Translational Control in Early Sea Urchin Embryogenesis: Initiation Factor eIF4F Stimulates Protein Synthesis in Lysates from Unfertilized Eggs of *Strongylocentrotus purpuratus*†

Alina C. Lopo,* Susan MacMillan, and John W. B. Hershey
Department of Biological Chemistry, University of California School of Medicine, Davis, California 95616
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ABSTRACT: We have used cell-free translation systems from unfertilized eggs and embryos of the sea urchin *Strongylocentrotus purpuratus* to analyze the mechanisms limiting protein synthesis in early embryogenesis. Unfertilized egg lysates supplemented with nuclease-treated reticulocyte lysate were stimulated 2–4-fold in incorporation of radioactive amino acid into protein. Thirty-minute zygote lysates supplemented in this way were not stimulated. These results suggested that a component limiting translation in the unfertilized egg lysate was provided by the nuclease-treated lysate and that this component was no longer limiting protein synthesis following fertilization. In view of these results, partially fractionated lysates and individual purified translational components from mammalian cells were tested for stimulation of the unfertilized egg lysate. A 100000*g* supernatant devoid of ribosomal subunits also stimulated the unfertilized egg lysate. Thus, the stimulation was not due to the addition of active ribosomal subunits but to soluble elements in the reticulocyte lysate. Of the soluble components tested, only the cap-binding protein complex eIF4F caused a dramatic stimulation of the unfertilized egg lysate (2–3.5-fold). The 30-min zygote lysate was not stimulated by eIF4F or by any of the other components tested, supporting the hypothesis that a block in the translational machinery is removed at fertilization. A rabbit reticulocyte shift assay was used to analyze whether mRNA is limiting in early development. When unfertilized egg lysate was added to the shift assay, there was no shift in radioactivity from 43S to 80S complexes, indicating the unfertilized egg mRNA is not available for translation. These results suggest that there are at least two mechanisms of translational control in early embryogenesis: one operating at the level of the translational machinery, via initiation factor 4F, and the other regulating mRNA availability.

Following fertilization in sea urchins, there is a rapid increase in the rate of protein synthesis that is maintained throughout early development (Epel, 1967; Goustin & Wilt, 1981). Since this translational activation was first described 35 years ago (Hultin, 1952), it has been extensively documented in the eggs and embryos of vertebrates and invertebrates alike (Davidson, 1982, 1986). In sea urchins, the increase in protein synthesis at fertilization begins several minutes after sperm–egg fusion (Epel, 1967). Within 1 h after fertilization, protein synthesis has increased to 10–15 times the rate in the unfertilized egg, and within several hours, this figure has increased to 100-fold. This increase in protein synthesis is reflected both in measurements of incorporation of radiolabeled amino acids into protein and in the recruitment of ribosomes into polysomes (Humphreys, 1969, 1971). Prior to fertilization, less than 1% of the large number of free ribosomes in the egg are in polysomes. By 2 h after fertilization, 20% have been recruited, and by 15 h, 60% are in polysomes (Humphreys, 1971).

Since the increase in the rate of protein synthesis following fertilization involves preformed mRNAs and ribosomes, regulation appears to be at the level of translation. In spite of the large body of information on the biochemical detail of the translational activation, there is only limited understanding of the molecular mechanism regulating this activation. The utilization of maternal mRNAs in cytoplasmic ribonucleoprotein (mRNP)[1] particles is an important feature of this event (Spirin, 1966; Gross, 1968; Raff, 1980, 1983). Two general hypotheses have been proposed to explain the translationally repressed states of the unfertilized egg and very early embryos: (1) mRNAs stored in the cytoplasm are "marked" and therefore rate limiting; (2) some aspect of the translational machinery limits protein synthesis.

Cell-free translation systems, particularly the rabbit reticulocyte lysate, have proven extraordinarily useful in the analysis of the individual reactions as well as the requirements of protein synthesis [e.g., see Jackson and Hunt (1983) and Eisenstein and Harper (1984)]. Several groups have developed cell-free systems and systems consisting of partially purified components from unfertilized eggs, and useful information has been derived from these studies [e.g., see Gambino et al. (1973) and Ilan and Ilan (1978)]. However, these systems have generally suffered from limited activity. An exception has been the cell-free system from unfertilized eggs of the sea urchin *Lytechinus pictus* developed by Winkler and Steinhardt (1981). Using this system, Winkler et al. (1985) presented evidence for more than one level of translational control operating at fertilization. Their evidence suggested that in the

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* Correspondence should be addressed to this author at the Division of Biomedical Sciences, University of California, Riverside, CA 92521-0121.

[1] Abbreviations: ASW, artificial seawater; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; eIF, eukaryotic initiation factor; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; m^{7}GTP, 7-methylguanosine 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; mRNP, cytoplasmic ribonucleoprotein; DTE, dithioerythritol.

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unfertilized egg translation is limited at the level of mRNA availability as well as at the level of the translation machinery.

We are interested in identifying the specific mechanisms regulating this translational activation. We have chosen two approaches to this problem: (1) a biochemical dissection of the translational machinery, with a special focus on initiation of protein synthesis (Lopo & Hershey, 1985b; Lopo et al., 1986); and (2) development and utilization of cell-free translation systems from eggs and embryos. The system we have chosen is the common purple sea urchin *Strongylocentrotus purpuratus*. Purification of translational components is feasible from *S. purpuratus* because of the very large quantities of gametes which are easily and inexpensively obtained. Work on the purification and characterization of the translational machinery in eggs and embryos of this species is already under way in our laboratory (Lopo & Hershey, 1985b; Lopo et al., 1986). A cell-free translation system from this species would provide an important, complementary research tool, since it would permit us to carry out both the purification work and mechanism studies on the same species.

In this paper, we use cell-free translation systems from unfertilized eggs, 30-min zygotes, and hatched blastulae of *S. purpuratus* to show that translational initiation factor eIF4F, the cap-binding complex, plays a significant role in translational control in the unfertilized egg. We also present evidence for a second mechanism of translational control operating at fertilization, which regulates mRNA availability.

Materials and Methods

General Methods. (*A*) *Reagents.* All reagents were from Sigma Chemical Co. except as follows. Artificial seawater (ASW;[1] "Instant Ocean") was from Aquarium Systems, Inc. RNasin, the ribonuclease inhibitor from human placenta, was from Amersham.

(*B*) *Protein Determination.* Protein concentration was determined by using the method of Bradford (1976) with the protein assay dye reagent concentrate from Bio-Rad Laboratories, Richmond, CA.

(*C*) *SDS–PAGE.* Polyacrylamide gel electrophoresis was as described by Laemmli (1970). For autoradiograms, Kodak X-Omat AR film was used.

Cells. Gametes were obtained from adult sea urchins (*Strongylocentrotus purpuratus*) by pouring 0.5 M KCl into opened body cavities. Eggs were pooled and washed by settling in 5 volumes of ASW at 4 °C. The settled eggs were dejellied by resuspension in 10 volumes of pH 4.5 ASW (4 °C) for 5 min, with gentle stirring. The pH was neutralized by addition of 1 M Hepes, pH 7.4. The eggs were then washed twice in about 10 volumes of 4 °C ASW and held at 4 °C until used (but never for longer than 4 h).

To prepare unfertilized egg lysates, unfertilized eggs were obtained as described above and processed as outlined below [see (B) under Cell-Free Translations]. To obtain 30-min zygotes, unfertilized eggs were resuspended to a final concentration of 1% in 15 °C ASW containing 50 mM 3-amino-1,2,4-triazole to prevent hardening of the fertilization envelope (Foerder & Shapiro, 1977) and thus permit homogenization. The eggs were fertilized and incubated 30-min at 15 °C, and zygotes were collected by gentle hand centrifugation and immediately chilled. To obtain hatched blastulae, unfertilized eggs were fertilized and incubated as a 0.5% suspension at 15 °C until >90% were hatched (about 20 h) in ASW containing 50 mg/L each penicillin and streptomycin. To collect the hatched embryos, the culture was chilled by adding frozen ASW pellets and then passed through a 52-μm Nitex filter.

Cell-Free Translations. (*A*) *pH 7.2 Buffer.* The pH 7.2 buffer is a modification of the pH 7.4 buffer described by Winkler and Steinhardt (1981). The pH 7.2 buffer is 40 mM NaCl, 106 mM potassium gluconate, 263 mM glycerol, 300 mM glycine, 50 mM Hepes, 10 mM EGTA, 7.3 mM $CaCl_2$, 0.52 mM $MgCl_2$, and approximately 50 mM KOH added to adjust the pH to 7.2. The pH of the buffer was adjusted at 15 °C. This combination of Ca^{2+}, Mg^{2+}, and EGTA yields a final concentration of 5×10^{-7} M free Ca^{2+} and 0.5 mM free Mg^{2+}, at pH 7.2, 15 °C. Prior to use, 1 mg/mL soybean trypsin inhibitor and 0.5 mg/mL reduced glutathione were added to the buffer. These values produced lysates of all three stages maximally active in protein synthesis [the complete characterization and optimization of these lysates are described in a separate manuscript (A. C. Lopo et al., unpublished results)].

(*B*) *Lysate Preparation.* The procedure we used is a modification of the method described by Winkler and Steinhardt (1981) for *Lytechinus pictus*. Two milliliters of unfertilized eggs or embryos of the appropriate stage was washed twice in 6 volumes of ice-cold pH 7.2 buffer with soybean trypsin inhibitor and glutathione and then resuspended 1:1 in the same buffer. RNasin was added to a final concentration of 300–350 units/mL, and the cell suspension was transferred to a 7-mL stainless-steel homogenizer. It was necessary to include RNasin in the lysates to obtain consistently active lysates. This is presumably due to the high level of endogenous ribonucleases in *S. purpuratus* eggs and embryos. The cells were broken with three to four strokes. There is a distinct change in color of the suspension to a milky yellow when the cells break. The homogenate was immediately transferred to 1.5-mL capped tubes and centrifuged 5 min at 15000*g*. Following a 1-h dialysis against pH 7.2 buffer with reduced glutathione (0.5 mg/mL), the supernatant was frozen as 100–400-μL aliquots in liquid nitrogen and stored at −80 °C (for up to 6 months) or in liquid nitrogen. The protein concentration was 18–20 mg/mL for lysates of all three stages prepared as described above.

The greatest variability in the activity in lysates of a given stage was among the blastula lysates. We are not certain of the cause. This may be due to breakdown of polysomes during the concentration of the blastulae, since this can be a rather lengthy process because they are swimming. Recently (A. C. Lopo and J. W. B. Hershey, unpublished results), we have found that similar results to those presented here are obtained with blastulae collected prior to hatching. These prehatched blastula lysates also show less variation in activity and incorporate radiolabeled amino acid at a consistently higher level than the lysates prepared from hatched blastulae. This is probably due to the more rapid preparation of the lysate.

(*C*) *Cell-Free Translation Systems.* The lysates were rapidly thawed, and the desired volume was transferred to a sterile 10 × 75 mm culture tube and kept on ice. Buffer or the experimental sample was added to the lysate (in a 10% volume), and the mixture was allowed to equilibrate to 15 °C for 5 min. All translations were performed at 15 °C, except where noted. A 10% volume of master mix was then added. The time of master mix addition was considered the start of the translation (time zero). Samples were taken by using a Drummond precision pipet (0–10 μL) at the desired time intervals. The sample was diluted into 0.5 mL of distilled water, and 1 mL of 20% TCA containing 1 mg/mL unlabeled methionine was added immediately. The samples were then stored on ice for 1 h, heated to 90 °C for 20 min, chilled, and collected on GF/A filters (Whatman). The filters were washed

with 5–10 mL of cold 10% TCA, rinsed with about 5 mL of 95% ethanol, and dried, and radioactivity was determined by using a Beckman LSC 9000 instrument. The master mix was prepared fresh each time from stock solutions to a final concentration in the lysate of 2 mM ATP, 0.8 mM GTP, 20 mM creatine phosphate, 10 units/mL creatine phosphokinase, 50 μM glucose 6-phosphate, 50 μM each of 19 amino acids (minus the label), 0.2 μM [^{35}S]methionine (specific activity 1100 Ci/mmol), 0.1 mg/mL leupeptin, and 0.1 mg/mL pepstatin. Magnesium chloride (2.8 mM final concentration) was included in the stock solutions to account for nucleotide binding. Inclusion of leupeptin and pepstatin was required to prevent an apparent degradation of labeled protein, as indicated by a decrease in radiolabel incorporated after about 45 min of incubation.

Purified Translational Components. Purified eIF4F and eIF2B were prepared essentially as described by Grifo et al. (1983) and Konieczny and Safer (1983). Other purified initiation factors from rabbit reticulocytes or HeLa cells were prepared as described in Benne et al. (1970). Rabbit reticulocyte lysate and nuclease-treated reticulocyte lysate were prepared as described by Jackson and Hunt (1983). Globin mRNA was purified as described Benne et al. (1979). pH 5 enzyme fraction [the dissolved pH 5 precipitate of a postribosomal (100000*g*) supernatant, which contains tRNAs, aminoacyl-tRNA synthetases, and elongation and termination factors required for complete translation] was prepared as described by Falvey and Staehelin (1970) for rabbit reticulocyte and by Lopo et al. (1986) for sea urchin (*S. purpuratus*) eggs and embryos. [^{35}S]Met-tRNA$_i$ was prepared as described by Stanley (1974).

Shift Assays. Shift assays were performed as described by Darnbrough et al. (1973) with modifications. This assay measures the shift in radioactivity from 43S preinitiation complexes into 80S initiation complexes in an elongation-inhibited lysate. A reticulocyte lysate was elongation-inhibited using 0.1 mM emetine and incubated for 3 min at 30 °C. [^{35}S]Met-tRNA$_i$ was added, and the mixture was incubated another 2 min. The experimental sample, not exceeding 10% of the total volume (100 μL), was then added. The mixture was further incubated at 30 °C, and after 2 min, the reaction was stopped by adding 5 volume of chilled buffer. The sample was layered onto 15–40% sucrose gradients (250 mM KCl, 25 mM $MgCl_2$, 50 mM Hepes, pH 7.2, 2 mM DTE, 0.1 mM emetine, and 10 mM benzamidine) and centrifuged in a Beckman SW60 rotor at 4 °C for 2 h and 10 min. The gradients were fractionated by pumping from the bottom using a small capillary, and individual fractions (ca. 30) were analyzed for absorbance at 260 nm and for radioactivity by counting with a 1:9 mixture of water/Aquamix in a Beckman LSC 9000 instrument.

Results

The lysates we have prepared initiate protein synthesis (Figure 3, discussed below) and respond to external stimuli in many ways similar to the intact eggs and embryos [the complete characterization and optimization of these lysates are described in a separate manuscript (A. C. Lopo et al., unpublished results)]. Thus, these lysates should be useful tools for the analysis of the mechanisms limiting protein synthesis at fertilization.

Is the Translational Machinery Limiting during Early Embryogenesis? If a translational component other than mRNA is limiting, supplementing the lysates with whole or partial fractions of an active translation system may provide sufficient amounts of the missing component to stimulate the

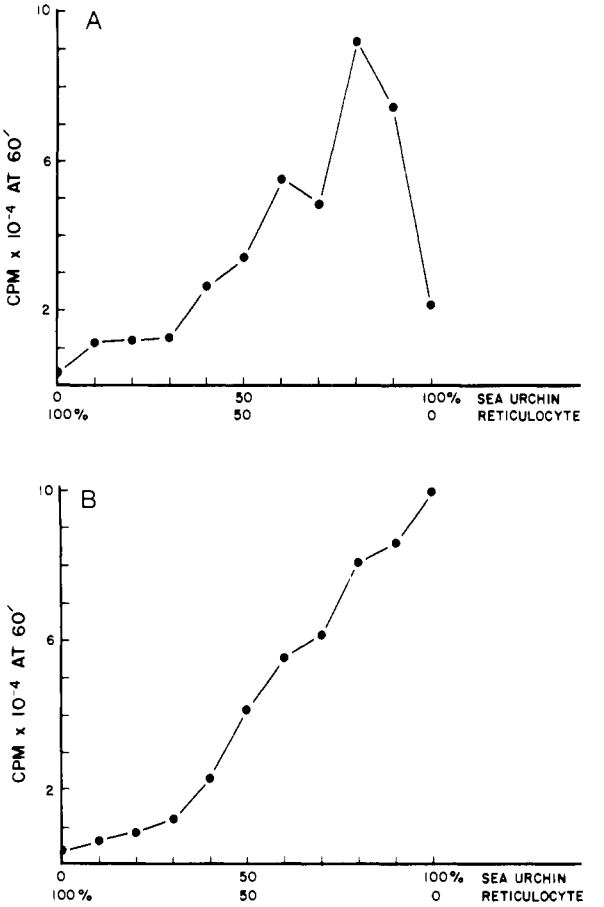

FIGURE 1: Response of the *S. purpuratus* lysates to nuclease-treated rabbit reticulocyte lysate. Sea urchin lysates were partially replaced with varying amounts of nuclease-treated reticulocyte lysate (abscissa), and incorporation of [^{35}S]methionine at 60 min was determined as described (Materials and Methods). The radiolabel concentration was constant for all time points. Sample volume, 10 μL. (Panel A) Unfertilized egg lysate; (panel B) 30-min zygote lysate. Although there is some variability from preparation to preparation, the stimulation by the nuclease-treated reticulocyte lysate is always severalfold.

lysates. To test this, the *S. purpuratus* lysates were supplemented with nuclease-treated rabbit reticulocyte lysate, which contains no mRNA but is otherwise intact with respect to the translational machinery. Although evolutionarily distant, the translational components of the two species are completely interchangeable (Lopo et al., 1986).

When the unfertilized egg lysate was supplemented with a small amount (10–30%) of nuclease-treated reticulocyte lysate, there was a 2–4-fold stimulation of incorporation of radiolabel into protein (Figure 1A). The degree of stimulation varied with different preparations of the sea urchin lysate or the reticulocyte lysate. Since the reticulocyte lysate used to supplement the unfertilized egg lysate was nuclease treated, the stimulation was due entirely to translation of sea urchin mRNA. However, when the 30-min zygote lysate was supplemented in the same way, no stimulation was observed (Figure 1B). These results suggest that the nuclease-treated lysate provides a component that is limiting translation in the unfertilized egg lysate. The absence of stimulation of the postfertilization lysates by reticulocyte lysate suggests that the component provided by the reticulocyte lysate is no longer limiting protein synthesis following fertilization.

It is interesting to note that in the translations composed of 10–40% sea urchin and 60–90% reticulocyte lysate (left side of the graph), there is only limited incorporation of radiolabel. The fraction of unfertilized egg or embryo lysate added in these

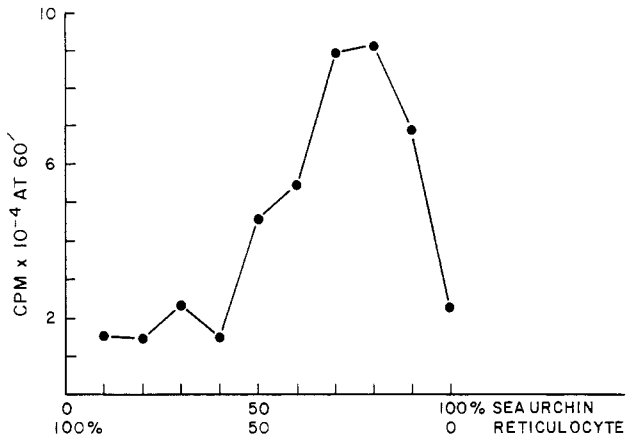

FIGURE 2: Response of the *S. purpuratus* unfertilized egg lysate to a high-speed supernatant from nuclease-treated reticulocyte lysates. The unfertilized egg lysate was partially replaced with varying amounts of high-speed supernatant (S-100) from nuclease-treated reticulocyte lysate (abscissa), and incorporation of [^{35}S]Met at 60 min was determined as described under Materials and Methods. The concentration of radiolabel was constant for all time points.

cases contains amounts of mRNA expected to stimulate the reticulocyte lysate to a degree much greater than observed. This could be due to the presence of an inhibitor of a specific step in the initiation pathway present in the sea urchin lysate. A nuclease-sensitive inhibitor of translation has recently been reported by Mandley and Lopo (1987). An inhibitory activity has also been reported by Hansen et al. (1987). A second possibility is that much of the sea urchin mRNA is unavailable to the reticulocyte translational machinery. This question is explored below in the studies using the shift assay.

Limiting Translational Component May Be an Initiation Factor. To find out whether the stimulation was due to the addition of active ribosomal subunits or soluble elements in the reticulocyte lysate, we prepared a high-speed supernatant (100000*g* or S-100) from the nuclease-treated reticulocyte lysate. This high-speed supernatant, which is devoid of ribosomal subunits, was then used to supplement the unfertilized egg lysate as described above. Here, too, there was a 2–4-fold stimulation of incorporation (Figure 2), similar to that seen with the nuclease-treated lysate. In view of these results, we tested subfractions of lysates or individual purified translational components from mammalian cells for stimulation of the unfertilized egg and 30-min zygote lysates. The lysates were supplemented with sea urchin and mammalian pH 5 enzyme fraction, purified salt-washed mammalian ribosomes, or initiation factors from HeLa cells or rabbit reticulocytes. The addition of a pH 5 fraction [the dissolved pH 5 precipitate of a postribosomal (100000*g*) supernatant, which contains tRNAs, aminoacyl-tRNA synthetases, and elongation and termination factors required for complete translation] from reticulocytes stimulated the unfertilized egg lysate only marginally (to 148%), whereas comparable fractions from sea urchin eggs or embryos were without significant effect (Table I). Likewise, mammalian ribosomal subunits did not affect the unfertilized egg lysate. We next tested individual initiation factors purified from HeLa cells. The addition of seven initiation factors (eIF2, eIF2B, eIF3, eIF4A, eIF4B, eIF4C, or eIF5) resulted either in no effect or in a very minor stimulation (up to 140%; Table I). In particular, eIF2 produced no stimulation, in contrast to earlier results with an *L. pictus* lysate in which exogenous eIF2 stimulated protein synthesis slightly to 57% (Winkler et al., 1985). When the cap-binding protein complex, eIF4F, is tested, a much more dramatic stimulation is observed: addition of eIF4F stimulates 2–3.5-fold. The extent of stimulation is not altered significantly by adding various combinations of other initiation factors implicated in mRNA binding to 40S ribosomal subunits (eIF4A, eIF4B). The 30-min zygote lysate was *not* stimulated by eIF4F or by any of the other components tested (Table I). These results suggest that, in the unfertilized egg lysate, but not in the postfertilization lysate, eIF4F is limiting protein synthesis at the level of initiation. Addition of eIF4F in combination with other mRNA-binding initiation factors did not stimulate beyond the levels when eIF4F alone was added.

Table I: Effect of Purified Mammalian Translational Components on Sea Urchin Lysates[a]

translational component[b]	unfertilized egg	30-min zygote
control[c]	100	100
pH 5 (reticulocyte; 68 μg)	148	126
pH 5 (unfertilized egg; 16 μg)	113	104
pH 5 (30-min zygote; 43 μg)	102	85
pH 5 (hatched blastula; 43 μg)	94	102
eIF2 (8 μg)	93	120
eIF2B (2 μg)	104	118
eIF2 + eIF2B (8 μg + 2 μg)	126	119
eIF3 (5 μg)	115	115
eIF4A (1.2 μg)	81	127
eIF4B (1.2 μg)	133	116
eIF4C (0.2 μg)	140	106
eIF5 (1.4 μg)	136	110
eIF4F (1 μg)	253	126
eIF4F (1 μg)	326	113
eIF4F (1.5 μg)	356	111
eIF4F (1.5 μg)	195	92
40 S (0.2 unit)	106	96
60 S (0.50 unit)	113	109
40 S + 60 S (0.2 unit + 0.50 unit)	89	121

[a] Purified mammalian translational components were added to the three sea urchin lysates, and incorporation of radioactive amino acid into protein at 60 min was determined. Stimulation is expressed as percent stimulation over control. All translations were carried out at 15 °C. The values of each translational component added are representative of individual experiments. Varying amounts of these components were added without significantly affecting the level of incorporation. The sources of mammalian translational components were the following: pH 5, rabbit reticulocytes; eIF2, eIF2B, eIF3, eIF4A, eIF4C, and eIF5, rabbit reticulocytes; eIF4B and eIF4F, HeLa cells; 40S and 60S ribosomal subunits, rat liver. [b] The amounts of initiation factors, reticulocyte pH 5 enzyme fraction, or ribosomal subunits added are based on the levels used in the defined rabbit reticulocyte globin synthesis assay for measuring protein synthesis stimulation (Benne et al., 1979). Values given in parentheses are in micrograms of protein per 100-μL cell-free translation system for the initiation factors and pH 5 enzyme fractions added and in A_{260} units per 100-μL cell-free translation system for the ribosomal subunits added. The amounts of sea urchin pH 5 enzyme fraction added were derived from optimal values in the globin synthesis assay (Lopo et al., 1986). [c] Control values (in cpm/10 μL); unfertilized egg = 28 494, 30-min zygote = 75 078.

Is Messenger RNA a Limiting Component? The large stores of mRNA in the cytoplasm of animals eggs led to the formulation of the "masked message" hypothesis (Spirin, 1966; Raff, 1980), which states that the stored mRNA in the egg is inactive due to a masking factor(s) associated with it and that the carefully regulated removal of this masking factor at fertilization and during early development allows the mRNA to be translated. If messenger RNA is a limiting component, supplementing the lysates with mRNA should result in an increase in the total incorporation of radiolabeled amino acid. Purified globin mRNA (to a final globin mRNA concentration of 100–150 μg/mL) was added to the unfertilized egg and 30-min zygote sea urchin lysate. Both lysates utilize the globin

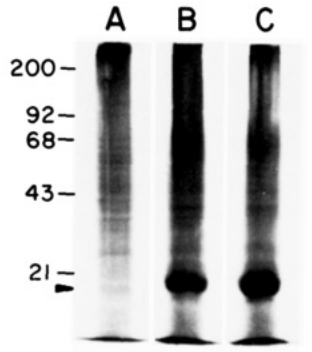

FIGURE 3: Lysates from *S. purpuratus* utilize exogenous mRNA. Purified globin mRNA (133.3 μg/mL) was added to unfertilized egg and 30-min zygote lysates, and the mixtures were incubated for protein synthesis as described under Materials and Methods. Samples (2.5 μL) were taken for SDS–PAGE (Laemmli, 1979) and autoradiograms prepared. Lane A, unfertilized egg (buffer); lane B, unfertilized egg lysate (globin mRNA); lane C, 30-min zygote (globin mRNA).

mRNA, as evidenced by autoradiography of the translation products (Figure 3). This also shows clearly that the lysates are capable of initiation. Overall, protein synthesis in the unfertilized egg lysate is not stimulated by the exogenous mRNA, suggesting that message availability is not the primary control imposed on translation in the unfertilized egg. However, the 30-min zygote lysate is stimulated about 30% by the exogenous mRNA. This is approximately equivalent to a 2-fold increase over the level of mRNA in the lysate. The stimulation of translation by exogenous mRNA in the 30-min zygote lysate suggests that message availability may play a role in limiting protein synthesis postfertilization.

We have used an initiation assay ("shift assay"; Darnbrough et al., 1973) to ask whether message is also limiting in the unfertilized egg of *S. purpuratus*. This assay measures the shift in radioactivity from labeled 43S preinitiation complexes into 80S initiation complexes following addition of mRNA (for additional details on the shift assay, see Materials and Methods). The main advantage of using the shift assay is that it specifically assays for the message-binding step of initiation. Furthermore, it is a very rapid assay, since the reaction is stopped within 2 min of adding the experimental sample. This eliminates effects on the sample of long-term incubation, as may occur in 60-min incubations routinely used to assay for overall protein synthesis.

When purified globin mRNA is added to the reticulocyte lysate, the expected shift in radioactivity occurs (Figure 4A). However, when unfertilized egg lysate was added, there is no shift in the radioactivity from 43S complexes, suggesting the mRNA in the sea urchin preparation is not being "recognized" by the reticulocyte lysate (Figure 4B). In contrast, adding the blastula lysate causes the shift (Figure 4C). We believe that the blastula lysate is active in the shift assay because it contains much more mRNA undergoing translation. This mRNA is likely to join complexes and shift readily.

Discussion

We have developed and characterized cell-free translation systems from unfertilized eggs, 30-min zygotes, and hatched blastulae of *S. purpuratus*. They are capable of initiating protein synthesis on both endogenous and exogenous mRNAs and produce full-length polypeptide products. These in vitro systems from both unfertilized eggs and postfertilization stages provide the means to analyze differences in the mechanism of protein synthesis before and after fertilization.

The results presented here show that translational control at fertilization is effected at two levels: at the level of the activity of the protein synthetic machinery and at the level of mRNA availability. That the protein synthetic machinery may be limiting is demonstrated by the experiments in which nuclease-treated reticulocyte lysate was added to the sea urchin lysates, with the resulting stimulation of the unfertilized egg lysate. It is significant that the 30-min zygote lysate was not stimulated by the reticulocyte lysate. An attractive interpretation is that there is a limiting component provided to the unfertilized egg lysate by the reticulocyte lysate that is no longer limiting postfertilization. Therefore, there is no stimulation when the mammalian lysate is added to embryo extracts. In similar supplementation experiments using a cell-free system from unfertilized eggs of *Lytechinus pictus*, Winkler et al. (1985) observed a similar, though somewhat smaller, stimulation. Experiments with the 30-min zygote lysate have not been reported with *L. pictus*. Finally, Colin and Hille (1986) microinjected globin mRNA into intact unfertilized eggs and did not observed an increase in the level of protein synthesis, thereby supporting the view that the activity of the translational machinery may be limiting in the unfertilized egg.

The evidence presented here suggests that eIF4F may be the component provided to the unfertilized egg lysate by the

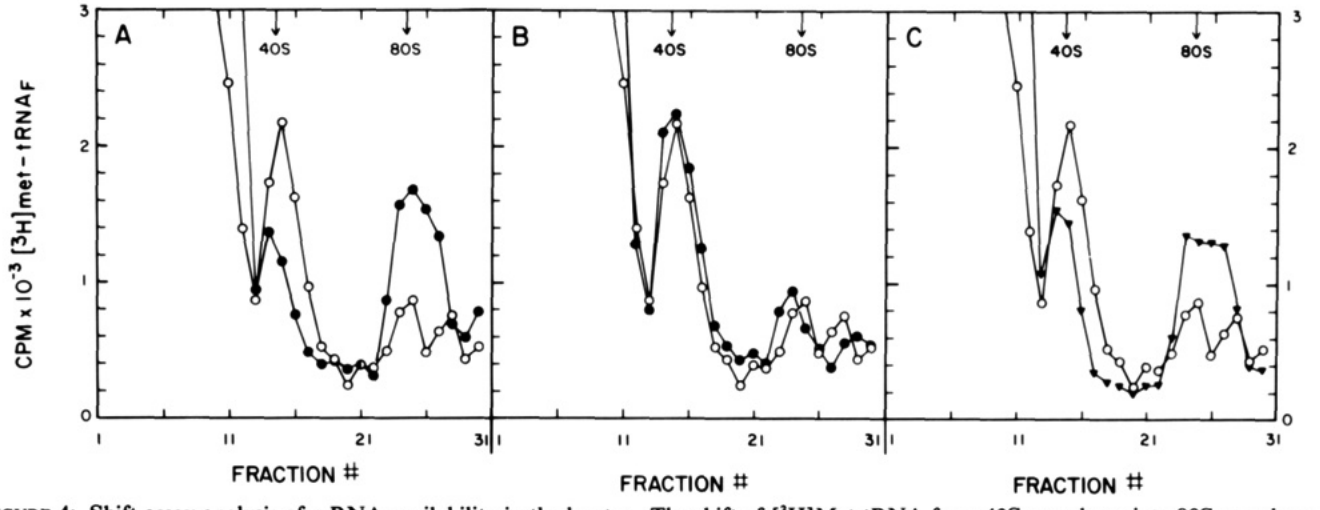

FIGURE 4: Shift assay analysis of mRNA availability in the lysates. The shift of [^{3}H]Met-tRNA from 43S complexes into 80S complexes in an elongation-inhibited reticulocyte lysate was measured by separating the incubation mixtures in sucrose gradients and determining the radioactivity in individual fractions (abscissa). For additional details, see Materials and Methods. (Panel A) (○) Buffer control; (●) globin mRNA. (Panel B) (○) Buffer control; (●) unfertilized egg lysate. (Panel C) Blastula lysate (10%); (○) buffer control; (▲) blastula lysate (10% volume). All incubations were at 37 °C.

nuclease-treated reticulocyte lysate. Since the stimulation by eIF4F is comparable to that by the whole lysate, it is possible but not certain that eIF4F activity may be the only translational component limiting in the egg. Mammalian eIF4F (the cap-binding protein complex, CBP-II) is composed of three subunits: p220, p46 (eIF4A), and p28 (eIF4E or CBP-1). It binds to the 5′ terminus of capped mRNAs and is implicated in melting out mRNA secondary structure (Ray et al., 1985). Stimulation by eIF4F is interesting in the context of previous work by Winkler et al. (1983a,b), who presented evidence for an absolute requirement for the 5′-methylated cap structure in sea urchins. In related experiments using the *Lytechinus pictus* unfertilized egg cell-free system, Winkler et al. (1985) found modest stimulation with eIF2 (1.57-fold). These researchers did not test eIF4F, which was not available to them at that time. eIF4F levels may be limiting for protein synthesis in mammalian cells (Duncan et al., 1987). It has been identified as the limiting initiation factor involved in discrimination of mRNAs for translation (Ray et al., 1983). Panniers et al. (1985) have shown that eIF4F is involved in the translational shutoff in heat-shocked cells. We have recently shown that the p28 subunit of HeLa eIF4F is a phosphoprotein and that the extent of phosphorylation is altered following heat shock of HeLa cells (Duncan et al., 1987). The regulation of eIF4F activity by phosphorylation following fertilization of sea urchin eggs is an attractive hypothesis that currently is being studied (Waltz and Lopo, unpublished results).

The results presented here also are consistent with a second mechanism of translational control operating at fertilization and during early development, i.e., the regulation of mRNA availability. Our results using the shift assay support the idea that mRNA availability is also limiting protein synthesis in the unfertilized egg. This is consistent with what is seen in vivo. At fertilization, mRNA begins to be mobilized into polysomes, but it is several hours before the rate of protein synthesis reaches its maximum level of about 100-fold that of the unfertilized egg.

Another approach used to address the question of mRNA "masking" has been to purify mRNPs and deproteinized mRNAs from unfertilized eggs and embryos and test their "translatability" [e.g., see Jenkins et al. (1978), Kaumeyer et al. (1978), Young and Raff (1979), Moon et al. (1981, 1982), and Raff et al. (1981)]. These experiments have yielded conflicting results, primarily because of the uncertainty that the isolated material is identical with what is found in the cell. A third, possibly more clear-cut approach to this problem has been the use of microinjection into *Xenopus* oocytes (Lingrel & Woodland, 1974; Laskey et al., 1977; Richter & Smith, 1981) and sea urchin eggs (Colin & Hille, 1986). Colin and Hille (1986) injected *S. droebachiensis* unfertilized eggs and zygotes with globin mRNA and found that it was utilized by both stages. The globin message competed more efficiently for the zygote translational machinery, although total protein synthesis was not stimulated by the injected globin mRNA in either unfertilized eggs or zygotes. This suggested that mRNA availability may not be limiting pre- or postfertilization. The microinjection experiments support our results with the unfertilized egg. Additional work (e.g., using different mRNAs) in both the in vivo and the cell-free systems is necessary to understand mRNA mobilization further.

The shift assay results presented here indicate that the levels of competent mRNAs are low and thereby support the idea that mRNA is also limiting *before* fertilization. On the other hand, the lysate mixing experiments support the idea that some mRNA is available to be utilized but that the limiting step involves a component of the translational machinery (likely eIF4F). How can these results be reconciled? The answer may be found in the time courses of the various assays. The shift assay is a very rapid assay. The reaction is stopped within 2 min of adding the experimental sample. The translation assays used to assess the stimulation by nuclease-treated reticulocyte lysate measure incorporation after 60 min. Analyzing the time course of incorporation shows that there is a lag before the stimulation can be detected, suggesting that there may be a lag during which changes are taking place in the lysate (an "unmasking"?) that then permit the stimulation to take place. This is not due to warming of the lysates, since the lysates are preequilibrated to the translation temperature (15 °C) prior to beginning the translation.

Two experiments indicate that the mRNA in sea urchin mRNPs is not recognized by the mammalian translational machinery: the lysate mixing experiments (Figure 1) in which 90% reticulocyte lysate is barely stimulated by 10% sea urchin lysate even though the amount of mRNA added was expected to provide a large stimulation and the shift assay experiments (Figure 4) in which sea urchin unfertilized egg lysate mRNAs fail to bind to 43S preinitiation complexes. Both experiments are clear indications that the sea urchin mRNAs are functionally repressed in vitro and suggest that they are masked. An alternate explanation is that an inhibitor of protein synthesis is present in the egg and 30-min zygote lysates that blocks protein synthesis on otherwise competent mRNAs. Recent evidence has demonstrated the presence of a nuclease-sensitive inhibitor of translation in unfertilized eggs of *L. pictus* (Mandley & Lopo, 1987). However, preliminary evidence indicates that this inhibitor is not operating by regulating message availability (Mandley and Lopo, unpublished observations). Jagus and co-workers (Hansen et al., 1987) have also reported the presence of a translational inhibitor in sea urchin eggs operating at a step subsequent to message binding.

Thus, dual controls limit the level of protein synthesis in sea urchin eggs. The translational machinery is limiting, at least in part, by the activity of eIF4F, but when this is relieved by addition of exogenous eIF4F, the overall system then becomes limited by the availability of mRNA. The system, therefore, is well-balanced, both machinery and mRNA being close to limiting. Following fertilization, the potential of the zygote to initiate is increased, but the overall activity for translation is primarily limited by mRNA availability, which increases in a steady, well-controlled way during the early cleavage stages of development.

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REFERENCES

Benne, R., Brown-Luedi, M. L., & Hershey, J. W. B. (1979) *Methods Enzymol. 60*, 15–35.

Brandis, J., & Raff, R. A. (1978) *Dev. Biol. 67*, 99–113.

Brandis, J., & Raff, R. A. (1979) *Nature (London) 278*, 467–469.

Colin, A. M., & Hille, M. B. (1985) *Dev. Biol. 115*, 184–192.

Danilchik, M. V., & Hille, M. B. (1981) *Dev. Biol. 84*, 291–298.

Darnbrough, C., Legon, S., Hunt, T., & Jackson, R. J. (1973) *J. Mol. Biol. 76*, 379–403.

Davidson, E. H. (1986) *Gene Activity in Early Development*, 3rd ed., Academic Press, New York.

Davidson, E. H., Hough-Evans, B. R., & Britten, R. J. (1982) *Science (Washington, D.C.) 217*, 17–26.
Duncan, R., Milburn, S., & Hershey, J. W. B. (1986) *J. Biol. Chem. 262*, 380–388.
Eisenstein, R. S., & Harper, A. E. (1984) *J. Biol. Chem. 259*, 9922–9928.
Epel, D. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 899–906.
Falvey, A. K., & Staehelin, T. (1970) *J. Mol. Biol. 53*, 1–19.
Foerder, C. A., & Shapiro, B. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4214–4218.
Gambino, R., Metafora, S., Felicetti, L., & Raisman, J. (1973) *Biochim. Biophys. Acta 312*, 377–391.
Goustin, A. S., & Wilt, F. H. (1981) *Dev. Biol. 82*, 32–40.
Grifo, J. A., Tahara, S. M., Organ, M. A., Shatkin, A. J., & Merrick, W. C. (1983) *J. Biol. Chem. 258*, 5804–5810.
Gross, P. R. (1968) *Curr. Top. Dev. Biol. 1*, 1–46.
Hansen, L. J., Huang, W.-I., & Jagus, R. (1987) *J. Biol. Chem. 262*, 6114–6120.
Hille, M. B., & Albers, A. A. (1979) *Nature (London) 278*, 469–471.
Hille, M. B., Hall, D. C., Yablonka-Reuveni, Z., Danilchik, M. V., & Moon, R. T. (1981) *Dev. Biol. 86*, 241–249.
Hille, M. B., Danilchik, M. V., Colin, A., & Moon, R. T. (1985) *Belle W. Baruch Libr. Mar. Sci. No. 15*, 91–124.
Hultin, T. (1952) *Exp. Cell Res. 3*, 494–501.
Humphreys, T. (1969) *Dev. Biol. 20*, 435–458.
Humphreys, T. (1971) *Dev. Biol. 26*, 201–208.
Ilan, J., & Ilan, J. (1978) *Dev. Biol. 66*, 375–385.
Jackson, R. J., & Hunt, T. (1983) *Methods Enzymol. 96*, 50–74.
Jenkins, N. A., Kaumeyer, J. F., Young, E. M., & Raff, R. A. (1978) *Dev. Biol. 63*, 279–298.
Kaumeyer, J. F., Jenkins, N. A., & Raff, R. A. (1978) *Dev. Biol. 63*, 266–278.
Konieczny, A., & Safer, B. (1983) *J. Biol. Chem. 258*, 3402–3408.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laskey, R. A., Mills, A. D., Gurdon, J. B., & Partington, G. A. (1977) *Cell (Cambridge, Mass.) 11*, 345–351.
Lingrel, J. B., & Woodland, H. R. (1974) *Eur. J. Biochem. 47*, 47–56.
Loeb, J. (1916) *The Organism as a Whole*, University of Chicago Press, Chicago, IL.
Lopo, A. C., & Hershey, J. W. B. (1985a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1801.
Lopo, A. C., & Hershey, J. W. B. (1985b) *J. Cell Biol. 101*, 229a.
Lopo, A. C., Lashbrook, C. C., Infante, A. A., Infante, D., & Hershey, J. W. B. (1986) *Arch. Biochem. Biophys. 250*, 162–170.
Mandley, E. N., & Lopo, A. C. (1987) *Biochem. Biophys. Res. Commun. 145*, 921–926.
Moon, R. T., Moe, K. D., & Hille, M. B. (1980) *Biochemistry 19*, 2723–2730.
Moon, R. T., Danilchik, M. V., & Hille, M. B. (1982) *Dev. Biol. 93*, 389–403.
Panniers, R., Stewart, E. B., Merrick, W. C., & Henshaw, E. C. (1985) *J. Biol. Chem. 260*, 9648–9653.
Raff, R. A. (1980) *Cell Biol. 4*, 107–136.
Raff, R. A. (1983) in *Time, Space and Pattern in Embryonic Development* (Jeffery, W. R., & Raff, R. A., Eds.) pp 65–85, Liss, New York.
Raff, R. A., Brandis, J. W., Huffman, C. J., Koch, A. L., & Leister, D. E. (1981) *Dev. Biol. 86*, 265–271.
Ray, B. K., Brendler, T. G., Adya, S., Daniels-McQueen, S., Miller, J. K., Hershey, J. W. B., Grifo, J. A., Merrick, W. C., & Thach, R. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 663–667.
Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C., & Thach, R. E. (1985) *J. Biol. Chem. 260*, 7651–7658.
Richter, J. D., & Smith, L. D. (1981) *Cell (Cambridge, Mass.) 27*, 183–191.
Spirin, A. S. (1966) *Curr. Top. Dev. Biol. 1*, 1–23.
Stanley, W. M. (1974) *Methods Enzymol. 29*, 530–547.
Whitaker, M. J., & Steinhardt, R. A. (1982) *Q. Rev. Biophys. 15*, 593–666.
Wilt, F. H. (1977) *Cell (Cambridge, Mass.) 11*, 673–681.
Winkler, M. M. (1982) in *Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions* (Nuccitelli, R., & Deamer, D. W., Eds.) pp 325–340, Liss, New York.
Winkler, M. M., & Steinhardt, R. A. (1981) *Dev. Biol. 84*, 432–439.
Winkler, M. M., Steinhardt, R. A., Grainger, J. L., & Minning, L. (1980) *Nature (London) 287*, 558–560.
Winkler, M. M., Bruening, G., & Hershey, J. W. B. (1983a) *Eur. J. Biochem. 137*, 227–232.
Winkler, M. M., Lashbrook, C., Hershey, J. W. B., Mukherjee, A. K., & Sarkar, S. (1983b) *J. Biol. Chem. 258*, 15141–15145.
Winkler, M. M., Nelson, E. M., Lashbrook, C., & Hershey, J. W. B. (1985) *Dev. Biol. 107*, 290–300.
Young, E. M., & Raff, R. A. (1979) *Dev. Biol. 72*, 24–40.