Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 6359–6363, September 1987
Biochemistry

Inhibitor of eukaryotic initiation factor 4F activity in unfertilized sea urchin eggs

(translational control/mRNA utilization)

W.-I. HUANG*, L. J. HANSEN*, W. C. MERRICK†, AND R. JAGUS*‡

*Department of Microbiology, Biochemistry and Molecular Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261; and †Department of Biochemistry, Case Western Reserve School of Medicine, Cleveland, OH 44601

Communicated by Harland G. Wood, May 21, 1987

ABSTRACT **Extracts from unfertilized sea urchin eggs contain an inhibitor of translation that inhibits protein synthesis in cell-free translation systems from sea urchin embryos or rabbit reticulocytes. The inhibitory effects of egg extracts can be reversed by the addition of mammalian eukaryotic initiation factor 4F (eIF-4F) in both sea urchin embryo and reticulocyte systems, suggesting that the inhibitor inactivates this initiation factor. The accumulated data suggest that the ability of eIF-4F to recycle may be compromised. The addition of eIF-4F to cell-free translation systems from unfertilized sea urchin eggs also stimulates protein synthesis. However, the stimulation does not increase protein synthetic activity in the egg cell-free translation system to the levels observed in those produced from 2-hr embryos. This suggests that, although the unfertilized egg contains an inhibitor of eIF-4F and reduced levels of eIF-4F activity, inactivation of this component is only one of the factors involved in the low rate of maternal mRNA utilization found prior to fertilization.**

The unfolding of morphogenetic events in the newly fertilized egg depends heavily on the utilization of mRNAs deposited in the oocyte (1, 2). The mechanism(s) that regulates translation of this maternally accumulated mRNA is poorly understood. However, the inability of the unfertilized egg to translate the stored mRNA has been attributed to at least three causes: (*i*) proteins associated with mRNA prevent translation, a hypothesis referred to as "masking" (3, 4); (*ii*) the rate of translation is limited by the availability or activity of translation components other than mRNA (5, 6); (*iii*) maternal mRNA requires additional modification or processing (7–9).

Following fertilization, there is a dramatic increase in the rate of protein synthesis utilizing maternal mRNA (10, 11). However, although previous investigations have demonstrated that the egg appears to have all the necessary components of the protein synthetic machinery (1, 12, 13), a satisfactory explanation of the mechanism of protein synthesis activation has not been presented.

Previous studies, using cell-free translation systems from sea urchin eggs and 2-hr embryos, showed that unfertilized sea urchin eggs contain an inhibitor of translation (14). This inhibitor reduces mRNA utilization in cell-free translation systems from sea urchin 2-hr embryos and from rabbit reticulocytes (14). The egg inhibitor acts at the level of initiation to reduce 60S-subunit interaction with the 48S, mRNA-containing, preinitiation complex, resulting in the accumulation of this normally short-lived intermediate (14).

The present study indicates that the inhibitory effects of egg extracts in reticulocyte lysate can be reversed by added eukaryotic initiation factor 4F (eIF-4F), which promotes mRNA binding to the small ribosomal subunit (15). In view of the function of eIF-4F, the accumulation of a mRNA-containing initiation intermediate, described in the earlier report (14), is somewhat surprising and suggests that the egg inhibitor may prevent the normal recycling of eIF-4F.

The role of the inhibitor of eIF-4F activity in the maintenance of low protein synthetic rates prior to fertilization seems to be complex. Although translation systems from unfertilized eggs contain an inhibitor of eIF-4F, protein synthetic rates are only stimulated modestly by added eIF-4F, suggesting that eIF-4F inactivation is only one of the factors involved in the regulation of mRNA utilization prior to fertilization.

MATERIALS AND METHODS

Handling of Gametes and Embryos. Spawning, *in vitro* fertilization, and culturing of *Strongylocentrotus purpuratus* gametes were performed as described (14).

Preparation of Cell-Free Translation Systems. The preparation of cell-free translation systems from sea urchin eggs and embryos is described in detail by Hansen *et al.* (14). Pelleted, dejellied eggs and embryos were resuspended and rinsed twice in 105 mM potassium gluconate/50 mM Hepes/40 mM NaCl/1 mM $Mg(CH_3CO_2)_2$/10 mM EGTA/300 mM glycine/220 mM glycerol/1 mM spermidine/1 mM dithiothreitol, pH 7.2. The cells were homogenized in equal volumes of the same buffer supplemented with RNAsin (Promega Biotec, Madison, WI) at 300 units/ml, soybean trypsin inhibitor at 1 mg/ml, ovomucoid at 1 mg/ml, and lysolecithin at 250 μg/ml. The cells were disrupted in a chilled, tight-fitting stainless-steel Dounce homogenizer. The homogenates were centrifuged at 15,000 rpm, Sorvall SS34, for 10 min at 4°C. The supernatants were removed and dialyzed against the rinse buffer at 4°C for 3 hr, with buffer changes every hour. Glucose 6-phosphate was added to 500 μM prior to snap-freezing of aliquots. Rabbit reticulocyte lysate was prepared as described (16).

Cell-Free Translation. Sea urchin egg and embryo cell-free translation assays contained 0.9 vol of egg or embryo lysate and other components at the following final concentrations: 3 units of creatine phosphokinase (Boehringer Mannheim) per ml, 10 mM phosphocreatine, 0.75 mM ATP, 0.1 mM GTP, 1.85 mM $Mg(CH_3CO_2)_2$, 150 μM amino acids minus methionine, and 0.5 μM (50 μCi) [^{35}S]methionine (800–1200 Ci/mmol; New England Nuclear, translation grade; 1 Ci = 37 GBq). The translation systems were incubated at 20°C, and 5-μl aliquots were removed at various times for determination of trichloroacetic acid-precipitable radioactivity. For experiments in which egg and embryo extracts were mixed, egg lysate was added to replace part of the 0.9 vol normally represented by embryo extract but is expressed as a percent-

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Abbbreviation: eIF, eukaryotic initiation factor.

‡To whom reprint requests should be addressed.

age of the total volume. In control incubations, dialysis buffer replaced embryo extract to the same final percentage.

The incorporation of [^{14}C]valine in the rabbit reticulocyte translation system was assayed as described (16). All reticulocyte incubations also contained RNAsin (300 units/ml), EGTA (2 mM), and soybean trypsin inhibitor (1 mg/ml) to ensure that the inhibitory effects of egg extract do not represent spurious RNase or protease activities. For experiments in which egg extracts were included, the egg lysate was first dialyzed against 25 mM KCl/10 mM NaCl/20 mM Hepes, pH 7.2/1 mM $Mg(CH_3CO_2)_2$. Egg extract was added to replace part of the 0.8 vol normally represented by reticulocyte lysate but is expressed as a percentage of the total volume. In control incubations, dialysis buffer was substituted for egg extract to the same final percentage.

Purification of Initiation Factors. eIF-4A, eIF-4B, and eIF-4F were purified from rabbit reticulocyte lysate as described (15, 17); eIF-2 and eIF-2B were purified from the same source as described (18). The activities of eIF-2 and eIF-2B were checked for biological activity by measuring their abilities to restore activity to a hemin-deficient rabbit reticulocyte translation system. The biological activities of eIF-4A, -4B, and -4F were checked by their abilities to retain mRNA on nitrocellulose filters and promote RNA-dependent ATP hydrolysis.

RESULTS

eIF-4F Stimulates Reticulocyte Protein Synthesis in the Presence of the Egg Inhibitor. Fig. 1 shows the effects of egg extract on [^{14}C]valine incorporation in the rabbit reticulocyte translation system. At levels of egg extract representing 15% of the volume of the incubation mix, protein synthesis continues at the same rate as in its absence for the first 10–12 min and then declines. This inhibition has been described previously and shown to be due to a defect at the level of initiation (14). In contrast to the inhibitory effects of egg extracts, those prepared from 2-hr embryos show negligible effects on translation (14).

Each step in the initiation sequence is known to be promoted by specific protein factors called initiation factors (recently reviewed in ref. 19). Modulation of initiation rates by changing specific initiation factor activity has been widely documented in mammalian systems. It seems highly probable that regulatory mechanisms similar to those operating in other eukaryotes play a role in translational control in the developing sea urchin. Consequently, initiation factor regulation is a logical potential site for translational modulation by the egg inhibitor. To evaluate this, several purified initiation factors were added back to the reticulocyte translation system along with the egg inhibitor.

Table 1 shows the effects of adding a variety of purified initiation factors to a rabbit reticulocyte translation system incubated with or without the egg inhibitor. Initiation factor concentrations were chosen to be between 5 and 15 times their endogenous level in reticulocyte lysate. The addition of eIF-2, eIF-2B, eIF-4B, and a fraction containing eIF-2, eIF-3, eIF-4C, and eIF-5 did not stimulate protein synthesis. Added eIF-4A gave a slight stimulation. In contrast, eIF-4F gave marked stimulation of activity in reticulocyte lysate incubated with egg extract. In reticulocyte lysate incubated without egg extract, the addition of eIF-4F had no effect. The addition of eIF-4A together with eIF-4F did not potentiate stimulation (results not shown).

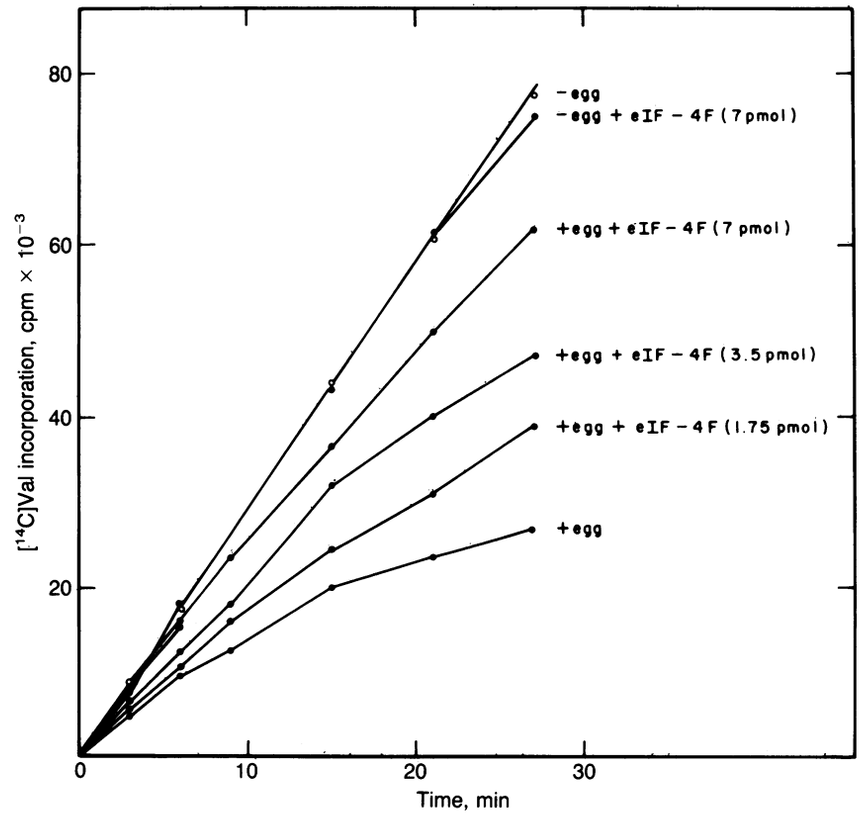

FIG. 1. Effect of eIF-4F on inhibition of translation in a reticulocyte translation system by sea urchin egg extracts. Supernatants of egg extracts (10,000 g) were prepared as described. Reticulocyte lysate was incubated in the presence or absence of dialyzed egg extracts (15%) and in the presence or absence of increasing quantities of purified eIF-4F. The amounts of eIF-4F indicated in the figure reflect the amount added per 50-μl incubation. Protein synthetic activity of reticulocyte lysate was assayed at 30°C under standard conditions as described (14). Aliquots (5 μl) were removed at the time indicated and assayed for the incorporation of [^{14}C]valine into trichloroacetic acid-precipitable material. A time course of incorporation is shown here.

Table 1. Effect of purified translational initiation factors on inhibition of protein synthesis by egg extracts in reticulocyte lysate

Egg extract	eIF added	[^{14}C]Valine incorporation, cpm/5 μl per 27 min
Without	—	76,359
With	—	27,256
Without	eIF-2	76,526
With	eIF-2	26,892
Without	eIF-2B	76,875
With	eIF-2B	26,783
Without	eIF-4A	77,540
With	eIF-4A	32,027
Without	eIF-4B	76,460
With	eIF-4B	27,848
Without	eIF-4F	76,898
With	eIF-4F	60,098
With	eIF-2, -3, -4C, -5	25,432

Reticulocyte lysate was incubated for 27 min in the presence or absence of dialyzed egg extract (15%) and in the presence or absence of purified initiation factors. The final concentrations of factors in the incubation mix were: eIF-2, 200 pmol/ml; eIF-2B, 100 pmol/ml; eIF-4A, 1.79 nmol/ml; eIF-4B, 500 pmol/ml; eIF-4F, 140 pmol/ml; and a fraction containing eIF-2, eIF-3, eIF-4C, and eIF-5, 500 μg/ml. Protein synthetic activity was assayed as described; 5-μl aliquots were removed at various times and assayed for trichloroacetic acid-precipitable ^{14}C-labeled material.

eIF-4F is one of the factors involved in the interaction of mRNA with the small ribosomal subunit (15, 20). This factor is composed of three dissimilar subunits with molecular mass of 220, 46, and 24–26 kDa (15, 20). It has been reported to be present at lower levels than other initiation factors in rapidly growing HeLa cells (21) and is only present in rabbit reticulocyte translation systems at levels between 0.02 and 0.05 per ribosome (22). Therefore, eIF-4F could be a limiting factor in mRNA utilization. Fig. 1 shows the effect of increasing quantities of eIF-4F on the inhibition of translation by egg extract. Added eIF-4F reduced the inhibition by egg extracts at all levels added. The addition of 7 pmol of eIF-4F, a level 7–15 times the endogenous eIF-4F level, prevented significant inhibition. The addition of eIF-4F to reticulocyte lysate without added egg extract had no stimulatory effect.

Fig. 2 shows the effect of adding eIF-4F back to reticulocyte lysate at various times after inhibition by egg extract has been established. In addition to preventing the inhibition of translational activity in reticulocyte lysate by egg extracts, eIF-4F also could rescue the activity of egg-inhibited reticulocyte lysate.

eIF-4F Also Prevents Protein Synthesis Inhibition by Egg Extracts in a Translation System from 2-hr Embryos. Egg extracts also inhibit translation in cell-free systems prepared from sea urchin embryos. Fig. 3 shows the effects of 15% (vol/vol) egg extract on [^{35}S]methionine incorporation in the translation system prepared from 2-hr embryos. The addition of egg extract resulted in the inhibition of translation with similar kinetics to that observed in the reticulocyte system. Fig. 3 also shows that the addition of eIF-4F could reduce significantly the inhibition of translation by the egg inhibitor in the cell-free translation system from 2-hr embryos. This suggests that egg extracts inhibit embryo translation systems by a similar mechanism to that observed in the reticulocyte system.

In addition, these data show that reticulocyte eIF-4F could be utilized by the sea urchin translational machinery. Lopo *et al.* (23) have previously demonstrated that many sea urchin egg and embryo translational components are active in heterologous assays for mammalian factors. Their demonstration of conservation of function supports the use of rabbit initiation factors in probing sea urchin translation systems for defects in the initiation machinery. However, it is reassuring to find direct confirmation of this for eIF-4F.

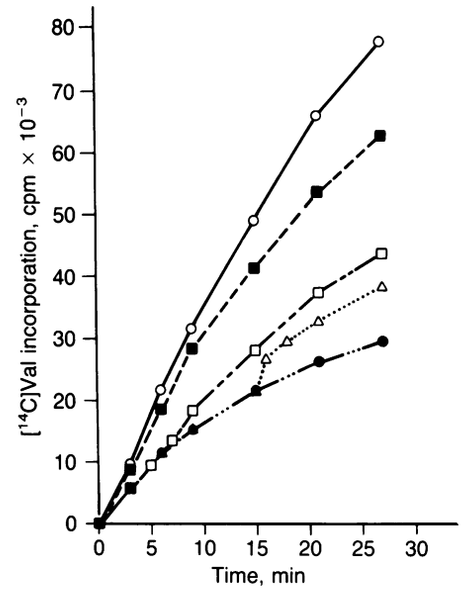

FIG. 2. Effect of delayed addition of eIF-4F on inhibition of translation in a reticulocyte translation system by sea urchin extracts. Reticulocyte lysate was incubated in the presence or absence of dialyzed egg extracts (15%). The equivalent of 7 pmol of eIF-4F per 50 μl was added to the incubation mix at 0, 5, and 15 min of incubation. Protein synthetic activity of reticulocyte lysate was assayed at 30°C under standard conditions as described (14). A time course of incorporation is shown here. ○, Without egg; ●, ■, □, △, with egg at 0 min; ■, with eIF-4F at 0 min; □, with eIF-4F at 5 min; △, with eIF-4F at 15 min.

Relevance of the Inhibitor of eIF-4F to the Low Rates of mRNA Utilization Prior to Fertilization. The accumulated data suggest that the egg contains an inhibitor of eIF-4F activity, and the question that arises is how relevant this inhibitor is to the low level of mRNA utilization in the egg. This question was addressed by adding purified eIF-4F to a cell-free translation system derived from unfertilized eggs. Unlike the translation systems derived from 2-hr embryos, which contain similar levels of ribosomes and mRNA, those derived from eggs show very low protein synthetic rates that reflect the rates observed *in vivo* (14). As shown in Table 2, the addition of eIF-4F only stimulated protein synthetic rate in the egg translation system by approximately 40%. This level of stimulation was seen consistently in all egg preparations examined (results not shown). Increasing the amount of eIF-4F added did not give significant additional stimulation (results not shown). However, this level was approximately 10 times lower than that observed in the translation systems derived from 2-hr embryos. Since eIF-4F could restore protein synthetic activity to an egg-inhibited embryo translation system, as shown in Fig. 3, this result cannot reflect the inability of rabbit eIF-4F to promote mRNA binding to the small ribosomal subunit in the sea urchin system. One reason that the stimulation by eIF-4F in the egg cell-free system was much less than that observed in the embryo or reticulocyte system could be that the factor(s) leading to the inhibition of eIF-4F is present in much higher levels in the egg. However, these data could also suggest that eIF-4F inactivity is not the only lesion in initiation occurring in the unfertilized egg.

Added eIF-4F did not stimulate protein synthetic activity of the cell-free translation system from 2-hr embryos. This lack of effect of added eIF-4F on the embryo system indicates that this rare initiation factor is retained during preparation of the

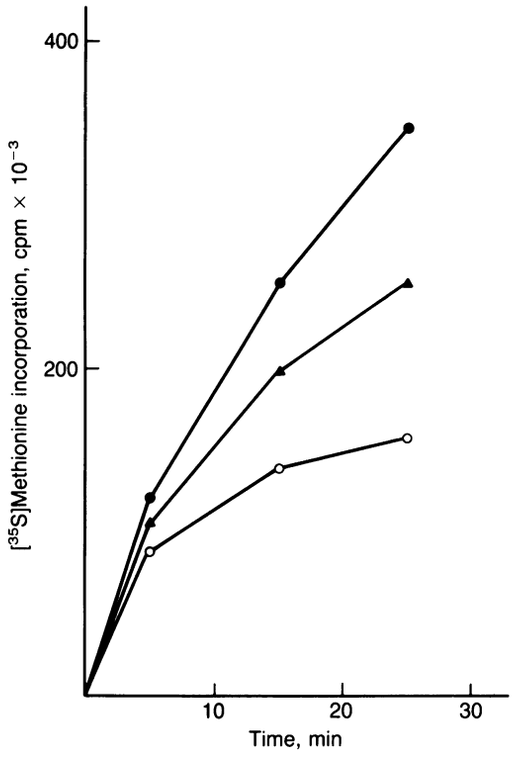

FIG. 3. Effect of eIF-4F on inhibition of translation in a cell-free system from 2-hr embryos by sea urchin egg extracts. Cell-free translation systems from *S. purpuratus* eggs and 2-hr embryos were prepared as described and dialyzed against 105 mM potassium gluconate/50 mM Hepes, pH 7.2/40 mM NaCl/1 mM Mg-$(CH_3CO_2)_2$/10 mM EGTA/300 mM glycine/220 mM glycerol/1 mM spermidine/1 mM dithiothreitol. The systems were incubated at 20°C as described in the presence or absence of egg extract (15%) and in the presence or absence of eIF-4F (7 pmol/50 μl). Aliquots (5 μl) were assayed for [^{35}S]methionine incorporation into trichloroacetic acid-precipitable radioactivity. A time course of incorporation is shown here. ●, Two-hour embryos incubated in the absence of both egg and eIF-4F; ▲, in the presence of both 15% egg and eIF-4F; ○, in the presence of 15% egg and absence of eIF-4F.

cell-free extracts and that factor loss is unlikely to account for the stimulation by eIF-4F observed in the egg system.

When eIF-4F was added to the egg translation system along with purified globin mRNA (30–300 μg/ml), some additional stimulation was seen—to approximately 50% higher levels than was observed in the egg alone. However, this level of protein synthesis is approximately one-eighth that found in the embryo system. The addition of globin mRNA alone had no effect. Even when (almost) all other available initiation factors were added along with mRNA, the level of translational activity in the egg system remained about one-seventh that in the 2-hr embryo system. These data suggest that, although the egg contains an inhibitor of eIF-4F activity, which is at least partially responsible for the low level of translation prior to fertilization, eIF-4F inactivation is only one of the factors involved in the regulation of maternal mRNA utilization.

No other single initiation factor added either alone or in conjunction with eIF-4F stimulated protein synthesis in the translation system from unfertilized eggs (results not shown). No confirmation of the results of Winkler *et al.* (13), showing slight stimulation of an egg cell-free translation system by added eIF-2, has been obtained. However, these investigators did not demonstrate that added eIF-2 lacked stimulatory effect on the embryo system, and it remains possible that the eIF-2 stimulation reported reflected the loss of the factor during preparation of the translation system.

Table 2. Effect of purified translational initiation factors and globin mRNA on protein synthetic activity of cell-free translation systems from sea urchin eggs and 2-hr embryos

Sea urchin system	Addition		Incorporation, %	
	mRNA, μg/ml	eIF	% Egg	% Embryo
Egg	—	—	100	7.67
	30	—	105	8.00
	150	—	106	8.13
	300	—	105	8.00
	—	eIF-4F	140	10.67
	30	eIF-4F	152	11.70
	150	eIF-4F	160	12.27
	300	eIF-4F	155	11.88
	30	eIF mixture*	180	14.00
Embryo	—	—		100
	30	—		100
	150	—		100
	—	eIF-4F		101
	30	eIF-4F		101
	150	eIF-4F		102

Cell-free translation systems from *S. purpuratus* eggs and embryos were prepared as described and incubated in the presence or absence of eIF-4F at 140 pmol/ml, eIF-4A at 1.79 nmol/ml, eIF-4B at 500 pmol/ml, and/or a fraction at 500 μg/ml containing eIF-2, eIF-3, eIF-4C, and eIF-5. The systems were incubated under standard conditions and assayed for [^{35}S]methionine incorporation into trichloroacetic acid-precipitable radioactivity. The 30-min time point is shown here, expressed as a percentage of the figure obtained for egg or 2-hr embryo.

*eIF-2, -3, -4A, -4B, -4C, -4F, -5.

DISCUSSION

Evidence for an inhibitor of eIF-4F activity in unfertilized sea urchin eggs has been presented. This inhibitor is not present in 2-hr embryos. Inactivation of this inhibitory activity could account, in part, for the rise in mRNA utilization that occurs after fertilization, although it is clearly not the only factor maintaining the low rates of mRNA utilization prior to fertilization. Limiting concentrations of eIF-4F have been shown to regulate translation in mammalian cell-free translation systems (24). In addition, regulation of mRNA utilization by modulation of eIF-4F activity has also been reported from mammalian systems. The classic example of inactivation of eIF-4F is during poliovirus infection, which apparently results in proteolysis of the 220-kDa peptide of eIF-4F (25, 26). eIF-4F inactivation has also been reported to occur during the heat shock response by HeLa (21, 27) and mouse Ehrlich ascites cells (28). During heat shock, the 24-kDa cap binding peptide of eIF-4F becomes dephosphorylated, although no difference in the *in vitro* activities of phosphorylated and nonphosphorylated forms of the factor have been found (21).

Although the inhibition of initiation caused by the egg factor seems to be at the level of eIF-4F activity, the lesion in initiation observed takes place after mRNA binding to the small ribosomal subunit (14). This is a surprising finding, since eIF-4F is known to promote this step of initiation (15). One possible explanation is that, in the presence of the egg inhibitor, eIF-4F accumulates in a functionally unavailable form in the mRNA-containing preinitiation complex. Since eIF-4F levels are very low (21, 22), the availability of active eIF-4F would rapidly decrease. Results from our earlier studies show that, in the egg-inhibited reticulocyte system, approximately 10% of the total mRNA pool and less than 5% of the small ribosomal subunits become trapped in 48S preinitiation complexes, presumably along with the small inactivated pool of eIF-4F. When excess eIF-4F is added to

reticulocyte lysate, in the presence of the egg inhibitor, there is still an excess of available mRNA and small ribosomal subunits to allow initiation to occur, until the excess added eIF-4F also becomes inactivated.

The calculated level of 48S preinitiation complexes that accumulate in egg-inhibited reticulocyte lysate, based on a mRNA content of 6–8 pmol of globin mRNA per 100 μl of lysate (29), is 0.6–0.8 pmol per 100 μl of lysate, which is equivalent to 0.6–0.8 pmol/20 pmol of ribosomes. This figure is close to the published estimates of eIF-4F levels in reticulocyte lysate (22). These calculations along with the accumulated data suggest that the total pool of eIF-4F could be trapped in the 48S preinitiation complexes.

The model suggested above for eIF-4F inactivation is amenable to investigation. Further investigations into the mechanism of action of the egg inhibitor should increase our understanding of the role of eIF-4F in the initiation sequence and the mechanisms by which its activity can be modulated, as well as improve our appreciation of the regulation of maternal mRNA utilization during embryogenesis.

Thanks are extended to Richard Abramson and Thomas Dever for purification of the initiation factors. This work was supported by National Institutes of Health Grants GM-33631 and GM-26796 to R.J. and W.C.M., respectively, and American Cancer Society Fellowship PF-2748 to L.J.H.

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