Characterization of translation systems *in vitro* from three developmental stages of *Strongylocentrotus purpuratus*

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We have developed and characterized cell-free systems active in translation from unfertilized eggs, 30-min zygotes and hatched blastulae of the sea urchin *Strongylocentrotus purpuratus*. The ion concentrations selected for preparation of the lysates were 150 mM-K⁺, 40 mM-Na⁺, 40 mM-Cl⁻, 5×10^{-7} M free Ca²⁺ and 1 mM free Mg²⁺. It was necessary to include the ribonuclease inhibitor RNasin in the preparations to obtain full activity consistently. The pH optimum was 7.2 and was extremely sharp for the three *S. purpuratus* lysates. The temperature optima of the three lysates were remarkably similar to those of the intact unfertilized egg and embryos. Lysates from unfertilized egg and 30-min zygotes showed a temperature optimum at 15 °C. The hatched blastula lysate showed a broader temperature optimum with a shift to about 20 °C. The optimized lysates incorporated radiolabelled amino acids into polypeptides for up to 90 min. The polypeptides synthesized ranged in M_r from 200 000 to 20 000, suggesting that the mRNA in the lysates was intact and capable of directing the synthesis of complete polypeptides. Furthermore, the three lysates were capable of initiation, as demonstrated by inhibition of initiation using the inhibitors edeine and 7-methylguanosine 5'-triphosphate (m⁷GTP). At 15 °C, the transit times for the three lysates were: unfertilized egg, 40 min; 30-min zygotes and hatched blastula lysates, 20 min. These transit times are similar to those of intact eggs and embryos, and significantly, reflect the two-fold increase in elongation rate seen following fertilization in intact embryos. Thus, these lysates display many features and characteristic responses typical of intact eggs and embryos, indicating that the lysates should be useful tools for the analysis of translation control in early embryogenesis.

INTRODUCTION

Fertilization in sea urchins is characterized by a series of programmed metabolic changes taking place in rapid, precise sequence following sperm-egg fusion. Prominent among these is a dramatic increase in the rate of protein synthesis (Epel, 1967; Goustin & Wilt, 1981), which is maintained throughout early embryogenesis and is necessary for successful development. Since its discovery over 35 years ago (Hultin, 1952), this translational activation has been extensively documented in eggs of vertebrates and invertebrates alike (Davidson *et al.*, 1982; Davidson, 1986). Within 1 h after fertilization, protein synthesis increases to 10–15 times the rate in the unfertilized egg, and within several hours, the rate has increased 100-fold (Epel, 1967; Goustin & Wilt, 1981), as demonstrated by both measurements of incorporation of radiolabelled amino acids into protein and in the recruitment of free ribosomes into polysomes (Humphreys, 1969, 1971). Less than 1% of the large number of free ribosomes in the unfertilized egg are in polysomes. By 2 h after fertilization, 20% of the ribosomes have been recruited, and by 15 h, 60% are in polysomes (Humphreys, 1971).

The unfertilized egg apparently contains all the necessary components for translation (Davidson *et al.*, 1982; Hille *et al.*, 1985). Extensive biochemical analyses have revealed that a key feature of this activation is the

precisely regulated utilization of large stores of mRNA laid down during oogenesis in the cytoplasm (Raff, 1980, 1983). In addition, there is an approximately two-fold increase in the rate of elongation (Brandis & Raff, 1978, 1979; Hille & Albers, 1979) following fertilization. Thus, the increase in translational activity is not only due to recruitment of mRNA into polysomes, but also to increased efficiency of translation of these messages. However, in spite of the large body of information on the biochemical detail of the translational activation, there is only limited understanding of how the stored message is 'mobilized'.

Our current understanding of eukaryotic translational control is due, in large part, to studies using cell-free translation systems, particularly the rabbit reticulocyte lysate. For the past twenty years this cell-free system has proved very useful in the analysis of the individual reactions as well as the requirements of protein synthesis (e.g. Zucker & Schulman, 1968; Jackson & Hunt, 1983). Although cell-free translation systems and partially fractionated systems have been developed from eggs (Gambino *et al.*, 1973), and useful information has been derived from these studies (e.g. Ilan & Ilan, 1978), 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 & Steinhardt (1981). Using this system, Winkler *et al.* (1985) demonstrated that there is more than one

Abbreviations used: ASW, artificial sea water; eIF, eukaryotic initiation factor; m⁷GTP, 7-methylguanosine 5'-triphosphate; PAGE, polyacrylamide-gel electrophoresis.

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level of translational control operating at fertilization. More recently, Mandley & Lopo (1987), also using lysates from *L. pictus*, identified a nuclease-sensitive negative control element operating at fertilization.

We are interested in elucidating the mechanisms which control the activation of protein synthesis at fertilization. We are using a dual approach to the problem: (1) a biochemical dissection of the translational machinery, with a 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 practicable 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.

Here we characterize cell-free systems active in translation from unfertilized eggs, 30-min zygotes and hatched blastulae of *S. purpuratus*. We and others have used these *S. purpuratus* lysates to good advantage in the analysis of the activation of protein synthesis at fertilization (Lopo & Hershey, 1985a; Hansen *et al.*, 1987; Lopo *et al.*, 1988).

A preliminary report of this work was presented at the Federation of American Societies for Experimental Biology Annual Meeting in Anaheim, California, in April 1985 (Lopo & Hershey, 1985a).

EXPERIMENTAL

General methods

Reagents. All reagents were from Sigma Chemical Company except as follows. Artificial sea water (ASW; 'Instant Ocean') was from Aquarium Systems, Inc., Mentor, OH, U.S.A. RNasin, the ribonuclease inhibitor from human placenta, was from Amersham International.

Protein determination. Protein concentration was determined using the method of Bradford (1976) with the Protein Assay Dye Reagent Concentrate from Bio-Rad Laboratories, Richmond, CA, U.S.A.

SDS polyacrylamide-gel electrophoresis. This was as described by Laemmli (1970). For autoradiograms, Kodak X-Omat AR film was used.

Cells

Gametes were obtained from adult sea urchins (*S. purpuratus*) by pouring 0.5 M-KCl into opened body cavities. Eggs were pooled and washed by settling in 5 vol. of ASW at 4 °C. The settled eggs were dejellied by resuspension in 10 vol. of ASW (4 °C; pH 4.5) 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 vol. of ASW at 4 °C, 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

described (see 'Lysate preparation'). To obtain 30-min zygotes, unfertilized eggs were resuspended to a final concentration of 1% in ASW at 15 °C 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 for 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 in a 0.5% suspension at 15 °C in ASW containing 50 mg/l each of penicillin and streptomycin until > 90% were hatched (about 20 h). To collect the hatched embryos, the culture first was chilled by adding frozen ASW pellets, then passed through a 52 µm Nitex filter.

Cell-free translations

pH 7.2 buffer. The pH 7.2 buffer is a modification of the pH 7.4 buffer described by Winkler & Steinhardt (1981). The pH 7.2 buffer contains 40 mM-NaCl, 106 mM-potassium gluconate, 263 mM-glycerol, 300 mM-glycine, 50 mM-Hepes, 10 mM-EGTA, 7.3 mM-CaCl₂, 0.52 mM-MgCl₂, and approx. 50 mM-KOH added to adjust the pH to 7.2. The pH of the buffer was adjusted at 15 °C. This combination of Ca²⁺, Mg²⁺ and EGTA yields a final concentration of 5×10^{-7} M-free Ca²⁺ and 0.5 mM-free Mg²⁺ at pH 7.2 and 15 °C. The influence of pH on these values was determined using Program Chelate (courtesy of Richard Steinhardt, University of California, Berkeley, U.S.A.). For experiments requiring different pH values, the amounts of reagents added were adjusted to give the same final concentrations of free Ca²⁺ and free Mg²⁺ according to Program Chelate. Prior to use, 1 mg of soybean trypsin inhibitor/ml and 0.5 mg of reduced glutathione/ml were added to the buffer.

Lysate preparation. The procedure we used is a modification of the method described by Winkler & Steinhardt (1981) for *L. pictus*. Unfertilized eggs or embryos of the appropriate stage (2 ml) were washed twice in 6 vol. of ice-cold pH 7.2 buffer with soybean trypsin inhibitor and glutathione, 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. The cells were broken with three to four strokes. There is a distinct change in the colour of the suspension to a milky yellow when the cells break. The homogenate was immediately transferred to 1.5 ml capped tubes and centrifuged for 5 min at 15,000 *g*. Following dialysis for 1 h against pH 7.2 buffer with reduced glutathione (0.5 mg/ml) and polyethylene glycol (2 g/l), the supernatant was frozen as 100–400 µl aliquots in liquid N₂ and stored at –80 °C (for up to 6 months) or in liquid N₂. The protocol for preparation of the lysates is summarized in Fig. 1.

There are several other parameters that should be considered in preparation of the *S. purpuratus* lysates. Dejelling the eggs properly is an important consideration. Jelly is likely to be inhibitory to protein synthesis, due to the high density of negative charges. The use of a fairly tight-fitting pestle in the homogenizer is also important. We used a stainless steel Dounce homogenizer with a clearance of 0.127 mm (Kontes) and found this the most satisfactory. Over 95% of the cells should be homogenized within four strokes. A homogenizer with an

Fig. 1. Protocol for preparation of lysates from *S. purpuratus* eggs and embryos

excessively tight fit will rupture the nuclei. This can be readily identified because the resulting lysate is a very deep yellow and will have a gelatinous consistency.

Cell-free translation systems. The lysates were rapidly thawed, and the desired volume transferred to a sterile 10 mm × 75 mm culture tube, and kept on ice. Buffer or the experimental sample were added to the lysate (in a 100% 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 (5 µl, 7.5 µl or 10 µl, depending on the experiment) were taken using a Drummond precision pipette (0–10 µl) at the desired time intervals. The sample was diluted into 0.5 ml of distilled water, and 1 ml of 20% trichloroacetic acid containing 1 mg of unlabelled methionine/ml 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% trichloroacetic acid, rinsed with about 5 ml of 95% ethanol, dried and radioactivity determined using a Beckman LSC 9000. The master mix was prepared fresh each time from stock solutions to give final concen-

trations in the lysate of 2 mM-ATP, 0.8 mM-GTP, 20 mM-creatine phosphate, 10 units of creatine phosphokinase/ml, 50 µM-glucose 6-phosphate, 19 amino acids (minus the radiolabel) (50 µM), 0.2 µM-[³⁵S]methionine (specific radio activity 1100 Ci/mmol), 0.1 mg of leupeptin/ml, and 0.1 mg of pepstatin/ml. MgCl₂ (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 labelled protein, as indicated by a decrease in radiolabel incorporated after about 45 min of incubation.

Transit time determinations. A modification of the procedure of Fan & Penman, (1970; see Nielsen & McConkey, 1980) was used in determining transit times. Lysates from each developmental stage were used for translations, and protein synthetic activity was measured by [³⁵S]methionine incorporation, as described above. In addition, aliquots were taken at 10 min intervals, diluted with 2 vol. of gradient buffer, and layered over 4 ml of 15% sucrose in gradient buffer and centrifuged for 1 h 50 min at 54000 rev./min in a Beckman SW60 rotor ($r_{av.} = 91.7$ mm). The gradient buffer used in the transit time determination experiments contained 250 mM-KCl, 25 mM-MgCl₂, 50 mM-Hepes, pH 7.2, 2 mM-dithioerythritol, 50 µg of cycloheximide/ml, 0.5 mg of heparin/ml and 10 mM-benzamidine. Following centrifugation the supernatant was removed, and the polysomal pellet was resuspended in 0.5 ml of gradient buffer. Trichloroacetic acid (20%, 1 ml) containing 1 mg of methionine/ml was added to the polysomal fraction, and the samples were processed for liquid scintillation counting as described above.

RESULTS AND DISCUSSION

Preparation of translationally active lysates from *S. purpuratus*

In preparing the lysates active in translation from *S. purpuratus*, we used as a starting point the conditions outlined by Winkler & Steinhardt (1981) for preparation of unfertilized egg cell-free systems from *L. pictus*. Under these conditions we obtained completely inactive *S. purpuratus* lysates. This was not totally unexpected, since *S. purpuratus* has been problematic for cell-free translation work and RNA purification because of high levels of endogenous ribonucleases. Although numerous inhibitors of ribonucleases are available, they generally are also inhibitory for translation. The exception is RNasin, the ribonuclease inhibitor from human placenta. We found that it was possible to prepare lysates active in translation from any developing stage of *S. purpuratus* if RNasin was included in the homogenizing medium (Table 1). Deleting the inhibitor yields inactive lysates (Table 1). Furthermore, the inhibitor must be added prior to homogenization; addition of the RNasin immediately following homogenization yields inactive lysates (Table 1). It should be emphasized, however, that to get fully active lysates consistently it is also necessary to work in a scrupulously clean environment and use acid-washed, baked glassware, sterile plasticware and sterile solutions whenever possible.

Optimization of the lysates

Ion concentration optima of the lysates. Because of the importance of ion concentration in protein synthesis in

Table 1. Effect of RNasin on incorporation by the *S. purpuratus* lysates

In delayed addition, RNasin was added immediately following homogenization; and in 'addition prior to homogenization', RNasin was added to the egg suspension immediately prior to homogenization as described in the Experimental section.

Type of lysate	Protein synthesis (c.p.m. incorporated at 60 min)		
	No RNasin	Delayed addition	Addition prior to homogenization
Unfertilized egg	1890	1731	13451
30-min zygote	1215	1974	79852
Hatched blastula	1561	1837	197372

Fig. 2. Free Ca^{2+} and Mg^{2+} concentration optima for the lysates

Protein synthesis was measured by [^{35}S]methionine incorporation for 60 min in an unfertilized egg lysate; each translation was carried out at a different free [Ca^{2+}] (a) or [Mg^{2+}] (b). The free Ca^{2+} and Mg^{2+} concentrations were carefully controlled using 10 mM-EGTA; pH was taken into account in determining the final values. Sample volume was 10 μl . For additional detail, see Materials and methods section.

general and in fertilization phenomena in particular (Whitaker & Steinhardt, 1982), optima were determined for several key ions. It should be noted that in carrying out these optimization experiments, the incubation mixtures were sampled at 10 min intervals for the duration of the experiment. The incubations were carried out for at least 60 min, and in many cases for 75–90 min. In all cases studied, the shapes of the curves obtained for each individual incubation were similar, although the final total incorporation varied.

Because the transient increase in free intracellular Ca^{2+} following fertilization (Steinhardt *et al.*, 1977) has been proposed to play a role in translational control (Winkler *et al.*, 1980), and because of evidence of effects of Ca^{2+} on protein synthesis in mammalian cells (Brostrom *et al.*, 1983), we were highly interested in establishing whether the free Ca^{2+} concentration affected the elongation rate or total rate of protein synthesis in the egg and embryo lysates. The ion concentrations selected for preparation of the lysates were 150 mM K^+ , 40 mM Na^+ , 40 mM Cl^- (results not shown), and 0.5 mM free Mg^{2+} (Fig. 2b). We could not find an effect of Ca^{2+} on translation in our lysates over the physiological range of 5×10^{-7} – 5×10^{-4} M (Fig. 2a). However, incorporation of radiolabel was inhibited if the free Ca^{2+} concentration was increased to 1 mM (results not shown). We have chosen 5×10^{-7} M-free Ca^{2+} as the final concentration in the lysates based on previously reported measurements of free Ca^{2+} in eggs and embryos (Whitaker & Steinhardt, 1982). The transient nature of the increase in cytoplasmic free Ca^{2+} makes this a difficult parameter to assess, and a difficult one to study using the cell-free translation systems.

The estimated free Mg^{2+} concentration used in lysate preparation is 0.5–1 mM (Fig. 2b). Although the lower levels of 0–0.1 mM free Mg^{2+} produced lysates with slightly higher activity, the actual free and total Mg^{2+} concentrations are uncertain, since a sizeable amount of Mg^{2+} may be retained in bound form. The concentration of 0.5–1 mM is lower than measurements of intracellular Mg^{2+} in the sea urchin *L. pictus* (Sui & Shen, 1986).

pH optima of the lysates. Within 2 min. of sea urchin sperm–egg fusion there is an intracellular alkalinization of approximately 0.5 pH units, from 6.84 to 7.26 (Shen & Steinhardt, 1978; Winkler *et al.*, 1980). The role of this alkalinization has been extensively studied and linked by several groups to the activation of protein synthesis 5 min after fertilization (Winkler *et al.*, 1980; Winkler, 1982), although the molecular basis for this is not understood. Lysates were prepared from unfertilized eggs, 30-min zygotes and blastulae using buffers adjusted in 0.1 unit increments from pH 6.4 to 7.6. The pH adjustments did not involve significant alterations in the concentration of K^+ . The cells were washed, homogenized and dialysed against buffer of the same pH as that used in homogenization. In all cases, the free Mg^{2+} and free Ca^{2+} concentrations were adjusted to give the same free ion concentrations (see Materials and methods section). Each lysate was then tested for protein synthetic activity at the same pH used in its preparation. Three separate pH determinations were carried out for each developmental stage using lysates prepared from eggs obtained from different females on different days.

The optimum pH for lysates from all stages of development is 7.2 (Fig. 3). In all experiments, the pH

Fig. 3. Determination of pH optimum for the unfertilized egg lysates

Protein synthesis was measured by [³⁵S]methionine incorporation for 60 min, each translation was carried out using a lysate prepared at a different pH. Final pH of each lysate was as indicated. The effect of pH on free Ca²⁺ and free Mg²⁺ concentrations was taken into account in determining the final values. Sample volume was 10 μ l.

optimum was equally sharp. Unfertilized egg lysates prepared by homogenizing eggs in pH 6.8 buffer, then adjusting the buffer to pH 7.2, incorporated more radioactive label into protein at pH 7.2 than at 6.8. However, this was never to the same extent as the case of unfertilized egg lysates prepared using 7.2 buffer (results not shown). The pH was checked after incubation for lysates from all three stages. It remained constant, within 0.03 pH units. The optimum pH for the *L. pictus* unfertilized egg cell-free system developed by Winkler & Steinhardt (1981) is 7.4. It should be noted that with *S. purpuratus* unfertilized egg and embryo lysates, we observed an exceedingly sharp pH optimum, whereas Winkler & Steinhardt (1981) observed a more gradual increase in activity with pH in *L. pictus* unfertilized eggs. The difference in the temperatures used for translation of lysates (15°C for *S. purpuratus* and 20°C for *L. pictus*) from the two different species could sufficiently affect pH to lead to different optimal pH in the two species.

Glucose 6-phosphate. The effect of glucose 6-phosphate on the sea urchin lysates was also tested. In the absence of glucose 6-phosphate, there was an increased variability in the total incorporation by the lysates for a given set of conditions. When glucose 6-phosphate was included to a final concentration of 25–50 μ M, the variability was eliminated. The sensitivity is dependent on the specific lysate preparation. Higher concentrations of glucose 6-phosphate consistently inhibited incorporation. No other hexose phosphates were tested. Jackson *et al.* (1983) have demonstrated a requirement for sugar phosphates in the rabbit reticulocyte lysate. In their experiments, absence of glucose 6-phosphate led to a reduction in the level of 43S preinitiation complexes.

Temperature. Fertilized *S. purpuratus* eggs will not cleave above 18°C. This temperature sensitivity is

Fig. 4. Temperature optima for the three *S. purpuratus* lysates

Protein synthesis was measured by [³⁵S]methionine incorporation for 60 min as described in Materials and methods section, except that different temperatures were used. The effect of temperature on pH was taken into account in preparing the buffers such that each reaction mixture was at pH 7.2. Sample volume was 10 μ l. ○, Unfertilized egg; ●, 30-min zygote; △, blastula.

gradually lost so that eight-cell stage embryos tolerate 18–19°C, and hatched blastulae are able to develop at 20–25°C (Loeb, 1916; A. C. Lopo, unpublished work). The temperature optima of the lysates are remarkably similar to those seen in the intact unfertilized egg and embryo. Unfertilized egg and 30-min zygote lysates show a temperature optimum at 15°C; incorporation decreases rapidly at temperatures above 18–20°C (Fig. 4). The hatched blastula lysate, on the other hand, shows a broader temperature optimum and a shift to about 20°C, although translation is also greatly inhibited at temperatures nearing 30°C. Thus, the lysates we have prepared display changes in temperature sensitivity with developmental stage similar to those of the intact embryos, again indicating that the lysates mimic the intact embryos in many ways. At these temperatures, the lysates remain active for at least 90 min. We routinely use 15°C as the incubation temperature for all lysates. We do not know at this time what confers this particular sensitivity to temperature, and whether in the intact cell the only process affected is protein synthesis.

Characterization of the lysates

Translation products. The optimized lysates from sea urchin unfertilized eggs, 30-min zygotes and hatched blastulae incorporate radiolabelled amino acids into polypeptides in the range of M_r 20000 to >200000, measured by SDS/PAGE (Fig. 5). This indicates that the mRNA in the lysates is intact and capable of directing the synthesis of complete proteins. It also shows that the translational apparatus is capable of synthesizing high molecular mass products, suggesting that premature termination does not occur frequently in the sea urchin lysates.

Although the gel profiles show a full range of molecular mass products, the patterns show the 'ladders' characteristic of proteolytic degradation. Proteinase inhibitors are included in the homogenization buffers and in the master mix added to the lysates at the beginning of each experiment. However, proteolytic degradation is a significant problem in preparations of

Fig. 5. Translation products of the lysates

The lysates were incubated for 60 min as described in Materials and methods. Samples containing 5×10^4 c.p.m. of incorporated [^{35}S]methionine were analysed by SDS/PAGE (gel concentration, 10%). The gels were dried and autoradiograms prepared by a 5-day exposure. A photograph of the autoradiogram is shown. The position of migration of molecular mass markers (in kDa) is shown on the left. Lane *a*, unfertilized egg lysate; lane *b*, 30-min zygotes; lane *c*, blastula.

sea urchin eggs and embryos (e.g., Yablonka-Reuveni & Hille, 1983). In spite of our efforts to control proteolytic activity, there are evidently additional proteinases that are not being successfully inhibited.

The spectrum of translation products synthesized by the lysates of all three stages is highly similar. This is not surprising in view of the findings of Brandhorst (1976) showing that there are few qualitative differences in the two-dimensional gel patterns of synthesized polypeptides in unfertilized eggs and pre-gastrula embryos.

Time course of translation. At 15°C, the optimized lysates may continue incorporation of label into protein

for up to 90 min, although this varies with the preparation. There is also variability in the rate of incorporation during an incubation. The variability appears to be dependent on the preparation and the stage of development used as the source of the lysate.

The greatest variability with respect to the rate of incorporation of radiolabel during the incubation is observed in the unfertilized egg lysate. Approximately 50% of the unfertilized egg lysates show a lag of 20–30 min before there is a significant increase in incorporation of label (Fig. 6*a*, open circles). In the remaining 50% of the lysates, incorporation is linear from the time of master mix addition (Fig. 7*a*, closed symbols). This curious variability is dependent on the preparation of unfertilized egg lysate used. It is never seen in the lysates from 30-min zygotes or hatched blastulae. All samples of a given lysate preparation display virtually identical kinetics. Incorporation of radiolabel is sensitive to $m^7\text{GTP}$ and edeine in both types of unfertilized egg lysates. At this time we have no information on the cause of this curious variability. Supplementing these lysates with purified mammalian initiation factors does not relieve the lag or change the kinetics of incorporation (A. C. Lopo & J. W. B. Hershey, unpublished work; see also Lopo *et al.*, 1988). Interestingly, a similar lag in activation was reported in ribosomes prepared from unfertilized eggs of this species by Danilchik & Hille (1981). However, we have no information on whether the lag seen in our experiments is due to the same phenomenon.

The lysates initiate protein synthesis. One of the most important criteria of the usefulness of a cell-free translation system is its ability to initiate translation. To establish that the three different lysates from *S. purpuratus* are capable of initiation, each one was incubated with two different inhibitors of initiation, edeine and $m^7\text{GTP}$. Edeine inhibits initiation by blocking the joining of the 60 S subunit with the 48 S initiation complex; $m^7\text{GTP}$ inhibits cap-dependent initiation. With both inhibitors there was a reduction in the total amount of incorporation in all lysates (Fig. 6). In the unfertilized egg lysate,

Fig. 6. The *S. purpuratus* lysates initiate new polypeptide chain synthesis

Each of the lysates was incubated as described in Materials and methods, except that the initiation inhibitors $m^7\text{GTP}$ and edeine were included in the translation medium. Protein synthesis was measured by [^{35}S]methionine incorporation; samples were taken at 10-min intervals. Sample volume was 10 μl . (*a*) Unfertilized egg lysate; (*b*) 30-min zygote; (*c*) blastula. $\circ$, Control; $\square$, 0.5 mM- $m^7\text{GTP}$; $\bullet$, 1 μM -edeine.

Fig. 7. Transit time determinations of the three lysates.

Transit times were determined for each lysate as described in detail in the Materials and methods section. Protein synthesis was measured by $[^{35}\text{S}]$ methionine incorporation in total (■) or polysomal (○) fractions; samples were taken at 10-min intervals. Sample volume was 10 μl . (a) Unfertilized egg lysate; (b) 30-min zygote; (c) blastula.

incorporation was reduced approximately to background levels (Fig. 6a). This indicates that most of the protein synthesis was due to *in vitro* initiation events, and that there are few pre-formed polysomes capable of run-off, as expected for the unfertilized egg. In the 30-min zygote and blastulae lysates, there is residual incorporation in the initiation-inhibited lysates. This incorporation is likely due to continued elongation on pre-formed polysomes present in the 30-min zygotes and hatched blastulae, and is expected based on estimates of numbers

of ribosomes in polysomes in the literature (Humphreys, 1969, 1971). Approximately 45% of the incorporation after 60 min incubation in the 30-min zygote lysate, and 33% of that in the blastula lysate, is due to new initiation.

It is interesting to note that both edeine and m^7GTP inhibited the lysates to the same degree. This suggests that all actively translating mRNAs in sea urchin eggs are capped, as was demonstrated by Winkler *et al.* (1983) in *L. pictus* unfertilized egg lysates.

Transit times. Transit times (the time required to translate an 'average' mRNA) have been determined for the three lysates. For these measurements we modified the procedure of Fan & Penman (1970; Nielsen & McConkey, 1980; described in detail in the Materials and methods section). The original Fan & Penman (1970) protocol calls for the determination of total and completed radiolabelled protein in the system. In practice, there is often contamination of the completed protein factors by the nascent protein fraction, i.e. the polypeptide chains still being synthesized (Nielsen & McConkey, 1980). We found this problem to be particularly true with the *S. purpuratus* lysates. We do not know whether this was due to limited proteolysis, residual ribonuclease activity, or premature termination. We discovered that a way to circumvent the problem was to measure total and nascent protein. Nascent protein was measured by collecting polysomal fractions centrifuged through a sucrose-high salt buffer. The nascent protein (or polysomal) fraction should be completely labelled in one transit time, and will remain constant after that (Fan & Penman, 1970). The pelleting of the polysomes through 250 mM-NaCl and 15% sucrose presumably minimizes contamination by the soluble protein fraction.

All transit time measurements were done at 15 °C. By this procedure, the polysomal fractions reach a constant level of radioactivity, or one transit time, as follows: unfertilized egg, approx. 40 min (36–42 min); fertilized egg and hatched blastula, approx. 20 min (15–24 min; Fig. 7). These times are similar to those of the corresponding intact embryonic stages. More significantly, these times reflect the two-fold increase in elongation rate seen following fertilization in intact eggs and embryos (Humphreys, 1969; Brandis & Raff, 1978; Hille & Albers, 1979; but see also Goustin & Wilt, 1981).

Brandis & Raff (1979) further characterized this increase in elongation rate and observed that in ammonia-activated embryos the increase in the rate of elongation seen at fertilization does not take place. In ammonia-activated eggs protein synthesis is only partially activated (Epel *et al.*, 1974). Brandis & Raff (1979) concluded that the increase in translational efficiency operates via an intracellular pH-independent mechanism. We also report an approximately two-fold increase in elongation rate between the unfertilized egg and 30-min zygote lysates, similar to that reported for intact eggs and embryos (Humphreys, 1969; Brandis & Raff, 1978, 1979; Hille & Albers, 1979). The *S. purpuratus* unfertilized egg lysate, although at pH 7.2, does not show an increase in elongation rate. Thus, our results are consistent with the observations of Brandis & Raff (1978, 1979), since we do not see this increase in elongation rate in the *S. purpuratus* unfertilized egg lysate, even when translation is taking place at pH 7.2.

Table 2. Protein synthetic activity of the lysates

Results are expressed as pg/min per embryo. An 'embryo' in the lysate is the number of eggs or embryos estimated to have contributed to a given volume of lysate, based on our lysate preparation protocol and on a value of 2.9×10^6 eggs/ml (Vacquier & Payne, 1973). The following assumptions were made in obtaining the lysate values: the relative weight percentage value for methionine was assumed to be 1; and the average sea urchin polypeptide was assumed to be 5×10^4 kDa (Brandhorst, 1976). The free methionine concentration in the lysates was determined to be 25 μ M, by isotope dilution and by amino acid analysis.

Stage	Lysate (pg/min/embryo)	<i>In vivo</i> (pg/min/embryo)
Unfertilized egg	0.03	0.11
30-min zygote	2.0	7.2
Hatched blastulae	0.7	8.3

* Regier & Kafatos (1977).

Polysome profiles of the blastula lysate indicate a polysome size of 3–5, somewhat smaller than the average size of polysomes in the intact embryo (results not shown). Due to the very small numbers of polysomes in unfertilized eggs and 30-min zygotes, we have been unable to establish an accurate polysome size in these lysates.

The relative protein synthetic activity of the lysates (expressed as pg/min/embryo) when compared to the intact eggs or embryos, is approx. 27% for the unfertilized egg and 30-min zygote lysates, and 89% for the blastula lysate (Table 2). Although we have observed higher activity values in blastula lysates, this relatively low value is more in line with the norm. The most variable determinations of protein synthetic activity are obtained with the blastula lysates. This may be due to breakdown of polysomes during the concentration of the embryos and preparation of the lysate, which can be a rather lengthy process because of the swimming of the embryos. Recently (A. C. Lopo & J. W. B. Hershey, unpublished work), we have found that similar results to those presented in this manuscript with respect to such parameters as ion concentrations, transit times, and temperature dependence are obtained with blastulae collected prior to hatching. However, lysates prepared from pre-hatched blastulae are more consistently efficient, possibly due to improved speed of preparation of the lysate.

In this paper, we characterize cell-free translation systems from unfertilized eggs, 30-min zygotes and hatched blastulae of *S. purpuratus*. These cell-free translation systems from eggs and embryos provide the means to analyse differences in the mechanisms regulating protein synthesis before and after fertilization. The key advantages of using cell-free translation systems to analyse translational control is that they provide a means for identifying and analysing the individual reactions of protein synthesis. In addition, a cell-free system can be used to isolate the process of protein synthesis from the remainder of cellular processes, while still retaining the complexity of the process itself. For a cell-free translation system to be useful, it should meet the following

conditions: (1) it should respond to external stimuli in the same way as the intact cell; (2) it should translate full-length polypeptides, i.e. not display premature termination; (3) it must be capable of initiation of new polypeptide chains. The *S. purpuratus* lysates we describe in this paper meet these criteria of usefulness.

The molecular mechanisms regulating protein synthesis in early sea urchin development are of significant interest for two compelling reasons. First, an understanding of translational control in embryogenesis is vital to our understanding of the regulation of early development. Secondly, the sea urchin system presents an excellent model for the analysis of the molecular mechanisms regulating mRNA at the level of protein synthesis. We are successfully using these cell-free translation systems from *S. purpuratus* unfertilized eggs and embryos to present evidence for multi-step regulation of protein synthesis at fertilization (Mandley & Lopo, 1987; Lopo *et al.*, 1988).

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