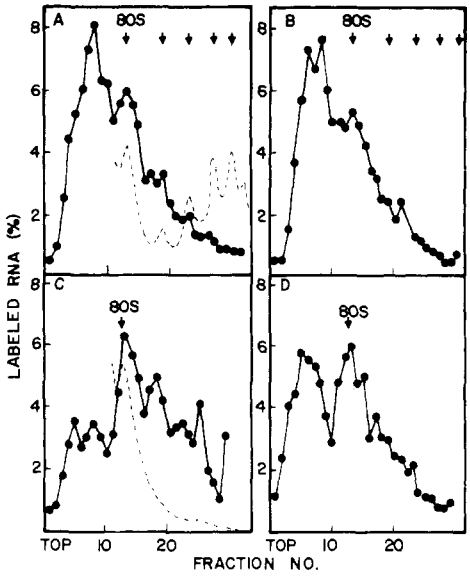

FIGURE 5: Ribosome-binding assay for [^{3}H]uridine-labeled poly(A+) RNA from polysomes and free RNPs in untreated and nuclease-treated reticulocyte lysates. Approximately 5000 cpm (1.1 μg) of a solution of poly(A+) RNA from either polysomes or free RNPs was added to the standard untreated reticulocyte lysate (A and B) or to the nuclease-treated lysate (C and D). The final volume was 100 μL in all cases. Incubation and processing were as described under Methods. In parts A and B is shown the ability of polysomal RNA (A) and free RNP RNA (B) to bind ribosomes in the untreated lysate. In parts C and D is shown the binding of the same RNAs (polysomal-C and free RNP-D) in the nuclease-treated lysate. The percentage of total counts per minute in each fraction is plotted on the ordinate. A cavity in these curves at about fraction 10 was taken as the dividing line between unbound and polysome-bound radioactive mRNA to determine the total degree of RNA binding. When ATA, a potent inhibitor of polypeptide initiation, is used, most of the radioactivity is in the region of the gradient to the left of fraction 10 (data not shown; see Figure 6, for example). The dashed curves in parts A and C show the A_{260} profiles of the control and nuclease-treated reticulocyte polysomes, respectively.

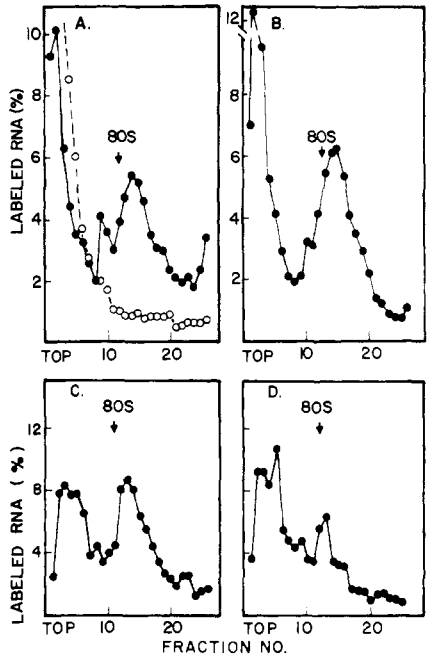

FIGURE 6: Ribosome-binding ability of 9S RNA from the early cleavage stage of polysomal (A) and free RNP RNA (B). Embryos were labeled with [^{3}H]uridine from 1 to 4 h of development, and total polysomal and free RNP RNA were obtained. The RNA was then sedimented on low ionic strength (A-5) sucrose gradients, and the region containing the prominent 9S peak was precipitated in ethanol. Ribosome-binding ability was assayed in a nuclease-treated wheat germ lysate as described under Methods. (A) Binding of polysomal 9S RNA; (B) binding of free RNP 9S RNA. The dashed line shows the binding of polysomal 9S RNA in the presence of 0.1 mM ATA. In parts C and D are shown the ribosome-binding abilities of total poly(A+) RNA in this system; (C) binding of polysomal poly(A+) RNA; (D) binding of free RNP RNA poly(A+) RNA.

to threefold). This may reflect some unknown feature in the binding assay system or the possibility that although "weaker" in ribosome binding the free RNP RNA may be weak in other translation processes as well.

Nonpolysomal Histone mRNA. The data presented thus far indicate that the population of mRNAs in the free RNPs is less efficient than that in polysomes. It would be very informative to test the translation efficiency of a specific mRNA isolated from both the polysomes and the free RNPs. This is not possible at present in this system; however, we have taken advantage of the high level of histone RNA synthesis in the very early embryo to examine this subset of messages. Accordingly, we have compared the in vitro ribosome-binding efficiencies of the newly made 9S RNA from free RNPs and polysomes prepared from embryos which were labeled between 1 and 4 h of development. Figures 6A,B show that approximately the same percentage of 9S RNA from either source is incorporated into polysomes: 53% of the 9S polysomal RNA and 50% of the 9S free RNP RNA. This study was performed by using a micrococcal nuclease-treated wheat germ cell-free system. The binding of the total poly(A+) RNA to ribosomes in this sytem is shown in Figures 6C,D. As in the case with the nuclease-treated reticulocyte system (Figures 5C,D), the extent of ribosome binding of the free RNP RNA is clearly less than the binding of polysomal RNA (35 and 59%, respectively). It therefore appears that although the total class of free RNP RNA is less efficient than the total polysomal RNA class, individual mRNAs have the same efficiency whether isolated from polysomes or free RNPs.

Test for Presence of 5′ Cap in Free RNP RNA. An explanation for the reduced translational efficiency of free RNP RNA might be the absence of an effective cap structure. To test this possibility, we have used the method of Hickey et al. (1976), who showed that the translation of capped mRNAs can be specifically inhibited by the cap analogue 7-methylguanosine 5′-monophosphate ($m^7G^5'p$). This inhibition occurs at the level of initiation and will not occur with other cap analogues. The extent of inhibition by $m^7G^5'p$ can therefore be taken as a measure of the relative extent of capping of the mRNA being translated.

The inhibition of translation of free RNP and polysomal mRNA by varying levels of $m^7G^5'p$ was compared (Figure 7). The extents of inhibition for each class of RNA translated are essentially identical. Some inhibition also occurs with the cap analogue m^7G, although both classes of mRNA are far more affected by the phosphorylated cap species, indicating that the process is specific for messages capped with the $m^7G^5'p$ structure. The inhibition curves are extremely similar to that obtained over the same inhibitor concentrations by Hickey et al. (1976) for poly(A+) Hela cell RNA, which is known to have a 5′ terminal $m^7G^5'p$ (Furuichi et al., 1975). The inhibition for polysomal poly(A+) RNA was expected because this RNA is also known to possess the $m^7G^5'p$ cap structure (Surrey & Nemer, 1976; Faust et al., 1976). Since poly(A+) RNA from free RNPs yields the same inhibition curve with $m^7G^5'p$, it apparently possesses the same cap structure as polysomal mRNA, although a direct analysis will be required to definitely ascertain the nature of the 5′ terminus. The result is not biased by possible differences in the salt conditions

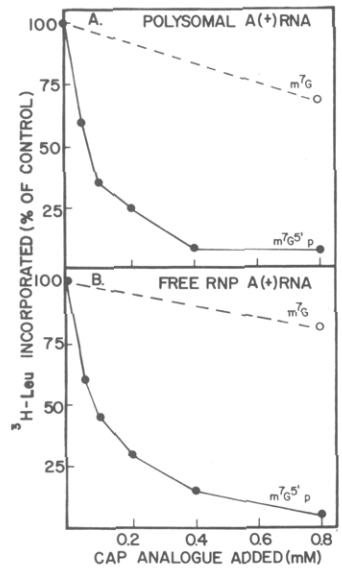

FIGURE 7: Inhibition of translation of poly(A+) free RNP and polysomal RNA by cap analogues in the wheat germ system. Polysomal RNA (A) and free RNP RNA (B) were assayed in the presence of 200 μM *S*-adenosylhomocysteine and varying concentrations of m^7G^5p (●—●) or m^7G (O---O). RNA concentrations which yielded optimal incorporation were used (about 50 μg/mL). Total control incorporations over background for the 25-μL incubations were 229 650 cpm with polysomal RNA and 76 225 cpm with free RNP RNA.

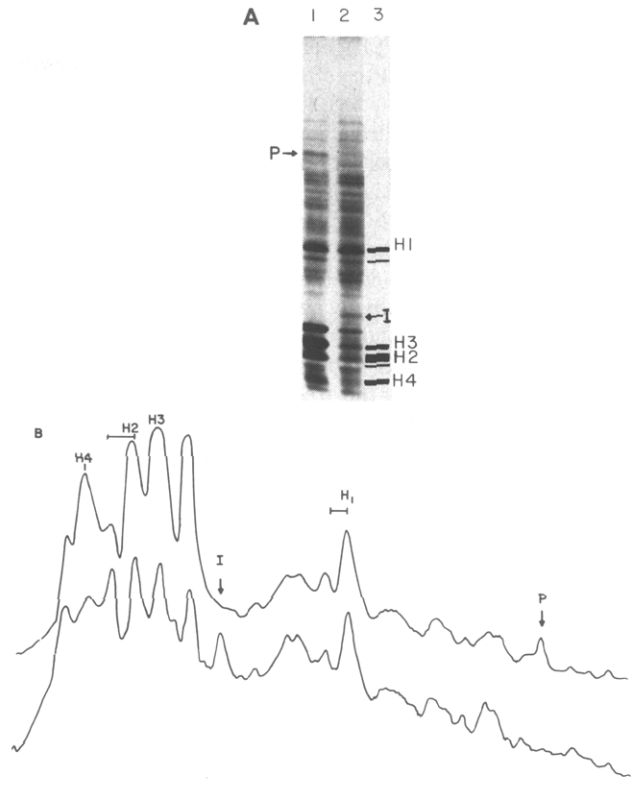

FIGURE 8: Gel electrophoresis of poly(A+) free RNP and polysomal mRNA translation products. A standard wheat germ translation assay was performed with amounts of poly(A+) RNA that yielded optimal incorporation (10–30-fold stimulation over background in this experiment). Samples containing a total of about 85 000 cpm in 25 μL were treated and electrophoresed as described under Methods. Calf thymus histones were run on a parallel gel. (A) Translation products are from poly(A+) polysomal RNA (lane 1) and poly(A+) free RNP RNA (lane 2). The banding of the calf thymus histones is indicated in lane 3. (B) Tracings of densitometer scans of the exposed gel fluorograph patterns of part A. The upper curve is the scan of the translation products of polysomal RNA; the lower curve is for the free RNP RNA. The approximate positions of the calf thymus histones on the scans are indicated for reference.

required by the two classes of message, in translation, since both classes have the same potassium and magnesium requirements for optimal protein synthesis (unpublished data). In addition, possible endogenous methylation by enzymes in the wheat germ extract was prevented in all of these studies by performing the translation in the presence of 200 μM *S*-adenosylhomocysteine (Muthukrishnan et al., 1976).

Gel Analysis of Translation Products. The polypeptides synthesized in vitro by the poly(A+) RNA from polysomes and free RNPs were examined by electrophoresis on polyacrylamide gels. The one-dimensional patterns of the proteins synthesized by using RNA from the 14-h embryo are given in Figure 8. This gel analysis is part of a larger study comparing the mRNA populations in free RNPs and polysomes through early development (L. M. Rudensey and A. A. Infante, unpublished experiments). There are many bands present in the patterns of the translation products from both RNA preparations, and several prominent bands comigrate closely with histone standards run on parallel gels. The presence of histone mRNA in the poly(A+) RNA fraction has been shown recently by several laboratories (Ruderman & Pardue, 1978; Levenson & Marcu, 1976). An examination of the in vitro translation products leads to three major observations. (1) There is a great deal of similarity in the polypeptides synthesized, with virtually all bands present in the polysomal RNA translation profile also being produced in free RNP RNA directed translation. This heterogeneity in both classes agrees with the heterogenous and similar sedimentation patterns of the two RNA species (Figure 1). (2) There are obvious quantitative differences in the patterns (Figure 8B); for example, the extent of protein synthesis in the band designated as H1 relative to the other putative histone bands is much greater for the free RNP RNA compared to the polysomal RNA. (3) Finally, there are some apparent qualitative differences in the two patterns. There is at least one band (indicated as I) present in the translation products of the free RNP RNA which is virtually absent in the polysomal RNA. Also there is a band present from polysomal RNA (indicated as P) which appears absent in the translation products of free RNP RNA.

Discussion

The data presented show definitively that the RNA present in the free RNPs of sea urchin embryos contains templates for protein synthesis (Figures 2 and 3). This extends earlier work which established the ribonucleoprotein nature of this material and indicated that the RNA was very likely mRNA because of its DNA hybridization properties (Infante & Nemer, 1968), its sucrose gradient sedimentation profiles (Dworkin & Infante, 1976), and its ribosome-binding ability (Dworkin et al., 1977). We raised the possibility that the free RNPs represent an underutilized pool of templates which are in excess of what the translational apparatus of the cell can utilize. The gel analysis of proteins synthesized in vitro (Figure 8) using the templates in the free RNPs indicates that in fact there is not a restricted group of a few templates present in the free RNPs but rather a complexity similar to that found in polysomal RNA. In addition, the proteins synthesized from free RNP messages in vitro appear to be very similar to those made using polysomal mRNA. This is substantiated in two-dimensional gel analysis of the translation products (L. M. Rudensey, L. J. Heilmann, and A. A. Infante, unpublished experiments). It therefore appears that the free RNPs are indeed primarily a pool of excess messengers. However, various data indicate that there may not be a random seg-

regation of mRNA between the translating (polysomes) and nontranslating (free RNP) compartments. These results include (1) differences in the efficiency of translation of each mRNA class (Figures 2 and 3), (2) differences in their abilities to bind to ribosomes in vitro (Figures 5 and 6), and (3) several clear quantitative (and perhaps qualitative) differences in the products of in vitro translation of each mRNA class (Figure 8). Also, as shown previously, the free RNPs contain proportionately much less of the total 9S histone mRNAs than the polysomes (Dworkin & Infante, 1976). Furthermore, the poly(A) tails, although initially about 200 nucleotides in length for both the free RNP and polysomal mRNA, shorten more slowly on free RNP RNA (Dworkin et al., 1977). These differences suggest that the free RNPs do not represent a simple randomly chosen array of excess messages, but are a selected group, thus implying a distinct mechanism for the control by which messages are translated.

The major finding of this work is that the mRNA present in the free RNPs of sea urchin embryos at the blastula stage is not translated as efficiently as the polysomal RNA, in vitro. The simpliest explanation for this finding is that the RNA moiety of the free RNPs itself is less capable of being translated. Intrinsic differences in the abilities of specific mRNAs to be translated have been reported (Nuss & Koch, 1976; Sonenshein & Brawerman, 1976; Koch et al., 1977; Kozak & Shatkin, 1978; Kaempfer et al., 1978), and this proposal is analogous to that advanced by Lodish (1974) to explain the presence of α-globin mRNA in the free RNPs of reticulocytes. It is attractive to explain the reduced efficiency of the free RNP RNA on the basis of a nonrandom selection, in vivo, of "strong" mRNA into polysomes, thereby leaving behind a higher proportion of less efficient ("weak") mRNAs in free RNPs. However, there are other possible explanations. An extreme possibility is that exactly the same kinds and relative amounts of templates are present in the polysomes and free RNPs. In this case the observed reduced translational efficiency of the free RNP RNA would have to be due to a cellular modification of the RNA itself. A modification of mRNA has been implied as the reason for the reduced translatability of mRNA of tissue culture cells that are removed from monolayer conditions and grown in suspension (Farmer et al., 1978). It is clear that direct measurements of sequence complexities and abundancies through hybridization analysis must be done to fully establish whether different mRNAs may be distributed differentially in the cytoplasm as our translation data suggest.

There are other mechanisms which may be operating in vivo to form free mRNPs; these include a role for the protein moiety of the RNP as a regulator of ribosome binding (Vincent et al., 1977), the presence of inhibitors which may act on selected messages (Heywood & Kennedy, 1976; Stewart et al., 1977; Bester et al., 1975; Weissbach & Ochoa, 1976), and requirements for specific initiation factors (Golini et al., 1976; Kabat & Chappel, 1977; Ilan & Ilan, 1976; Heywood & Kennedy, 1976) which may determine the extent of translation of specific RNA templates. Any of these mechanisms may be involved in the sea urchin, where there is certainly some discrimination in vivo since at the time of cellular fractionation the mRNA is distributed between polysomes and free RNPs. It is of interest that a discrimination also occurs in vitro in heterologous systems where polysomal mRNA is more effectively translated. Thus, in systems as diverse as the wheat germ, rabbit reticulocyte, and the sea urchin, there are components which do not interact with the free RNP RNA as well as with the polysomal RNA. It is difficult to argue that the lack of specific components in the three systems is responsible; rather it appears more likely that an intrinsic feature in the RNA is responsible for this discrimination.

It is necessary to be cautious in interpreting in vitro amino acid incorporation data when comparing different mRNA preparations since high levels of RNA may be inhibitory (Figure 3). For example, we found that the addition of relatively low levels of any class of sea urchin message interferes with the translation of a saturating level of globin mRNA in either the wheat germ or nuclease-treated reticulocyte system. This appears to be due to some kind of mass competition, where urchin message translation begins to replace the translation of the highly efficient globin templates. Overall incorporation is reduced as the globin band on $NaDodSO_4$ gels becomes lighter and urchin protein bands begin to appear (data not shown). It is possible therefore that the differences between polysomal and free RNP RNA at saturating levels of RNA (Figures 2 and 3) may reflect a difference in the inhibition of the system by each RNA. Two results argue against this. First, the rate of incorporation is directly proportional to the RNA added for both RNA classes below saturating concentrations (40 μg/mL). Also, the rates of protein synthesis at low levels of RNA are clearly different and support the results at saturating levels.

We sought to eliminate the possibility that an inhibitor was present in the free RNP RNA preparations which accounted for its reduced ability to support polypeptide synthesis compared to polysomal RNA. Such an inhibitor associated with free RNP RNA has been reported by Bester et al. (1975), who have characterized it and refer to it as translation-control RNA (tcRNA). These small RNAs are postulated to bind to poly(A) segments of mRNA and interfere with initation. Although the presence of an inhibitor was sometimes found in our poly(A−) fraction from free RNPs, the results (Figure 4) show that mixtures of nonsaturating levels of poly(A+) RNA yield levels of protein synthesis which are equivalent to the sum of levels yielded by each RNA tested separately, thereby indicating the absence of an inhibitor. It might be argued that an inhibitor which acts stoichiometrically (Heywood & Kennedy, 1976) may be reducing the apparent rate of protein synthesis. This is unlikely since the maximal rates of incorporation (at saturating RNA levels) are different. This consideration would also apply to the possibilities that the free RNP RNA is less stable in the in vitro systems or forms nontranslatable aggregates which the polysomal RNA does not. We cannot eliminate the unlikely possibilities that products of degradation or the possible aggregates are themselves stoichiometric inhibitors.

Previous work in our laboratory has shown that when mRNA enters the cytoplasm, it is immediately distributed between polysomes and free RNPs (Dworkin & Infante, 1976). The data here provide evidence that this process of selection for translation may be based at least in part on the ribosome-binding abilities of the mRNA molecules, which may be in competition for limited components of the translational apparatus. This model would predict that a certain percentage of any given message should be present in both free RNPs and polysomes (the quantitative distribution depending on the ribosome-binding ability of the message) and that the translation efficiency of such an individual message should be constant regardless of which cytoplasmic compartment it is isolated from. This suggestion is strongly supported by the finding that the ribosome-binding ability of the putative 9S histone mRNA (from cleavage stage embryos) is the same whether isolated from polysomes or free RNPs (Figure 6).

The presence of free RNPs and a nonrandom distribution of mRNA in polysomes and free RNPs may be postulated to be critical factors in assuring the synthesis of adequate levels of specific proteins. This hypothesis would suggest that the balanced production of specific proteins through early development depends not only upon the relative concentrations of specific mRNAs but also upon the relative efficiencies of the mRNAs to be translated. The kind of translational control being implied here for sea urchin messengers has been implicated with respect to histone mRNAs in the developing clam (Gabrielli & Baglioni, 1977). It has been suggested that the translational capacity in this sytem has been exceeded by the amount of available mRNA and that under these conditions the "stronger" histone messages are preferentially translated. We have found that the histone mRNA is in fact much more concentrated in the polysomes (Dworkin & Infante, 1976), suggesting that these templates are very efficiently utilized. Recently we have found, by gel electrophoresis of the 9S histone RNA from the blastula stage, that the free RNPs relative to polysomes are greatly enriched for H1 histone RNA. This difference may be the reason for the differences observed in the analysis of the in vitro translation products where the putative H1 histone relative to the other histones is greater with the free RNP RNA (Figure 8). If the hypothesis above is correct, it would be predicted that the H1 histone mRNA is less efficiently translated than the other histone mRNAs.

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