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'Unmasking' of stored maternal mRNAs and the activation of protein synthesis at fertilization in sea urchins

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Summary

The isolation and *in vitro* assay of maternal mRNPs has led to differing conclusions as to whether maternal mRNAs in sea urchin eggs are in a repressed or 'masked' form. To circumvent the problems involved with *in vitro* approaches, we have used an *in vivo* assay to determine if the availability of mRNA and/or components of the translational machinery are limiting protein synthesis in the unfertilized egg. This assay involves the use of a protein synthesis elongation inhibitor to create a situation in the egg in which there is excess translational machinery available to bind mRNA. Eggs were fertilized and the rate of entry into polysomes of individual mRNAs was measured in inhibitor-treated and control embryos using ^{32}P-labeled cDNA probes. The fraction of ribosomes in polysomes and the polysome size were also determined. The results from this *in vivo* approach provide strong evidence for the coactivation of both mRNAs and components of the translational machinery following fertilization. The average polysome size increases from 7.5 ribosomes per message in 15 min embryos to approximately 10.8 ribosomes in 2 h embryos. This result gives additional support to the idea that translational machinery, as well as mRNA, is activated following fertilization. We also found that individual mRNAs are recruited into polysomes with different kinetics, and that the fraction of an mRNA in polysomes in the unfertilized egg correlates with the rate at which that mRNA is recruited into polysomes following fertilization.

Key words: fertilization, protein synthesis, maternal mRNA, translational control, masked mRNA.

Introduction

Fertilization of the sea urchin egg results in a 20- to 40-fold increase in the rate of protein synthesis in the two hours following fertilization (Hultin, 1952; Epel, 1967; Regier and Kafatos, 1977). This increase in the rate of protein synthesis is largely mediated by the mobilization of stored maternal mRNAs into polysomes (Gross and Cousineau, 1963; Denny and Tyler, 1964; Humphreys, 1971). The molecular mechanisms that have been proposed to explain the activation of these stored maternal mRNAs can be conveniently divided into two non-mutually exclusive general categories. These are an increase in the availability of either (1) mRNA or (2) components of the translational machinery other than mRNA. (Availability is defined as being in a form or intracellular location where it can be utilized for translation). The discovery that mRNA exists in the cell as RNA–protein complexes (messenger ribonucleoprotein particles or mRNPs) led to the suggestion that associated proteins might be repressors of mRNA activity in the unfertilized egg (reviewed by Davidson, 1986). This theory has become termed the 'masked message' hypothesis (Spirin, 1966). Four previous studies have utilized the isolation and *in vitro* assay of mRNPs from unfertilized eggs to determine if protein synthesis is regulated at the level of repression of mRNPs (Jenkins *et al.* 1978; Ilan and Ilan, 1978; Moon *et al.* 1982; Grainger and Winkler, 1987). The conclusions from these studies oscillate between the idea that mRNA is masked or not masked in the unfertilized egg. Many of these discrepancies can be attributed to technical problems associated with the isolation or assay of mRNP activity.

Our approach to this question has been to develop an *in vivo* assay for studying mRNP activity that does not rely on mRNP isolation. Thus, we avoid the problem of artifactually altering the activity of the mRNP or components of the translational machinery (i.e. ribosomes, initiation factors) during the isolation or assay process. The results from our study clearly show that some form of 'masking' of mRNAs limits protein synthesis in the unfertilized egg. In addition, we find that protein synthesis is limited by the activity of some component(s) of the translational machinery. These experiments also reveal that the rate of mobilization of an mRNA into polysomes correlates with that mRNA's translational efficiency in the egg.

Materials and methods

Preparation of sea urchin post-mitochondrial supernatants and glycerol density gradient centrifugation

A 1% suspension of *Strongylocentrotus purpuratus* eggs in Jamarin U Artificial Sea Water (Osaka, Japan) was divided into two equal aliquots. Anisomycin (10 μM) was added to one aliquot of eggs 5 min prior to fertilization. Both anisomycin-treated and untreated eggs were fertilized by adding 1/20000 volume sperm. Eggs and embryos were incubated at 15°C with constant stirring. Post-mitochondrial supernatants (PMS) were prepared (Grainger and Winkler, 1987) and 20 OD_{260} units were adjusted to 0.1% Triton X-100 and layered onto 15–50% glycerol gradients in Cold IV buffer (0.25 M NaCl, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, 10 mM Pipes, pH 6.8) or Cold IV+ EDTA (10 mM EDTA) with a 7 ml 80% glycerol–EDTA pad (Kelso-Winemiller *et al.* 1989). Gradients were centrifuged for 5 h at 28000 revs min^{-1} in a Beckman SW 28 rotor at 4°C. Ten fractions were collected from each gradient by upwards displacement with 50% sucrose–30% glycerol (w/v). The optical density was monitored at 254 nm with an Isco recording spectrophotometer. Baker's yeast tRNA (Sigma) was added as a carrier to each fraction at a concentration of 10 $\mu g\,ml^{-1}$. 2.5 volumes of 95% ethanol were added to each gradient fraction and allowed to precipitate at −20°C overnight.

Isolation of RNA

Gradient fractions were centrifuged at 3600 *g* for 30 min. RNA pellets were resuspended in 0.5 ml STEN buffer (0.5% SDS, 10 mM EDTA, 50 mM NaCl, 10 mM Tris, pH 8), 250 $\mu g\,ml^{-1}$ proteinase K (Boehringer-Mannheim Biochemicals), and incubated at 40°C for 2 h. Gradient fractions were extracted twice with equal volumes of phenol:chloroform (50% phenol, 46% chloroform, 4% isoamyl alcohol (v/v), 0.1% 8-hydroxyquinoline (w/v), saturated with 1 M Tris, pH 8) and once with an equal volume of chloroform. 2.5 volumes of 95% ethanol were added to each gradient fraction and the RNA allowed to precipitate at −20°C overnight. Gradient fractions were centrifuged at 10000 revs min^{-1} in a microfuge and RNA pellets were resuspended in double-distilled H_2O and stored at −80°C.

Preparation of mRNA probes from a cDNA library

Probes used in hybridizations were prepared from cDNA clones selected from a 2 h (2-cell stage) *Strongylocentrotus purpuratus* cDNA library made from polysomal poly $(A)^+$ RNA using a modified version of the ribonuclease H method (Gubler and Hoffman, 1983). Double-stranded cDNA molecules were ligated into lambda Zap (Stratagene). Recombinant Bluescript plasmids were excised from lambda Zap according to Stratagene protocols. cDNA clones were hybridized to equal amounts of unfertilized egg RNA and mitochondrial RNA (Wells *et al.* 1982) to determine if any of them coded mitochondrial sequences. The signal was stronger with the unfertilized egg RNA in all cases. To determine if there was a large percentage of mitochondrial sequences coded by the cDNA used to prepare the library, labeled cDNA was hybridized to equal amounts of unfertilized egg and mitochondrial RNA. The signal from the mitochondrial RNA was barely detectable indicating that the library contained very few mitochondrial sequences. These cDNA clones coded for bonafide mRNAs based on three criteria: (1) presence of 3′ poly (A) tails, (2) mobilization into polysomes after fertilization, and (3) release from polysomes with EDTA treatment. cDNA clone 53 (encoded an 'A' type cyclin) hybridized to 4.9 kb and 6.6 kb transcripts. cDNA clone 12 hybridized to 4.5 kb and 5.8 kb transcripts. Construction of the plasmid Sp64-β1 coding containing the cDNA clone encoding β tubulin mRNAs was described by Harlow and Nemer (1987). Either T3, T7 or SP6 polymerase were used to generate radiolabelled RNA transcripts from cDNA clones as described by Stratagene protocols. 50 μCi of [α-^{32}P] CTP (~600 Ci $mmol^{-1}$: ICN) was added to a standard reaction volume of 25 μl. A typical reaction yielded between 30–60 ng of [^{32}P] RNA with a specific activity of 8×10^8 cts min^{-1} μg^{-1}. Random primed ^{32}P-labeled probes as described by Amersham protocols.

Determination of fraction of mRNA in polysomes

RNA isolated from each fraction was applied to Hybond N (Amersham) nylon membranes under vacuum with a slot blotter (Schleicher and Schuell) and baked one hour at 80°C (White and Bancroft, 1982). Slot blots were hybridized with radiolabeled probes as described by Hurley *et al.* (1989) except that hybridizations were incubated at 65°C, and 50 $\mu g\,ml^{-1}$ poly (A) RNA was added to the hybridization solution. Following hybridization, membranes were washed twice with 40 mM Na_3PO_4 (pH 7.2), 1 mM EDTA, 1% SDS, at 65°C. Membranes were wrapped in plastic wrap and autoradiographed with Kodak XAR-5 X-ray film. Autoradiographs were exposed for appropriate time intervals to remain within the linear response of the film as monitored by a standard curve of unfertilized egg RNA. Slot blot autoradiographs of gradient RNA fractions were scanned using a densitometer and the areas under the peaks integrated. Gradient fractions 1–6 contained nontranslated mRNA and fractions 7–11 contained polysomal RNA. Parallel gradients containing EDTA were used to determine the fraction of non-EDTA releasable mRNA in the polysome fraction of the gradient. The fraction of each mRNA in polysomes was calculated by subtracting the amount of non-EDTA releasable messenger RNA in polysome fractions from the polysomal messenger RNA in those fractions (7–11). This value was divided by the total amount of that messenger RNA in the gradient to give the fraction of mRNA in polysomes.

Determination of fraction of ribosomes in polysomes

The fraction of ribosomes in polysomes was determined using a technique described by Martin (1973). Post-mitochondrial supernatants were prepared from control and inhibitor-treated *Strongylocentrotus purpuratus* embryos at 15 min intervals following fertilization. Supernatants were treated with 10 $\mu g\,ml^{-1}$ ribonuclease A in high salt (0.7 M) homogenization buffer. Ribonuclease A hydrolyzes mRNA not protected by a ribosome converting polysomes into short message fragments bound to 80S ribosomes. These 80S ribosome–mRNA complexes are resistant to dissociation by high salt whereas free 80S ribosomes dissociate into 40S and 60S subunits. The amounts of 40S and 60S subunits and 80S ribosomes were quantified by fractionating homogenates on 15–40% sucrose gradients made up in high salt buffer (0.7 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM Hepes). The gradients were centrifuged in a SW 41 rotor at 41000 revs min^{-1}, for 4.3 h, at 4°C. Absorption peaks (260 nm) of the 40S and 60S subunits and 80S ribosomes were integrated and the fraction of ribosomes in polysomes calculated by dividing the amount of 80S ribosomes by the total sum of the 40S and 60S subunits and 80S ribosomes.

Determination of number of ribosomes bound per messenger RNA

Strongylocentrotus purpuratus control and inhibitor-treated post-mitochondrial supernatants were prepared and fractionated on 33 ml linear glycerol gradients in a Beckman SW 28 rotor at 28 000 revs min^{-1}, 2 h, 4 °C. RNA was isolated from 11 fractions and transferred to nylon membranes with a slot blotter. Slot blots were probed with either a ^{32}P-labeled RNA transcript of cDNA 53 or 12. Autoradiographs were scanned using a densitometer and the areas under the peaks integrated. A parallel gradient of homogenate from either 10 or 12 h embryos was run at the same time so that polysome sedimentation into the gradient could be visualized and measured. The fraction of the total RNA for each gradient fraction was multiplied by 'x' (where x=average polysome size) for each fraction and totaled to give the 'x' for the particular developmental period. Fraction 11 was not used to determine 'x'. Fraction 11 included a wash of the gradient tube which likely contained aggregated material.

Results

Previous studies utilized the isolation and *in vitro* assay of mRNPs from unfertilized eggs to determine if protein synthesis is regulated at the level of repression of mRNPs. Our approach has been to develop an *in vivo* assay that does not rely on mRNP isolation. Thus, we have avoided the problem of artifactually altering the activity of the mRNA (i.e. mRNPs) or components of the translational machinery (i.e. ribosomes, initiation factors) during the isolation or assay process. The principle of this assay is to create artificially a situation where there is additional active translational machinery in the egg. Thus, the ratio of active translational machinery to mRNA in the egg is increased. This can be accomplished by fertilizing eggs in the presence of saturating levels of a peptide elongation inhibitor. Under these conditions, as mRNAs move into polysomes, only one ribosome can bind each mRNA. (Note, we use the term polysome to describe one or more ribosomes bound to an mRNA molecule.) Further migration of the ribosome along the mRNA is blocked by the inhibitor (Fan and Penman, 1970; Lodish, 1971). If mRNA availability is the rate-limiting factor for egg protein synthesis, then we would expect that messenger RNAs would bind ribosomes with the same kinetics in control and inhibitor-treated embryos. However, if the activation of translational machinery is the rate limiting factor, then the mRNA of the inhibitor-treated embryos, which now have available extra translational machinery, would enter polysomes at a faster rate. If both mRNA and translational machinery are being activated simultaneously, we would expect results similar to those obtained if only mRNA is being unmasked. This assay is only valid when there is an excess of unutilized mRNA and translational machinery. For this reason our conclusions are only drawn from experimental measurements made 15–30 min following fertilization when both of these components are potentially available. By two hours post-fertilization most of the maternal mRNA has already been mobilized into polysomes.

To illustrate this assay, consider a hypothetical situation where 10 mRNAs and 100 ribosomes are in polysomes at time T. Assume each mRNA can bind 10 ribosomes so that when fully loaded the average polysome size is 10. Also assume that mRNAs show a linear recruitment into polysomes. In the first scenario, mRNA is limiting translation in the unfertilized egg, and is activated at fertilization. In this case, at time $T_{1/5}$, there would be 2 messages in polysomes in both control embryos and inhibitor-treated embryos. Control embryo polysomes would contain a total of 20 ribosomes with 10 ribosomes per mRNA, whereas inhibitor-treated polysomes would bind only 1 ribosome per mRNA with a total of 2 ribosomes in polysomes.

In contrast, consider an alternative scenario where translational machinery (i.e. ribosomes), and not mRNA, is limiting protein synthesis in the egg and is activated at fertilization. Then at time $T_{1/5}$, there would still be 2 messages in polysomes in the control embryos with 10 ribosomes attached to each. The inhibitor-treated embryos, however, would have available 20 active ribosomes, each of which could bind an mRNA. Thus, all 10 mRNAs would be in polysomes. In this situation where mRNA is not limiting protein synthesis, the inhibitor-treated embryos, which contain an excess of translational machinery, can mobilize mRNA into polysomes to a greater extent than control embryos. In a situation where both translational machinery and mRNA limit protein synthesis and are activated simultaneously, we would expect results similar to those if just mRNA is activated. We have made measurements similar to these examples described here. While our results are more complex than in this hypothetical situation, they indicate that both the availability of mRNA and translational machinery limit protein synthesis in the unfertilized egg.

Kinetics of messenger RNA entry into polysomes

To measure the mobilization of mRNAs into polysomes, we used labeled probes prepared from cDNA clones which are complementary to individual abundant mRNAs. These clones were selected from a cDNA library made from polysomal poly (A) RNA from 2 h (2-cell) *Strongylocentrotus purpuratus* embryos. cDNA clones coded for bonafide mRNAs on the basis of the following three criteria: (1) presence of 3′ poly (A) tails, (2) mobilization into polysomes after fertilization, and (3) release from polysomes with EDTA treatment.

One experimental difficulty encountered when measuring the kinetics of mRNA mobilization into polysomes involves the isolation of polysomal and nonpolysomal fractions of mRNA. Most often this is accomplished by density gradient centrifugation. However, the centrifugation conditions required to separate effectively these two populations of mRNAs pellets most of the polysomes. We developed a method that compresses the polysome region of the gradient into a small area (Kelso-Winemiller *et al.* 1989). This compression is accomplished by fractionating post-mitochondrial supernatants of sea urchin egg and embryo

homogenates on glycerol gradients containing an 80% glycerol–EDTA pad. When polysomes, which disaggregate in EDTA, reach this interface the ribosomes are released from the mRNA. Further migration into the gradient is greatly reduced and polysomes are not pelleted.

To be assured that individual mRNAs were behaving like the bulk of the total maternal message population, we measured the mobilization of several different mRNAs into polysomes in both control and inhibitor-treated embryos. ^{32}P-labeled RNA transcripts were prepared from cDNA clones and hybridized with RNA from gradient fractions. The fact that large untranslated mRNPs sediment in the same region of the gradient as polysomes presents a problem when determining the amount of polysomal mRNA. Since these mRNPs are stable in EDTA and polysomes are not, parallel gradients run in EDTA allowed the determination of the proportion of mRNA in the polysome region of the gradient that was untranslated mRNP (Fig. 2). The difference between the non-EDTA and EDTA polysome fractions gave the amount of polysomal mRNA in the faster sedimenting region of the gradient. For individual mRNAs, the amount of non-EDTA releasable mRNPs ranged from 0% to 17%. In Fig. 1A, RNA isolated from two separate embryo batches was probed with ^{32}P-labeled RNA transcripts to messenger RNA 53. We have subsequently identified this mRNA by sequence analysis as an 'A' type cyclin. This mRNA constitutes 0.32% of the mRNA in the egg. Each embryo batch consisted of eggs pooled from several females to reduce the possibility of individual variation. The non-EDTA releasable untranslated mRNPs were subtracted from the polysome region of the gradient and the values were averaged from the two separate experiments using different preparations of RNA. At 15 min post-fertilization, approximately 49% of the mRNA was in polysomes in the control embryos and 64% in the inhibitor-treated embryos. By 30 min, 68% and 74% of the mRNA was in polysomes in the control and inhibitor-treated embryos, respectively. In Fig. 1B, RNA isolated from one batch of embryos was probed with ^{32}P-labeled RNA transcripts to messenger RNA 12. This RNA constitutes 0.97% of the translatable mRNA in the egg. Non-EDTA releasable, untranslated mRNPs were subtracted from the polysome region of the gradient. Both the control and experimental embryos had approximately 55% of their mRNA in polysomes at 15 min. By 45 min, almost 90% of the mRNA had entered polysomes in both the control and experimental embryos. ^{32}P-labeled RNA transcripts complementary to β tubulin mRNA were also used to probe RNA isolated from one batch of embryos (Fig. 1C). Tubulin message shows a much slower rate of mobilization into polysomes. For example, only 28%

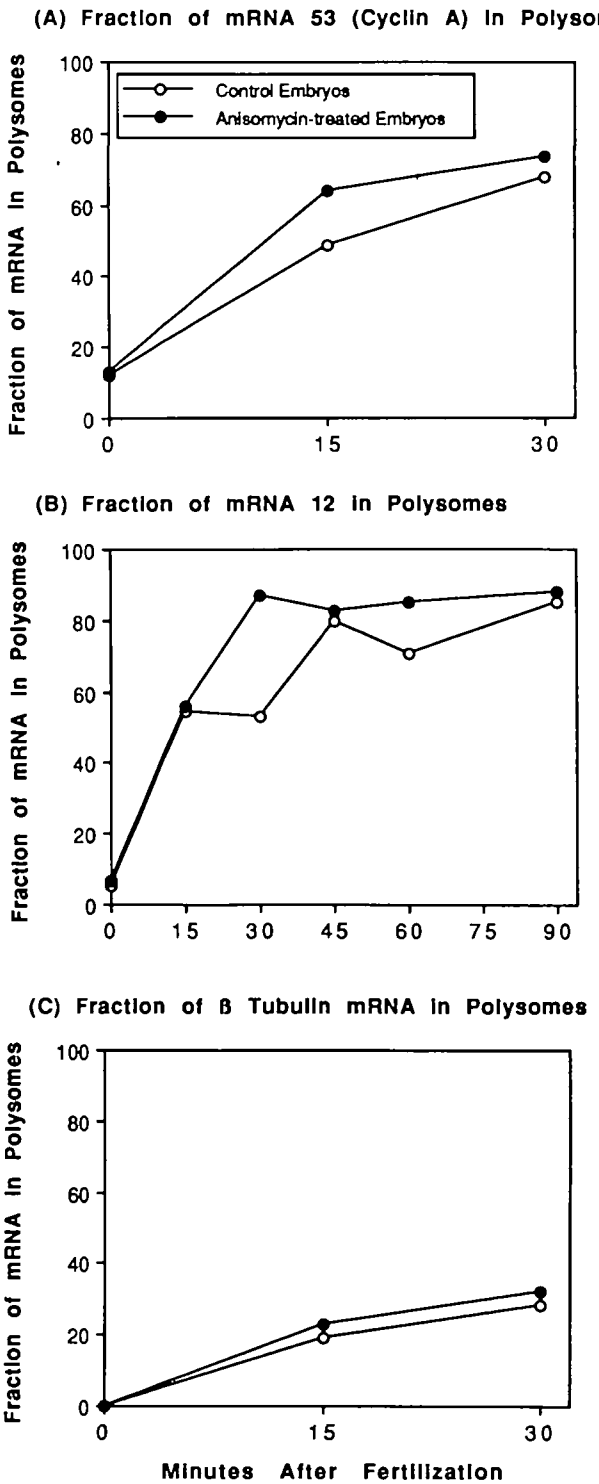

Fig. 1. Kinetics of mobilization of individual messenger RNAs into polysomes following fertilization. *Strongylocentrotus purpuratus* post-mitochondrial supernatants were fractionated on glycerol gradients containing a 80% glycerol–EDTA pad. RNA isolated from equal fractions was transferred onto nylon membranes with a slot blotter and hybridized with ^{32}P-labelled antisense RNA transcripts prepared from cDNA clones. Slot blot autoradiographs were scanned with a densitometer and the areas under the peaks integrated. (A) Average fraction of messenger RNA 53 in polysomes for two different embryo batches, (B) fraction of messenger RNA 12 in polysomes for 1 embryo batch, and (C) fraction of β tubulin messenger RNA in polysomes for 1 embryo batch. Solid circles denote inhibitor-treated embryos and closed circles denote control embryos.

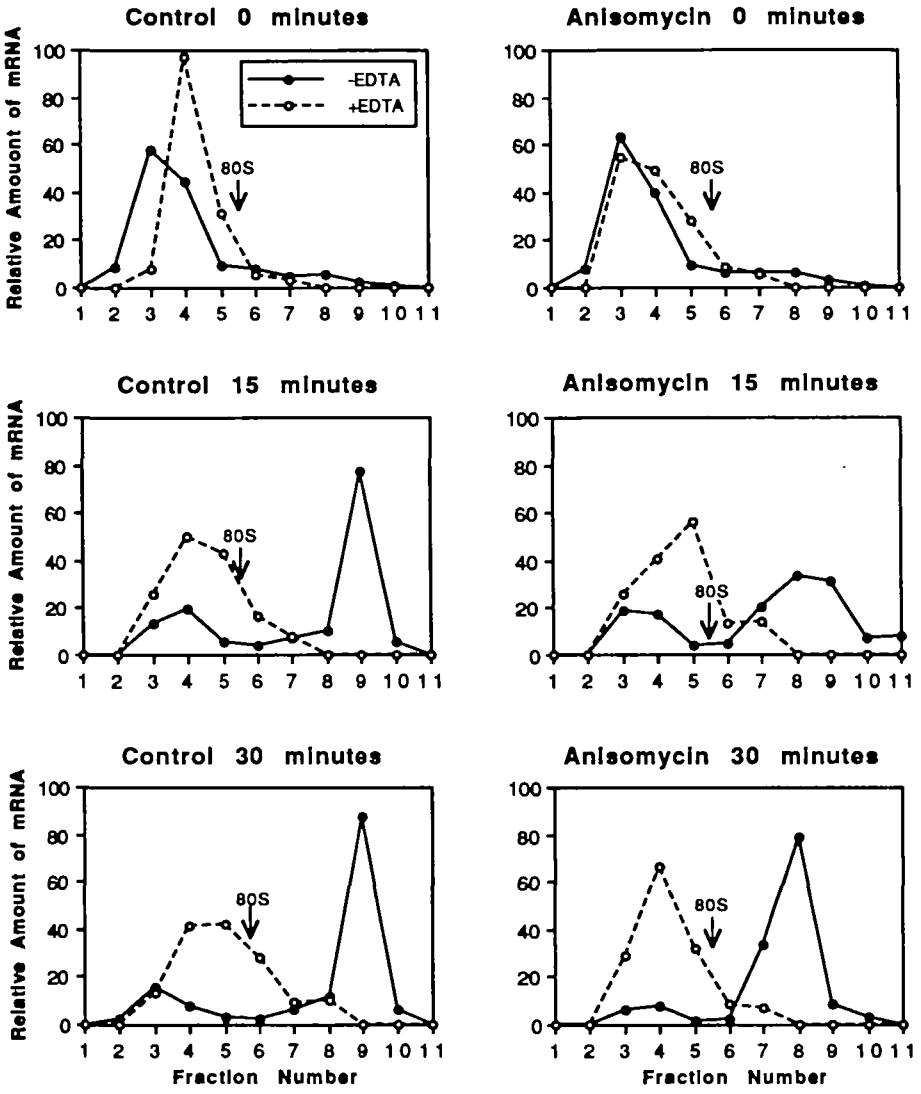

Fig. 2. Separation of polysomes from nontranslated mRNPs on glycerol gradients. *Strongylocentrotus purpuratus* post-mitochondrial supernatants prepared from control and anisomycin-treated embryos were fractionated on glycerol gradients containing an 80% glycerol–EDTA pad. RNA isolated from equal fractions was transferred onto nylon membranes with a slot blotter and hybridized with ^{32}P-labelled antisense RNA transcripts prepared from cDNA clone 53. Slot blot autoradiographs were scanned with a densitometer and the areas under the peaks integrated. Total relative amounts of RNA from each gradient were normalized to the 0 min control gradient. Solid lines indicate non-EDTA gradients and dashed lines denote gradients with 10 mM EDTA.

and 32% of β tubulin mRNA was in polysomes in control and inhibitor-treated embryos at 30 min post-fertilization. Similar rates of tubulin mRNA recruitment into polysomes occur in *Lytechinus pictus* (Alexandraki and Ruderman, 1985). Only a small portion of both a and β tubulin mRNAs are mobilized into polysomes within 30 min after fertilization. The mobilization kinetics of these three different messages into polysomes reveal that mRNAs have different rates of entry into polysomes.

It is interesting to note that approximately 10% of mRNAs 12 and 53 are in polysomes in the unfertilized egg, while tubulin is almost completely excluded from polysomes. In another study, we described the recruitment of two general classes of maternal mRNAs into polysomes at fertilization (Kelso-Winemiller and Winkler, unpublished data). We found that one class of mRNAs, which include 12 and 53, were recruited into polysomes very rapidly following fertilization. Over 50% of each of these mRNAs were in polysomes 30 min after fertilization. The other class of maternal mRNAs, which include tubulin, were mobilized into polysomes much more slowly. It appears that mRNAs that are recruited more rapidly into polysomes at fertilization are translated to a greater extent in the unfertilized egg. These results suggest that mRNA activation at fertilization is regulated by factors that show specificity for individual messages. It is not clear if these unknown 'factors' are the same as the 'masking' factor or are components of the translational machinery that interact with the message following unmasking. The fact that mRNAs in inhibitor-treated eggs show similar mobilization kinetics into polysomes as do controls suggests that the difference in activation of these two classes of mRNA lies at the level of 'unmasking'.

To confirm that these individual mRNAs were behaving like bulk of the messages, we also measured the rate of mobilization of the total mRNA population using labeled cDNA prepared from poly $(A)^+$ polysomal RNA from 2 h embryos. RNA isolated from two separate embryo batches was probed with ^{32}P-labeled cDNA (Fig. 3). We found that 25% of the bulk of mRNAs in control embryos and 30% of the bulk of mRNAs in inhibitor-treated embryos were in poly-

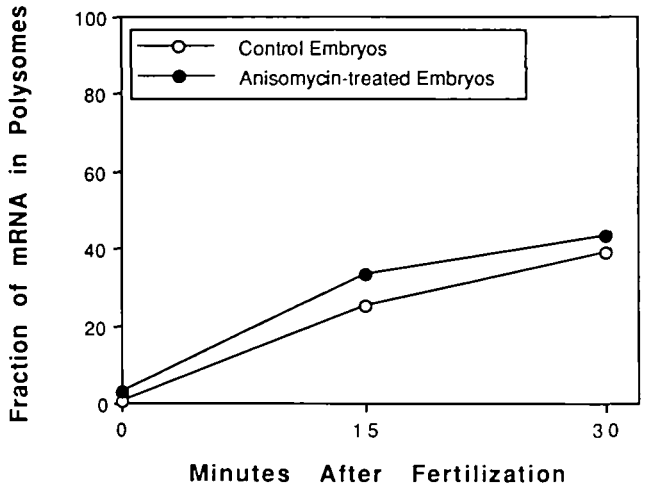

Fig. 3. Kinetics of mobilization of total mRNA into polysomes following fertilization. Slot blots described in Fig. 1 were hybridized with ^{32}P-labelled cDNA prepared from polysomal poly $(A)^+$ RNA from 2 h *Strongylocentrotus purpuratus* embryos. Closed circles denote the fraction of mRNA in polysomes in inhibitor-treated embryos. Open circles indicate the fraction of mRNA in polysomes in control embryos. Graph represents average of 2 experiments using two different embryo batches.

somes at 15 min post-fertilization. At 30 min 38 % and 43 % of the mRNAs were in polysomes in control and experimental embryos, respectively (Fig. 3). The rate of entry of the total mRNA is intermediate between the rate of mobilization of the two fast mobilizing mRNAs 53 and 12, and the slower mobilizing tubulin mRNA. This suggests that the mRNAs we studied provide a reasonable representation of the message population as a whole.

The *in vivo* approach that we used circumvents problems associated with biochemical isolation and assay of mRNPs. Our results suggest that mRNA availability limits protein synthesis in the unfertilized egg. The *in vivo* assay does not distinguish between mRNA and both mRNA and translational machinery becoming activated and it is likely that some component(s) of the translational machinery also limits protein synthesis in the unfertilized egg. This reasoning is based upon experiments that tested mRNP activity in cell-free translation systems (Winkler *et al.* 1985; Colin *et al.* 1987; Winkler, 1988). In contrast to previous *in vitro* studies, these experiments involved minimal biochemical manipulation of mRNPs. The results from these experiments clearly showed the involvement of both translational machinery and mRNA in the activation of protein synthesis at fertilization.

Measurements of mRNA entry into polysomes from inhibitor-treated embryos consistently show a slightly higher rate of mobilization as compared to mRNAs from untreated embryos (Figs 1 and 3). We speculate that at fertilization the activation of mRNA precedes slightly the activation of the translational machinery. Thus, mRNAs in inhibitor-treated eggs have excess unbound translational machinery available and are able to mobilize into polysomes slightly faster. Their rate of mobilization probably reflects the actual rate of unmasking. In contrast, in untreated embryos, where polysomes are fully loaded, there is a slight excess of active mRNAs over translational machinery. As new mRNAs become active, there is a lag time between mRNA activation and ribosome binding. This lag time decreases as more ribosomes become active. By 45 min post-fertilization, the control and inhibitor-treated embryos contain the same number of mRNAs in polysomes (Fig. 1B). Thus unmasking does not occur all at once following fertilization but continues over a period of time during early development.

Kinetics of ribosome mobilization into polysomes

To substantiate further the conclusion that mRNA availability limits protein synthesis, we used a second approach that involves measuring the rate of ribosome entry into polysomes. If mRNA availability is rate-limiting, untreated embryos should contain more ribosomes in polysomes than inhibitor-treated embryos by a factor proportional to the difference in polysome size between untreated and inhibitor-treated embryos. This is because mRNAs from inhibitor-treated embryos should bind only one ribosome. Note that whatever component of translational machinery is limiting will ultimately affect ribosome binding, and thus when considering ribosomes in this context we mean the limiting translational component.

The lack of concentrated OD_{260} absorbing material in the polysome region of the gradient makes it difficult to discern polysome peaks during sea urchin early development. In order to measure the amount of ribosomes in polysomes, we used a more sensitive procedure described by Martin (1973). Control and inhibitor-treated egg and embryo homogenates were treated with ribonuclease A, which hydrolyzes mRNA not protected by ribosomes, converting polysomes into short message fragments bound to 80S ribosomes. To distinguish free 80S ribosomes from those bound to mRNA, homogenates were treated with 0.7 M KCl. High salt dissociates free 80S ribosomes into their constituent 40S and 60S subunits, but does not affect message-bound 80S ribosomes. 40S and 60S subunits and 80S ribosomes were separated by centrifugation on high salt sucrose gradients.

Using this technique, the fraction of ribosomes in polysomes for inhibitor-treated and untreated embryos was determined for six different embryo cultures. The averages of the percentages for these six experiments is shown in Fig. 4. From 0 to 120 min following fertilization, the fraction of ribosomes in polysomes increased gradually from 1.8 % to 16.5 % in the control embryos. This is consistent with results from other investigators using different methods that show approximately 20 % of the ribosomes in polysomes by the first 2 h after fertilization (Humphreys, 1971; Goustin and Wilt, 1981). In the same time period, the fraction of ribosomes in polysomes in the anisomycin-treated embryos increased from 1.7 % to 9.3 %.

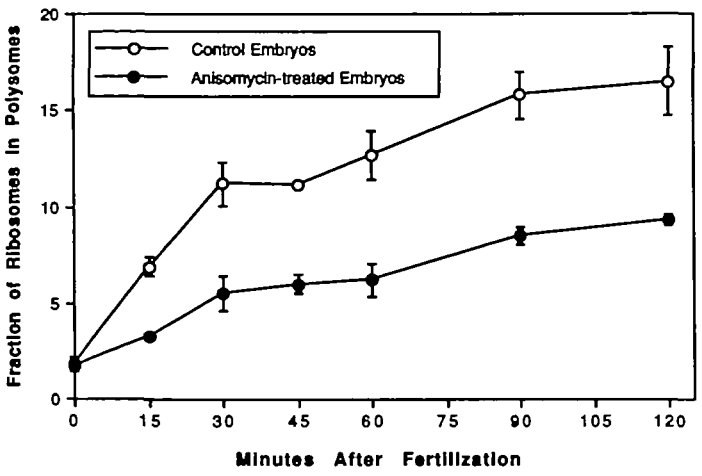

Fig. 4. Kinetics of ribosome entry into polysomes following fertilization. This figure shows the average fraction of ribosomes in polysomes for six embryo batches at 15 min intervals following fertilization. Error bars indicate standard error for each time point. Points lacking error bars had standard errors less than ±0.5. Solid circles indicate inhibitor-treated embryos and open circles denote control embryos.

On the average 2.0-fold more ribosomes were found in polysomes in untreated embryos compared to inhibitor-treated embryos (Fig. 4). Thus, ribosomes are mobilizing into polysomes to a greater extent in control embryos than in inhibitor-treated embryos, even with additional active translational machinery available in the inhibitor-treated embryos. We found that the average polysome size in untreated embryos is 7.5 ribosomes and in inhibitor-treated embryos is 3.2 ribosomes (Fig. 5) (The fact that polysomes from inhibitor-treated embryos bind more than a single ribosome will be discussed below). Thus, if mRNA availability limits protein synthesis, then polysomes of untreated embryos should contain 7.5/3.2 or 2.3-fold more ribosomes than inhibitor-treated embryo polysomes. The experimental (2.0 fold) and predicted (2.3 fold) values for the difference in polysome size are in close agreement clearly demonstrating that mRNA availability is a factor limiting protein synthesis in the unfertilized egg.

There are several possible explanations why mRNAs in inhibitor-treated embryos bind not the theoretical single ribosome, but an average of 3.2 ribosomes. First, anisomycin may not completely inhibit protein synthesis and movement of ribosomes down the mRNA, thus allowing additional ribosomes to bind. We measured both the rate of protein synthesis and the amount of labeled nascent chains in polysomes in inhibitor-treated embryos. Inhibitor-treated eggs failed to show any incorporation of the [^{35}S] methionine into proteins after fertilization and lacked labeled peptide chains in polysomes indicating that ribosomes are not 'creeping' down the messenger RNA (Shettles, B. and Winkler, M. M., unpublished data). Simultaneous treatment with saturating amounts of both emetine and anisomycin did not reduce the amount of ribosomes in polysomes over that seen with just one inhibitor. Emetine and anisomycin both inhibit elongation, but by different mechanisms (Vazquez, 1979). Anisomycin binds to the 60S subunit and blocks peptide bond formation, while emetine acts on the 40S subunit and prevents EF-2-dependent translocation by ribosomes. Thus, their effects would be expected to complement each other. These observations collectively suggest that anisomycin is completely inhibiting protein synthesis in treated eggs.

A more likely explanation is that more than one ribosome is binding each mRNA, even with the AUG initiator codon blocked by the first ribosome. Previous investigators have found that two ribosomes are able to bind to Tobacco Mosaic Virus and Turnip Yellow Mosaic Virus RNA leader sequences when they are incubated in a wheat germ extract with another elongation inhibitor, sparsomycin (Tyc *et al.* 1984; Filipowicz and Haenni, 1979). The first ribosome binds to the AUG initiator codon and the second ribosome binds upstream from the initiator codon in the 5′ untranslated region (≥69 nucleotides) of the mRNA. Similar formation of disomes, or trisomes, is probably occurring on sea urchin mRNAs, when embryos are incubated in the presence of anisomycin. There are several examples of eukaryotic mRNAs in which translation starts from an initiator AUG downstream from other non-initiating AUG codons (Racaniello and Baltimore, 1981; Darlix *et al.* 1982; Mulligan and Berg, 1981). Sequence analysis of an abundant cDNA clone from our 2 h *S. purpuratus* library revealed the presence of two additional AUG (out of frame) codons upstream from the AUG initiator codon (Yoon and Winkler, unpublished results). Thus, it is likely that binding of multiple ribosomes 5′ of the initiator AUG occurs in the sea urchin system.

Another issue raised by this work concerns changes in average polysome size following fertilization. We found that polysome size gradually increases during early development from 7.5 ribosomes in 15 min embryos to 9.8 ribosomes in 1 h embryos and by 2 h to 10.8 ribosomes (Fig. 5). A number of other investigators have also measured polysome size during development. Rinaldi and Monroy (1969) clearly showed with polyribosome profiles that the size of polysomes increases from 2 to 10 min and continues to increase up to the 2-cell stage (90 min). Martin and Miller (1983) determined polysome size from electron micrographs of *Lytechinus pictus* eggs and embryos. Their polysome size measurements show a decrease in polysome size following fertilization from an average 11.96 ribosomes per mRNA in eggs to an average 7.14 ribosomes per mRNA in 1 h embryos. It is possible that the centrifugation process used to spread polysomes on electron microscope grids might have artifactually selected for larger polysomes in the eggs. Humphreys (1969, 1971) concluded that polysome size does not change appreciably during the first 6 h of development (up to the 16-cell stage). However, inspection of

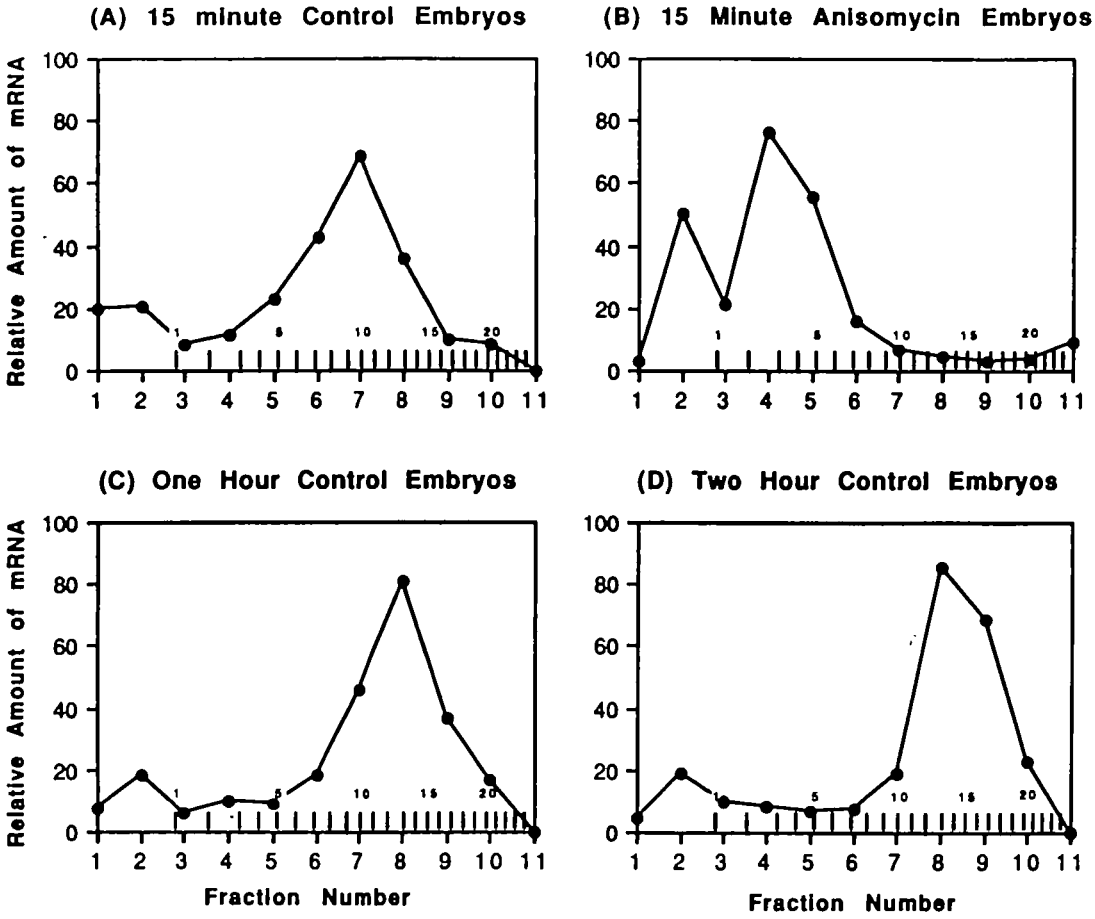

Fig. 5. Determination of number of ribosomes bound per messenger RNA. *Strongylocentrotus purpuratus* control and anisomycin-treated post-mitochondrial supernatants were prepared and fractionated on linear glycerol gradients. RNA was isolated from 11 fractions and transferred to nylon membranes with a slot blotter. Slot blots were probed with a ^{32}P-labelled RNA transcript of cDNA 53. Autoradiographs were scanned using a densitometer and the areas under the peaks integrated. A parallel gradient of either 10 or 12 h homogenate was run at the same time so that polysome sedimentation into the gradient could be visualized and measured. The sedimentation of polysomes in the gradient is indicated by the slash marks on the x-axis (i.e. 1=monosome, 5=polysome with 5 ribosomes bound, etc.). The fraction of the total RNA for each gradient fraction was multiplied by 'x' (where x=average polysome size) for each fraction and totaled to give the 'x' for the particular developmental period. Fraction 11 was not used to determine 'x'. Fraction 11 included a wash of the gradient tube which likely contained aggregated material. (A) 15 min control embryos, (B) 15 min anisomycin embryos, (C) 1 h control embryos, (D) 2 h control embryos.

Humphreys polysome profiles does reveal a slight shift to larger polysome size by 1 h following fertilization. This is consistent with our results which show that polysome size increases during the first 2 h of development by approximately 3 ribosomes per message. A possible explanation for this increase is that the ratio of active translational machinery to mRNA increases during this period. Earlier, we discussed that mRNA activation slightly precedes that of the translational machinery. As more translational machinery is activated during early development more ribosomes will be able to bind to messages. An alternative possibility is that polysomes require a long time period to reach the fully loaded state. Nelson and Winkler (1987) found that full loading of mRNAs in a reticulocyte lysate required at least 10 ribosome transit times. In either case, the increase in polysome size provides an additional piece of evidence consistent with the idea that translational machinery is activated continuously following fertilization.

Discussion

Our results support the hypothesis that both mRNA and some component or components of the translational machinery are limiting protein synthesis in the unfertilized egg of the sea urchin, *Strongylocentrotus purpuratus*. Following fertilization both of these are gradually made available for protein synthesis in the embryo. In the past, most hypotheses regarding the question as to what is limiting protein synthesis in the egg have fallen into two general categories. These are the limited availability of either (1) mRNA or (2) a component (s) of the translational machinery. These two hypotheses have often been presented as alterna-

tive possibilities. Humphreys (1971) postulated that if translational machinery was limiting in the egg and made available for protein synthesis at fertilization then polysome size would change. Since sea urchin eggs and embryos contain equal size polysomes (Humphreys, 1971), he concluded that mRNA and not translational machinery is made available for protein synthesis at fertilization. Other experimenters (Giudice, 1973; Danilchik and Hille, 1981) looking at the activity of various components of the translational machinery *in vitro* have come to the opposite conclusion that translational machinery rather than mRNA is limiting protein synthesis in the unfertilized egg.

However, these two hypotheses are not mutually exclusive. Several investigators have suggested that both translational machinery and mRNA are rate limiting for protein synthesis in the unfertilized egg (Winkler *et al.* 1985; Colin *et al.* 1987; Lopo *et al.* 1988; for a review see Clemens, 1987). mRNPs from both *Lytechinus pictus* and *Strongylocentrotus purpuratus* eggs do not bind efficiently to preinitiation complexes in reticulocyte lysate translation systems (Winkler *et al.* 1985; Grainger and Winkler, 1987; Lopo *et al.* 1988). This suggests that mRNAs in the egg are masked or unavailable for translation in the unfertilized egg. However, other experiments clearly show the involvement of translational machinery in the activation of protein synthesis at fertilization. Highly active cell-free translation systems have been developed to analyze the mechanisms limiting protein synthesis during early embryogenesis (Winkler and Steinhardt, 1981; Colin *et al.* 1987; Hansen *et al.* 1987; Lopo *et al.* 1989). These cell-free systems demonstrate rates of protein synthesis and regulatory characteristics approaching those observed *in vivo*. If protein synthesis in the unfertilized egg is limited by the supply of active mRNA, then the addition of exogenous message should stimulate protein synthesis. If protein synthesis in the unfertilized egg is limited by deficiencies in the cell's translational machinery then the exogenous message would be translated at the expense of endogenous message and overall protein synthesis would remain the same. Protein synthesis is not increased in unfertilized egg cell-free translation systems when exogenous mRNAs alone are added. However, protein synthesis can be stimulated by addition of eukaryotic initiation factors (eIF) eIF-2, eIF-4F, the guanine nucleotide exchange factor (GEF), or soluble components of post-ribosomal supernantants from reticulocyte lysates (Winkler *et al.* 1985; Colin *et al.* 1987; Huang *et al.* 1987; Lopo *et al.* 1988). The addition of increasing amounts of exogenous mRNA with purified initiation factors or S100 fractions from rabbit reticulocytes causes an even greater stimulation of protein synthesis (Colin *et al.* 1987; Winkler and Grainger, 1987). Thus initiation factors expand the capacity of the translational machinery which can then be utilized by the exogenous mRNA. Despite the expanded capacity of the translational machinery, the endogenous mRNAs still remain untranslated suggesting that they are in some repressed form unavailable to the translational machinery.

Other evidence for the involvement of initation factors in the activation of protein synthesis comes from the identification of inhibitor of translation in the egg (Hansen *et al.* 1987). Addition of this inhibitor to cell-free translation systems from sea urchin embryos or rabbit reticulocytes prevents translation. However, the inhibition can be reversed by the addition of exogenous eIF-4F to sea urchin embryo or reticulocyte cell-free systems (Huang *et al.* 1987). Experimental results indicate that phosphorylation of one subunit of eIF-4F at fertilization reverses the inhibitory effects (as referenced in Lopo *et al.* 1988). Using intact cells, rather than a cell-free system, Colin (Colin and Hille, 1987) also came to the conclusion that components of the translational machinery are limiting protein synthesis in the unfertilized sea urchin egg. Exogenous globin mRNA injected into unfertilized eggs of *Strongylocentrotus droebachiensis* and *L. pictus* had little effect on the overall rate of protein synthesis.

The frog *Xenopus laevis* is another organism in which the mechanisms for regulating protein synthesis during development have been intensely studied. At maturation, the frog oocyte undergoes a 2- to 4-fold increase in the rate of protein synthesis. The control of this protein synthesis increase has been studied principally by microinjecting mRNA into *Xenopus* oocyte cytoplasm. Like the sea urchin, the addition of exogenous mRNA does not stimulate overall protein synthesis. This suggests that a component of the translational machinery is limiting in the oocyte (Laskey *et al.* 1977; Richter and Smith, 1981). On the other hand, the microinjection studies of Lingrel and Woodland (1974) argue against translational machinery as the regulator of protein synthesis. Their experiments show that injection of exogenous message into oocytes does not result in a change in polysome size. Like Humphreys, they interpret their results to mean that protein synthesis is not regulated at the level of translational machinery since such events would lead to a change in polysome size. Recently, Audet *et al.* (1987) have reported that microinjection of initiation factor eIF-4A stimulates translation in *Xenopus* oocytes. However, no direct tests have been conducted on costimulation of protein synthesis by mRNAs and translational machinery components. It seems likely, however, that in *Xenopus* dual levels of control similar to those in the sea urchins are operating.

Recently, Calzone *et al.* (1988) have studied the regulation of protein synthesis in *Tetrahymena*. Growing *Tetrahymena* cells and starved-deciliated *Tetrahymena* cells show an increased rate of protein synthesis over starved *Tetrahymena* cells (Calzone *et al.* 1983). Since the polysome size and the peptide elongation rate remained constant during changes in the rate of protein synthesis, they suggested that messages could not be competing for a limiting initiation factor. They also argued against differential affinity of messages for a limiting initiation factor because essentially the same set of message sequences are found in polysomes before and after the change in the rate of protein synthesis. Thus, they concluded that the major mechanism that

regulates the rate of protein synthesis in *Tetrahymena* is the regulation of the number of messages made available for translation and not a change in the translational efficiency (polypeptides produced per mRNA per unit time) of the mRNAs. They concluded that unmasking of an untranslatable form of mRNA could explain the changes in protein synthesis seen between starved and growing *Tetrahymena* cells.

Our results show that the regulation of the increase in protein synthesis at fertilization is a complex process, which is controlled by components of the translational machinery as well as availability of the mRNA. We also find that mRNAs are not all simultaneously recruited into polysomes but show differential rates of mobilization. Some mRNAs mobilize into polysomes very rapidly at fertilization while others enter polysomes much more slowly. Those mRNAs that are recruited rapidly are translated to a greater extent in the egg. These results are consistent with masking factor(s) with different affinities for different mRNAs similar to the RNA-binding proteins which bind specific message sequences in *Xenopus* (Crawford and Richter, 1987). mRNAs that mobilize into polysomes with a faster rate would have a lower affinity for a masking factor than mRNAs that enter polysomes more slowly. Another possibility is that different mRNAs have different affinities for components of the translational machinery. For example, it is possible that in the egg there is only a limited supply of an active initiation factor which binds to individual mRNAs. At fertilization more of the initiation factor becomes available to bind to messages. A likely candidate for such an initiation factor could be eIF-4F, the 5′ cap binding protein. As discussed previously, eIF-4F activity is suppressed by an inhibitor in the egg which is inactivated at fertilization. Ray *et al.* (1983) identified eIF-4F as the limiting initiation factor implicated in discrimination of mRNAs for translation. Those messages with a higher affinity for the limited initiation factor would be preferentially translated in the egg and would be recruited into polysomes faster in the embryo. β tubulin exemplifies this hypothesis. It is barely translated in the egg and is recruited into polysomes at a slower rate than the bulk of the messages at fertilization. It is possible that classical initiation factors could act as mRNA recruitment factors and give results that appear similar to those caused by masking factors. This is reminiscent of models proposed by Lodish (1974) regarding competition of mRNAs for initiation factors. This type of model, however, requires a more complex set of assumptions than do unmasking models. In summary, our results show that mRNAs are made available for entry into polysomes following fertilization. We also conclude that components of the translational machinery are activated at fertilization and that their activation may be slightly preceded by that of the messenger RNA. It is the synergistic effect of these two processes that is responsible for the regulation of protein synthesis in the early sea urchin embryo.

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