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Isolation and Distribution of Elongation Factor 2 in Eggs and Embryos of Sea Urchins†

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ABSTRACT: The subcellular distribution of elongation factor 2 (EF-2) in eggs and early embryos of the sea urchin, *Strongylocentrotus purpuratus*, was studied by employing the diphtheria toxin dependent ADP-ribosylation of EF-2. When egg and embryo homogenates were fractionated by sedimentation, EF-2 was found associated with a low-speed pellet containing yolk, nuclei, and mitochondria. It also sedimented at 80 S and 5 S. No significant amounts of EF-2 were found on polyribosomes. The 5S form of EF-2 probably represents a monomeric unit of the factor as EF-2 had a molecular weight of 95 000 on sodium dodecyl sulfate–polyacrylamide gels. EF-2 could only be isolated intact if soybean trypsin inhibitor or EGTA was present. The total amount of EF-2 was similar in eggs and embryos. However, the distributions of the factor between the various fractions were substantially different for eggs and embryos. Also, a marked difference in the physical association of EF-2 with material in the low-speed pellet occurred after fertilization. Specifically, in eggs, 23% of the EF-2 was associated with the low-speed pellet; in cleavage-stage embryos, only 11% of the EF-2 was associated with the pellet. In eggs, 65% of the EF-2 sedimented as 80 S; by the 16-cell stage, this amount decreased to 44%. Concomitantly, the amount of EF-2 in the 5S fraction increased from about 8% in eggs to 44% in the 16-cell embryos. In addition, Triton X-100 was required for the extraction of EF-2 from the low-speed pellet of eggs, but not of embryos. We suggest that a redistribution of EF-2 after fertilization either may account for the increase in EF-2 activity observed by Felicetti et al. (1972) [Felicetti, L., Metafora, S., Gambino, R., & Di Matteo, G. (1972) *Cell Differ. 1*, 265–277] and, thus, be important in mediating the observed 2.5-fold increase in elongation rates after fertilization or may allow the activity of elongation factors to keep pace with the 50-fold increased rate of translation that occurs by the 2-cell stage.

Following fertilization of sea urchin eggs, there is a rapid and progressive increase of about 50-fold in the rate of protein synthesis (Nakano & Monroy, 1958; Epel, 1967; Gross et al., 1964; Regier & Kafatos, 1977). In the early stages of embryogenesis, this rate increase is independent of the synthesis of new ribosomes and mRNA. Although the major rate-limiting block in translation activity appears to be at the level of mRNA (Jenkins et al., 1978; Ilan & Ilan, 1978) or ribosome availability (Hille et al., 1981; Moon et al., 1982), there is also a 2.5-fold increase in the translational efficiency after fertilization (Hille & Albers, 1979; Brandis & Raff, 1978, 1979). Thus, the increase in translation after fertilization is a multiple of at least two factors. In addition, it is evident that, upon fertilization, numerous different molecular changes in the protein synthesis machinery occur. For instance, after fertilization, (1) the amount of free met-tRNA 40S ribosomal subunits increases (Hille et al., 1980), (2) the translational activity of ribosomes increases (Danilchik & Hille, 1981; Monroy & Tyler, 1967), (3) inhibitors of protein synthesis associated with egg ribosomes are less prevalent (Hille, 1974; Metafora et al., 1971), and (4) the activity of elongation factors increases severalfold (Felicetti et al., 1972). Some of these changes may have no direct effect on the rate-limiting step of protein synthesis. The mechanism of protein synthesis is, however, so complex that the large increase in translational

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activity may stress the limitations of many different molecules. Thus, some molecular components that are not rate limiting in eggs may increase in quantity or activity after fertilization in order that they *not* become rate limiting during development. These molecules would simply be keeping pace with the total increase in protein synthesis activity.

The importance that the mobilization of protein synthesis factors may play in allowing both elongation and initiation to keep up with this rapid increase in protein synthesis warrants a search for stored factors in unfertilized sea urchin eggs. We have chosen to study elongation factor 2 (EF-2)[1] because of the availability of a simple and highly specific assay and because the activity of the factor is known to increase 1.5-fold after fertilization (Felicetti et al., 1972).

EF-2 in the presence of GTP catalyzes the movement of nascent peptidyl-tRNA from the aminoacyl site to the peptidyl or donor site on ribosomes. This protein factor can be studied by taking advantage of its characteristic property of ADP-ribosylation in the presence of diphtheria toxin and NAD. The toxin catalyzes the transfer of one moiety of ADP-ribose from NAD to one molecule of EF-2, providing an easy and accurate means of determining the amount of EF-2 (Goor & Maxwell, 1970; Raeburn et al., 1971; Pappenheimer, 1977; Bermek, 1978). In this paper, we determined the quantity and subcellular localization of EF-2 in eggs and embryos. We also determined the molecular weight of EF-2 through early development.

Materials and Methods

Solutions. Buffers had the following designations and compositions unless otherwise mentioned: extraction buffer, 20 mM Pipes (Sigma), 80 mM KCl, 130 mM KOAc, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 1 mg/mL soybean trypsin inhibitor (SBTI, Sigma), pH 7.2 at 0 °C, adjusted with KOH; EDTA buffer, 50 mM Tris, 50 mM KCl, 0.6 mM EDTA, 1 mM DTT, and 10% glycerol, pH 7.2 at 4 °C, adjusted with HCl; gel sample buffer, 125 mM Tris, pH 6.8, adjusted with HCl at 23 °C, 10% SDS, 25% β-mercaptoethanol, 50% glycerol, and 0.004% bromophenol blue. All solutions, including seawater, were filtered through nitrocellulose filters with 0.45-μm pores.

Eggs and Embryos. Gametes of the sea urchin *Strongylocentrotus purpuratus*, collected from the Strait of Juan de Fuca in Northwestern Washington, were obtained by intracoelomic injection of 0.55 M KCl. Eggs were washed several times by hand centrifugation through filtered seawater (FSW). Fertilization was performed in FSW containing 50 mg of 3-amino-1,2,4-triazole (Sigma) per L to prevent hardening of the fertilization membrane (Showman & Foerder, 1979). The aminotriazole was removed after 30 min by hand centrifugation of the cultures through FSW. Only cultures with greater than 95% fertilization were used. All embryos were cultured at 14 °C with paddle stirring at a concentration of 0.5% (v/v) in FSW containing 100 mg/L streptomycin (Sigma). At desired stages, samples of embryos were collected by hand centrifugation, resuspended in FSW at a density of 6% (v/v), and rotated for 40 min at 14 °C in Erlenmeyer flasks, which allow a large surface for adequate aeration of the samples. These 40-min incubations are required to assure that the in vivo rates of protein synthesis are optimum before the cells are collected. Embryos, thus incubated, were rapidly harvested by a 20-fold dilution into extraction buffer (lacking SBTI and DTT) at −3 °C. Sudden cooling of embryos was used to prevent ribosome runoff observed during slow cooling (Davis, 1971). Embryos were then collected by centrifugation at 1500 g_{max} for about 1 min, washed once with 15 volumes of extraction buffer at −3 °C, and processed as described below. Unfertilized eggs used for analysis were washed with FSW, rotated for 40 min at a density of 6% (v/v), and then processed as described for embryos, except that eggs, because they are more fragile than embryos, were collected by centrifugation at 150g_{max} for about 1 min. Parallel experiments on unfertilized eggs treated with aminotriazole showed that it did not affect our results.

The concentrations of eggs and embryos were determined by diluting each sample with FSW and counting the eggs and embryos in a 0.05-mL disposable capillary micropipet with the aid of a dissecting microscope. At least six determinations were made of each sample.

Fractionation of Eggs and Embryos by Differential Centrifugation. Eggs and embryos, washed as described above, were suspended in 5–10 volumes of extraction buffer at −3 °C and homogenized by five gentle passages through a 20-gauge needle. Breakage of cells was confirmed by microscopic examination. Unless otherwise mentioned, extracts were centrifuged at 800g_{max} for 5 min followed by three additional centrifugations at 2400g_{max}, 12000g_{max}, and 20000g_{max} for 10 min each. The four pellets were dissolved in extraction buffer containing 25% glycerol and combined together in a final volume of 1 mL of buffer/10^6 eggs or embryos. This fraction, designated as the low-speed pellet, was frozen at −70 °C until it was further processed. The supernatant, obtained after the four centrifugations (postmitochondrial supernatant), was dialyzed against EDTA buffer and assayed for EF-2 as described below.

Solubilization of EF-2 from the Low-Speed Pellet. EF-2 bound to ribosomes and to other particles must be released from the particulate fraction in order to assay it quantitatively by the diphtheria toxin dependent ADP-ribosylation assay (Smulson et al., 1970; Smulson & Rideau, 1970; Gill & Dinius, 1973; Yablonka-Reuveni & Warner, 1979; Yablonka-Reuveni et al., 1983). To release the EF-2 from the frozen low-speed pellet, the suspension was thawed and diluted with extraction buffer to reduce the glycerol concentration to 10%. Triton X-100 and NH_4Cl were added to a final concentration of 1% and 0.75 M, respectively, by using stock solutions of 10% Triton X-100 and 3.75 M NH_4Cl in extraction buffer (final pH 7.2). The suspensions were vortexed gently and allowed to stand for 2.5 h with occasional mixing and then centrifuged at 20000g_{max} for 20 min at 1 °C. The pellet was discarded, and the resultant supernatant (NH_4Cl–Triton X-100 wash) was dialyzed against EDTA buffer and assayed for EF-2 as described below.

Sucrose Gradient Fractionation of Postmitochondrial Extracts. Sucrose gradient analysis was done to study the distribution of EF-2 in the postmitochondrial supernatant. Ten A_{260} units of postmitochondrial supernatants (0.2–1 mL) was layered on 11.4-mL sucrose gradients (15–40% w/v) layered over a 1.0-mL (50% w/v) sucrose pad. All sucrose solutions were made in extraction buffer lacking SBTI. Centrifugation was for 100 min at 189000g_{av} in an SW 40 rotor at 1 °C. Gradients were fractionated at 4 °C into 0.4-mL aliquots while the absorbance at 254 nm was measured by using an Isco Model UA-5 monitor. Fractions were stored at −70 °C until assayed for EF-2 as described below. It was found that

[1] Abbreviations: EF-2, elongation factor 2; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; SBTI, soybean trypsin inhibitor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; FSW, filtered seawater; BSA, bovine serum albumin.

freezing did not change the amounts of EF-2 detected in the gradient fractions as compared to fractions that were assayed immediately after gradient fractionation. In some experiments, in order to fractionate the postribosomal supernatant into various size particles, gradients of 5–30% (w/v) were used, with centrifugation at 189000g_{av} for 18 h.

Quantitation of Elongation Factor 2. The content of EF-2 in fractions obtained by differential centrifugation and by sucrose gradient fractionation was assayed by using the diphtheria toxin dependent ADP-ribosylation of EF-2 (Goor & Maxwell, 1970; Raeburn et al., 1971; Yablonka-Reuveni & Warner, 1979; Yablonka-Reuveni et al., 1983).

(A) Fractions obtained by differential centrifugation (low-speed pellet, postmitochondrial supernatant) were dialyzed against EDTA buffer and then variously diluted in EDTA buffer. Aliquots of the dilutions were then tested in a 0.1-mL reaction containing, in addition to the protein sample, 1.65 μg of diphtheria toxin (Connaught Laboratories lot D-298, 1800 Lf/mL, 13.2 mg/mL), 6.25 μg of BSA (Calbiochem), 0.25 mM $Mg(OAc)_2$, and 110 pmol of [^{3}H]NAD (New England Nuclear, 3.39 Ci/mmol) adjusted to 0.9 Ci/mmol with non-labeled NAD (Sigma) (1 pmol = 1032 cpm). The reaction mixture was incubated at 28 °C for 45 min and then stopped by the addition of 2 mL of 5.5% ice-cold trichloroacetic acid. No additional ADP-ribosylation was observed with longer incubations. Precipitated proteins were collected on nitrocellulose filters (0.45-μm pores) and washed 3 times with 3 mL of 5.5% ice-cold trichloroacetic acid. The filters were oven dried at 80 °C for 10 min and dissolved in 10 mL of Beckman Ready Solv HP scintillant, and their radioactivity was measured in a Beckman LS 8000 scintillation counter. The concentration of EF-2 was determined from the extent of [^{3}H]-ADP-ribosylation by assuming that one molecule of ADP-ribose is bound to one molecule of EF-2 in the presence of diphtheria toxin and excess NAD (Raeburn et al., 1971).

(B) Fractions obtained by sucrose gradient sedimentation were treated with EDTA to release EF-2 from ribosomes and other cosedimenting particles containing EF-2. Similar treatment was used by others for this purpose (Smulson et al., 1970; Smulson & Rideau, 1970; Traugh & Collier, 1971). Unless otherwise mentioned, aliquots of 0.1 and 0.05 mL were removed from the sucrose gradient fractions and were made 35 mM in EDTA with a stock solution of 175 mM EDTA in extraction buffer (final pH 7.2). The EDTA-treated samples were shaken for at least 1 h at 4 °C and then assayed by adding the same quantity of reactants as described above, except that $Mg(OAc)_2$ was not added.

SDS–Polyacrylamide Gel Electrophoresis of [^{3}H]ADP-Ribosylated Proteins. Fractions isolated by differential centrifugation were [^{3}H]ADP-ribosylated in the diphtheria toxin dependent assay as described above. The reaction was stopped by the addition of 0.25 volume of gel sample buffer. Mixtures were heated at 90 °C for 5 min, and aliquots of 0.025 mL were then analyzed on 10% polyacrylamide gel slabs containing 0.1% SDS made according to Laemmli (1970). All reagents were from Bio-Rad. Gels were stained in 50% methanol, 7.5% acetic acid, and 0.1% Coomassie brilliant blue R for about 1 h and then destained in 5% methanol and 7.5% acetic acid. Gels were treated with EN^{3}HANCE (New England Nuclear), and fluorography was made. The molecular weight of the [^{3}H]ADP-ribosylated polypeptides was estimated by using protein standards.

Results

Isolation and Molecular Weights of the [^{3}H]ADP-Ribosylated Polypeptides in Postmitochondrial Supernatants.

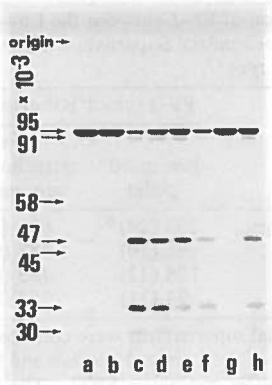

FIGURE 1: Effect of protease inhibitors on the breakdown of EF-2. Unfertilized eggs were washed with extraction buffer (lacking SBTI and DTT) as described under Materials and Methods and then divided into eight aliquots. Each aliquot was washed and homogenized in extraction buffer containing the inhibitors as indicated. Postmitochondrial supernatants were dialyzed against EDTA buffer containing the inhibitor (except for SBTI and ovomucoid which were not added to the dialysis buffer as they are not dialyzable). Aliquots of the dialyzed supernatants were [^{3}H]ADP-ribosylated in the diphtheria toxin dependent assay and then electrophoresed on SDS–polyacrylamide gels which were fluorographed. (Lane a) 1 mg/mL ovomucoid (Sigma); (lane b) 1 mg/mL SBTI (Sigma); (lane c) 1 mM *N*-ethylmaleimide; (lane d) 1 mM *o*-phenanthroline (Sigma); (lane e) 0.01 mM pepstatin (Sigma); (lane f) 1 mM phenylmethanesulfonyl fluoride (Calbiochem); (lane g) 1 mM EGTA; (lane h) buffer only. Molecular weights of the [^{3}H]ADP-ribosylated polypeptides are indicated. Standard proteins used for the determination of molecular weights are β-galactosidase (M_r 130000), phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), and carbonic anhydrase (M_r 30000).

Intact EF-2 as well as fragments of EF-2 containing the ADP-ribosylation site can undergo ADP-ribosylation in the presence of diphtheria toxin and [^{3}H]NAD (Collins et al., 1971; Yablonka-Reuveni & Warner, 1979). Therefore, we were able to localize both active and inactive EF-2 molecules and to determine their respective molecular weights. Several different extraction conditions were used to determine the native size of EF-2 in postmitochondrial supernatants from unfertilized eggs as analyzed on SDS–polyacrylamide gels. Two conditions resulted in the occurrence of a single ADP-ribosylated species of 95000 daltons as analyzed on SDS–polyacrylamide gels (Figure 1, lanes b and g): the addition of 1 mg/mL soybean trypsin inhibitor (SBTI) and the addition of 1 mM EGTA to homogenization buffer which contained physiological salt concentrations (Rothschild & Barnes, 1953; see Materials and Methods). SBTI is known to inhibit serine proteases and EGTA, Ca^{2+}-activated proteases. Other conditions resulted in various degrees of proteolysis of EF-2. In the absence of protease inhibitors, [^{3}H]ADP-ribosylated peptides with molecular weights of 33000, 47000, and 95000 were present in large quantities (Figure 1, lane h). In addition, four protein bands of 30000, 45000, 58000 and 91000 daltons were observed, some of which are barely visible in the photographic reproduction of the fluorograph of the polyacrylamide gel. Those protease inhibitors tested that fail to reduce the number of [^{3}H]ADP-ribosylated bands observed are *N*-ethylmaleimide, an inhibitor of SH-dependent protease (Figure 1, lane c), *o*-phenanthroline, an inhibitor of Zn^{2+}-dependent protease (Figure 1, lane d), pepstatin, an inhibitor of acidic proteases (Figure 1, lane e), and phenylmethanesulfonyl fluoride, an inhibitor of serine proteases (Figure 1, lane f) [mechanisms reviewed in Walsh (1974) and Laskowoski & Kato (1974)]. Ovomucoid, a trypsin inhibitor (Figure 1, lane a), did reduce, but did not eliminate, the breakdown of the 95000-dalton protein. When the gel used for the fluorograph

Table I: Distribution of EF-2 between the Low-Speed Pellets and Postmitochondrial Supernatants Prepared from Eggs and Early Embryos[a]

stage	EF-2 (pmol/10^6 eggs or embryos)		
	low-speed pellet	post-mitochondrial supernatant	total
unfertilized eggs	202 (24)[b]	638 (76)	840
zygotes	88 (10)	788 (90)	876
2- to 4-cell	108 (12)	795 (88)	903
8- to 16-cell	94 (11)	758 (89)	852

[a] Postmitochondrial supernatants were collected after four serial centrifugations as described under Materials and Methods by using pH 7.2 extraction buffer containing SBTI. The low-speed pellets were combined and treated with NH_4Cl and Triton X-100 as described. The content of EF-2 in the two fractions was determined by the diphtheria toxin dependent ADP-ribosylation assay. [b] Numbers in parentheses are percentages of the total amount of EF-2 at each stage.

shown in Figure 1 was stained with Coomassie blue for total protein, at least four polypeptides other than EF-2 showed degradation products in the absence of EGTA, SBTI, or ovomucoid (data now shown). Thus, a general degradation of polypeptides is indicated in the absence of the latter three inhibitors. In all subsequent studies, unless otherwise mentioned, we used SBTI in our extraction buffers. SBTI was preferred over EGTA to avoid a change in the concentration of ions in the extraction buffer.

The molecular weights of EF-2 at various stages of development (egg, zygote, 2- to 4-cell stage, 8- to 16-cell stage) were determined by using the extraction buffer containing SBTI. For these measurements, postmitochondrial supernatants and NH_4Cl–Triton X-100 washes of low-speed pellets were prepared, [^{3}H]ADP-ribosylated, and analyzed on flurographs of SDS–polyacrylamide gels. At all stages, the only [^{3}H]ADP-ribosylated polypeptide detected when SBTI was present was that with a molecular weight of 95 000 (Figure 2). This molecular weight is in agreement with the 93 000–105 000 range reported for EF-2 in rat liver, rabbit reticulocytes, hen oviduct, human tonsils, and *Artemia salina* [see Collins et al. (1971) and Robinson et al. (1974), Merrick et al. (1975), Comstock & Van (1977), Bermek (1978), and Yablonka-Reuveni & Warner (1979), respectively].

Distribution of EF-2 between the Low-Speed Pellet and the Postmitochondrial Supernatant Fractions from Eggs and Embryos. These studies compare the amount of EF-2 in the low-speed pellet material to that in the postmitochondrial supernatant. A low-speed pellet fraction was prepared from each stage by sequentially centrifuging homogenates at 800-, 2400-, 12000-, and 20000g_{max}. EF-2 was released from low-speed pellets by treatment with 0.75 M NH_4Cl–1% Triton X-100 before ADP-ribosylation of EF-2. Table I shows a comparison of amounts of EF-2 in a combined low-speed pellet and in the remaining postmitochondrial fractions of unfertilized eggs, zygotes (70 min at 14 °C), 2- to 4-cell embryos, and 8- to 16-cell embryos. The total amount of extractable EF-2

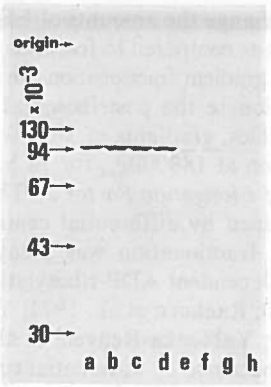

FIGURE 2: SDS–polyacrylamide gel analysis of [^{3}H]ADP-ribosylated EF-2 from the postmitochondrial supernatants and NH_4Cl–Triton X-100 washes of the low-speed pellets of eggs and embryos. Postmitochondrial supernatants and low-speed pellets were prepared as described under Materials and Methods in the presence of SBTI. Aliquots of the fractions were ribosylated, electrophoresed, and fluorographed. (Lanes a–d) Postmitochondrial supernatants; (lanes e–h) NH_4Cl–Triton X-100 washes of the low-speed pellets; (lanes a and e) unfertilized eggs; (lanes b and f) zygotes; (lanes c and g) 2- and 4-cell embryos; (lanes d and h) 8- to 16-cell embryos. The migration positions of molecular weight markers described in Figure 1 are indicated. Due to interfering proteins in the low-speed pellet, no more protein could be applied to lanes e–h. However, prolonged exposures of X-ray film showed no radiolabeled bands in these lanes except that at 95 000 daltons.

increased, on the average, only 5% in embryos compared to eggs (Table I, last column). However, after fertilization, the amount of EF-2 in the low-speed pellet decreased on the average by half, or 105 pmol, as shown by comparison of the various stages. Concomitantly, the amount of EF-2 in the postmitochondrial supernatants increased by 1.22-fold or 140 pmol. Thus, there is a large shift of EF-2 from a bound form in the low-speed pellet to a soluble form.

To determine whether the increase in the alkalinity of the cytoplasm after fertilization could cause the release of bound EF-2, we measured the distribution of EF-2 when eggs and embryos were extracted in a medium with the pH values similar to those before (pH 6.8) and after (pH 7.2) fertilization (Shen & Steinhardt, 1978). No significant differences were found in the distribution of EF-2 in solutions at these two different pH values at any stage from unfertilized eggs to 16-cell embryos. At both pH values, a shift of EF-2 from the low-speed pellet to the postmitochondrial supernatant was similar to that seen in Table I.

To determine whether the EF-2 in the low-speed pellet was associated with a particular size class of particles, each of the four pellets (800–20000g_{max}) was treated separately to release the EF-2. The results (Table II) did not demonstrate any clear association of EF-2 with any particular particle size for either unfertilized eggs or cleavage-stage embryos nor any change in the distribution of EF-2 among these size classes during early development.

Egg extracts differ from embryo extracts not only in the higher content of EF-2 in the nuclear mitochondrial pellet but

Table II: Distribution of EF-2 in Different Size Particles from the Low-Speed Pellets of Eggs and Early Embryos[a]

stage	EF-2 (pmol/10^6 eggs or embryos)			
	800g_{max} pellet	2400g_{max} pellet	12000g_{max} pellet	20000g_{max} pellet
eggs	39	55	58	39
zygotes	17	28	34	19
4- to 8-cell	18	30	22	17

[a] 800-, 2400-, 12000-, and 20000g_{max} pellets were prepared as described in Table I. The EF-2 was released from each pellet by using NH_4Cl–Triton X-100 treatment as described in Table I for the pooled low-speed pellet.

Table III: Effect of NH_4Cl and Triton X-100 on the Solubilization of EF-2 from the Low-Speed Pellet[a]

	% EF-2[b]			
		0.75 M NH_4Cl		0.75 M NH_4Cl + 1%
stage	no addition pH 7.2[c]	pH 6.8	pH 7.2	Triton X-100 pH 6.8 or 7.2[d]
unfertilized eggs	23	61	56	100
zygotes	36	99	103	100
2- to 4-cell	39	102	95	100
4- to 8-cell	35	100	101	100

[a] The low-speed pellet was prepared and assayed for the presence of EF-2 as described under Materials and Methods. Solubilization of EF-2 from the pellets was by the addition of NH_4Cl and Triton X-100 to a suspension of the pellet in extraction buffer as indicated for each data column. [b] The results are normalized for comparison, assuming 100% extraction in buffer containing 0.75 M NH_4Cl and 1% Triton X-100. [c] Extraction and resuspension of the low-speed pellet were done at the pH indicated. [d] The picomoles per 10^6 eggs or embryos for 100% EF-2 in the low-speed pellet are 210 (unfertilized eggs), 86 (zygotes), 99 (2- to 4-cell stage), 84 (4- to 8-cell stage) at pH 6.8 and 202 (unfertilized eggs), 88 (zygotes), 108 (2- to 4-cell stage), and 91 (4- to 8-cell stage) at pH 7.2.

also in the need for detergent to solubilize the EF-2 from this pellet. As shown in Table III, both NH_4Cl and Triton X-100 are required for complete release of EF-2 from the nuclear mitochondrial pellet of unfertilized eggs. However, only NH_4Cl is needed to achieve complete release of EF-2 from the zygote and embryo pellets. We also found that 0.5% Nonidet P-40 was as effective as 1% Triton X-100 in releasing EF-2 from these pellets whereas 0.1% SDS and 0.25% sodium deoxycholate did not enhance the amount of EF-2 released in the presence of 0.75 M NH_4Cl. The release of EF-2 did not depend on the pH of the extraction buffers. We also tested the possibility that dissociation of particles with EDTA was required for the release of EF-2 from the low-speed pellet. The addition of 15 mM EDTA to 0.75 M NH_4Cl or to 0.75 M NH_4Cl–1% Triton X-100 during the solubilization of EF-2 from the pellets did not enhance the amount of EF-2 detected (data not shown). The EF-2 that is soluble in the absence of both NH_4Cl and Triton X-100 (Table III, first column) could have been released mechanically during the handling of the pellet (pipetting, freezing and thawing, homogenization), or it may have been trapped in the low-speed pellet material. We suggest that the EF-2 which is released by NH_4Cl and Triton is bound or complexed with other components in the pellet and that the physical nature of this complex changes after fertilization.

Distribution of EF-2 in the Postmitochondrial Supernatants. We wished to determine whether EF-2 cosedimented with polyribosomes, monoribosomes, or other small particles, and whether this distribution differed in eggs and embryos. The postmitochondrial supernatants were, therefore, centrifuged on sucrose gradients under conditions which separated the monoribosomes and polyribosomes. The UV absorbance profiles of gradients for unfertilized eggs and 8- to 16-cell embryos were similar to those described by others (Infante & Nemer, 1967; Hille et al., 1981; Goustin & Wilt, 1981). The gradients showed that a large percentage of EF-2 cosedimented with monoribosomes. Analyses of several of the gradients are summarized in Table IV. While in eggs 65% of the total cellular EF-2 is in monoribosomes, this amount gradually decreases in the embryos to 40% by the 16-cell stage. At the same time, the amount of EF-2 in the postribosomal supernatant increases from 8% of the total cellular EF-2 in eggs to almost 45% in 16-cell embryos.

The amount of EF-2 in the polyribosomal region was small and constant throughout development, irrespective of the size of the polyribosome fraction. The majority of the EF-2 associated with polyribosomes was detected in the dimer region, suggesting that the EF-2 was not actually associated with polyribosomes but rather with monoribosomes that trail into the polyribosome region. To confirm that the polyribosomes contain very little, if any, EF-2, we overloaded gradients with postmitochondrial supernatant from eggs and 4- to 8-cell embryos. Only very small amounts of EF-2 were detected in the polyribosomal region (Figure 3).

The sedimentation coefficients of EF-2 in these postribosomal supernatants, made in the presence of protease inhibitors, were estimated by sucrose gradient analysis using shallow gradients that separated particles smaller than ribosomal subunits. In this region, almost all of the ADP-ribosylated peptides sediment as a single peak from extracts of both unfertilized eggs and 4- and 8-cell embryos (Figure 4). Using protein markers (not shown), we estimated the sedimentation value of this peak to be about 5 S and identical in both eggs and embryos. This value is similar to that determined by Comstock & Van (1977) for a single peptide of EF-2 from hen oviduct at infinite dilution ($4.92 s_{20,w}$) and by Yablonka-Reuveni et al. (1983) for EF-2 from *Artemia*. Thus, the 5S peak in the postribosomal supernatant of *S. purpuratus* eggs and embryos probably contains EF-2 which is not associated with itself or with other proteins.

Table IV: Distribution of EF-2 between Postribosomal Supernatant, Monoribosome, and Polyribosome Fractions Prepared from Eggs and Embryos[a]

		EF-2					
		% distribution in gradients[c]			% of total amount in eggs or embryos[d]		
expt[b]	stage	supernatant	mono-ribosomes	poly-ribosomes	supernatant	mono-ribosomes	poly-ribosomes
1	egg	11	84	5	8	65	4
	4- to 8-cell	32	62	6	28	55	5
2	egg	7	87	6	5	67	5
	4- to 8-cell	29	63	8	26	56	7
3	egg	13	82	5	10	63	4
	16-cell	50	44	6	45	39	5

[a] Postmitochondrial supernatants were fractionated on sucrose gradients under conditions described in Figure 3. The distribution of EF-2 was determined by the diphtheria toxin dependent assay. [b] In each experiment, eggs and embryos were derived from the same stock, and the manipulations were conducted in parallel. [c] For experiment 2, data from Figure 3 were used, and the fractions used for supernatant, monoribosomes, and polyribosomes are 1–5, 6–17, and 18–33, respectively. [d] Percent of the total amount of the three groups (supernatant, monoribosomes, polyribosomes) was calculated by assuming that in eggs and embryos an average of 77% and 89%, respectively, of the EF-2 occurred in the postmitochondrial supernatant. These values were calculated from the several experiments shown in Tables I and II.

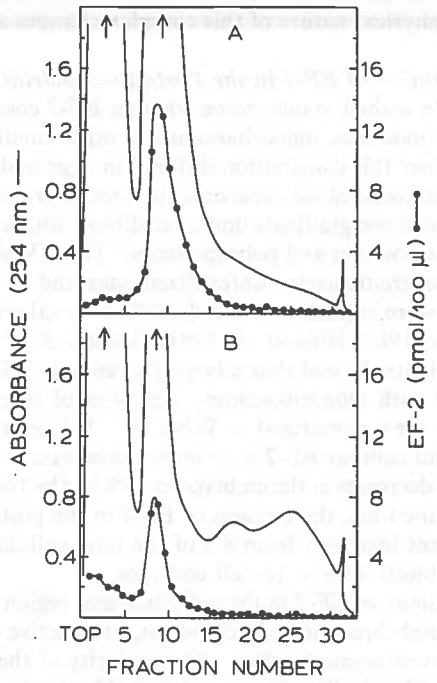

FIGURE 3: Sucrose gradient analysis of EF-2 in postmitochondrial supernatants from unfertilized eggs and embryos of *S. purpuratus* following applications of high quantities of supernatants. 50 A_{20} units of postmitochondrial supernatant from unfertilized eggs (A) and 33 A_{260} units from 4- to 8-cell embryos (B) were applied to gradients. Arrows indicate that the absorbance was off scale. Other conditions and the assay for EF-2 were as described under Materials and Methods.

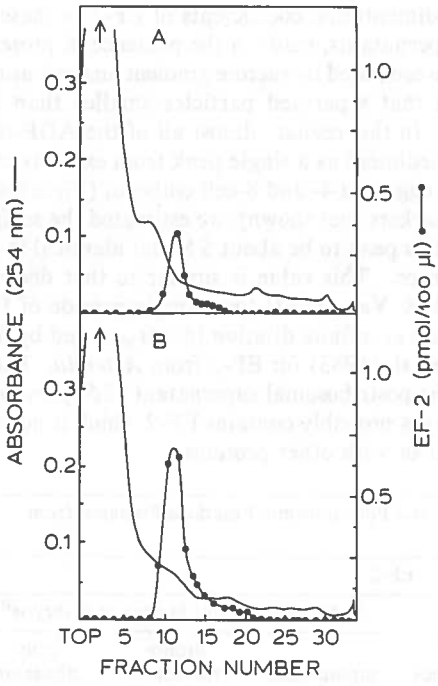

FIGURE 4: Sucrose gradient analysis of EF-2 in postribosomal supernatants from unfertilized and 4- and 8-cell embryos. Conditions were as described under Materials and Methods except the supernatants were layered on a 5–30% (w/v) sucrose gradient and centrifuged for 18 h at 189000g_{av}. (A) Unfertilized eggs; (B) 4- to 8-cell embryos.

Two aspects of the nature of the EF-2 which sediments with 80S monoribosomes were studied: the association of EF-2 with ribosomes and the lability of EF-2 to naturally occurring proteases. The association of EF-2 with ribosomes was tested by treatment of monoribosomes with NH_4Cl and with EDTA.

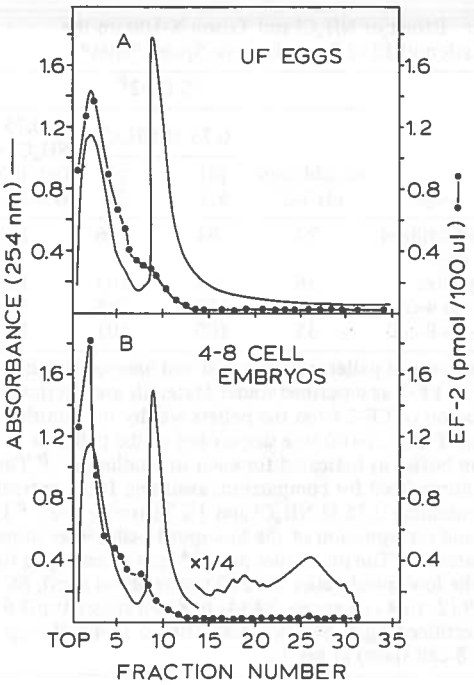

FIGURE 5: Sucrose gradient analysis of EF-2 in postmitochondrial supernatants from eggs and embryos following extraction in buffer lacking SBTI. Postmitochondrial supernatants were prepared from eggs (A) and 4- to 8-cell embryos (B) by using extraction buffer containing phenylmethanesulfonyl fluoride (1 mM) instead of SBTI. Conditions and assay of EF-2 are as described under Materials and Methods.

EDTA to a final concentration of 35 mM was added to postmitochondrial supernatants to dissociate the monoribosomes into subunits. NH_4Cl at a final concentration of 0.75 M was added to dissociate electrostatically bound factors from the monoribosomes and their subunits. With either treatment, EF-2 sedimented on sucrose gradients as a 5S particle: no EF-2 remained in the 80S region or occurred on ribosomal subunits (data not shown). These findings support the idea that EF-2 is bound to monoribosomes but cannot rule out the possibility that EF-2 is bound to another type of particle which disintegrates upon the addition of EDTA or NH_4Cl.

The lability of EF-2 to naturally occurring proteases was tested by analyzing products formed in the absence of protease inhibitors. As discussed previously, we observed that in the absence of protease inhibitors, EF-2 from unfertilized eggs is cleaved into fragments by endogenous proteases during homogenization (Figure 1). We also find that if protease activity is not inhibited in homogenates of unfertilized eggs and embryos, no EF-2 occurs on monoribosomes, as shown by a comparison of EF-2 distributions in Figure 5A and Figure 3A. Analysis of the postribosomal region of eggs (Figure 6A) shows that when no protease inhibitors were present, EF-2 appears in the supernatant as a complex pattern of macromolecules that sediments from about 5 S to 16 S (about 95000–500000 daltons; compare Figures 6A and 4A). Particles 5–9 S contained the degraded EF-2 as observed on gels. The source of the proteases in unfertilized eggs may be the cortical granules (Fodor, et al., 1975). Sedimentation of EF-2 with particles that range in size from 5 to 20 S has also been observed for *Artemia* (Yablonka-Reuveni et al., 1983) and rabbit reticulocytes (Collier & Traugh, 1969; Hradek & Dušek, 1978). The case for homogenates from sea urchin embryos prepared in the absence of the protease inhibitors SBTI and EGTA is somewhat different than for unfertilized eggs. Although EF-2

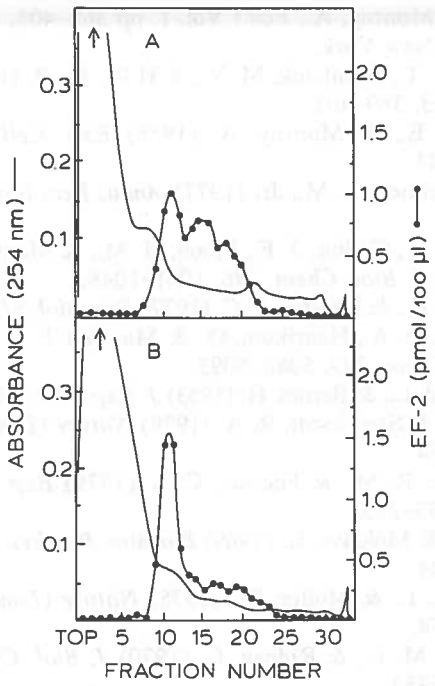

FIGURE 6: Sucrose gradient analysis of EF-2 in postribosomal supernatants from eggs and embryos after extraction in buffer lacking SBTI. Extraction conditions were as in Figure 5 and centrifugation conditions as in Figure 4. (A) Unfertilized eggs; (B) 4- to 8-cell embryos.

is released from the monoribosomes, it sediments predominantly as a 5S particle (Figure 6B) and electrophoreses as a 95 000-dalton peptide (data not shown). It is possible that the release of EF-2 from monoribosomes in homogenates of embryos, isolated in the absence of protease inhibitors, is similar to the natural, but slow, release of EF-2 from monoribosomes observed during early embryogenesis (Table IV).

Discussion

The potential importance that the mobilization of proteins might play in the activation of the metabolism of fertilized eggs of sea urchins has led us to study one such protein, peptide elongation factor 2 (EF-2), whose activity is increased rapidly after fertilization (Felicetti et al., 1972). Our measurements of the distributions of EF-2 between supernatant, monoribosome, and polyribosome fractions of eggs and embryos show that, although the total amount of EF-2 does not increase after fertilization, the amount of material in the 5S supernatant fraction increases dramatically during the cleavage stages. At the same time, the total amount of EF-2 on monoribosomes and in the low-speed pellet decreases. Very little, if any, EF-2 is ever found on polyribosomes. The molecular weight of the EF-2 protein in sea urchin eggs and embryos was found to be 95 000, and identical for all stages of development and for all subcellular locations, suggesting that the activation of EF-2 after fertilization is not accomplished by cleavage of a significant portion of EF-2.

Our finding of very low amounts of EF-2 on polyribosomes is not unexpected as it is consistent with studies on other cells suggesting that the EF-2 cycles on and off polyribosomes during polypeptide chain elongation. For instance, Siler & Moldave (1969) found that a single molecule of EF-2 catalyzed in vitro protein synthesis with several ribosomes. Smulson & Rideau (1970) showed that in HeLa cells most of the ribosome-associated EF-2 was bound to monoribosomes and very little of the factor was found associated with polyribosomes. Also, Chinali et al. (1982) found in rabbit reticulocytes and mouse liver that only the monoribosome fractions carry EF-2, while little factor is associated with polyribosomes.

Felicetti et al. (1972) observed that the in vitro activity of EF-2 in the postribosomal supernatant increases 1.5-fold within 5 min after fertilization of the sea urchin egg. A similar mechanism has been proposed by Yablonka-Reuveni & Warner (1979) and Slobin & Moller (1975) for the activation of elongation factors during the development of *Artemia* and by Hellerman & Shafritz (1975) for the activation of initiation factors in this organism. Also, Smulson and collaborators have observed that an increase in the amount of postribosomal EF-2 coincides with a decrease in ribosome-bound EF-2 and an increase in protein synthesis in HeLa cells following release from amino acid starvation (Smulson & Rideau, 1970; Smulson et al., 1970). The significance of the observed redistribution of EF-2 and the increase in the activity of elongation factors (Felicetti et al., 1972) after fertilization of sea urchin eggs is not known. Two possibilities are the following: (1) It is related to the increased translational efficiencies that occur after fertilization (Hille & Albers, 1979; Brandis & Raff, 1978); (2) it is required in order that sufficient quantities of elongation factors be present to keep pace with the increase in mRNA recruitment that occurs after fertilization. Without such an increase, EF-2 might become rate limiting during the 50-fold increase in the rate of peptide bond formation that occurs by the 2-cell stage. Release of a stored form of EF-2 after fertilization would prevent this rate limitation.

The importance of storage–release mechanisms following fertilization of eggs is also suggested by the number of enzymes associated with energy metabolism that are activated at fertilization. In these cases, the synthesis of new proteins also cannot account for the rapid changes [reviewed in Monroy & Tyler (1967) and in Epel (1978)]. Isono and co-workers (Isono et al., 1963; Isono, 1963) and Aune & Epel (1978) have shown that the pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase is released from a particulate form in eggs to a soluble form in embryos. As our observations on EF-2 are similar to those for glucose-6-phosphate dehydrogenase, we propose that the storage of proteins in particles may be a general property of sea urchin eggs.

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References

Aune, T. M., & Epel, D. (1978) *J. Cell Biol. 79*, 164a.
Bermek, E. (1978) *Prog. Nucleic Acid Res. Mol. Biol. 21*, 64–100.
Brandis, J. W., & Raff, R. A. (1978) *Dev. Biol. 67*, 99–113.
Brandis, J. W., & Raff, R. A. (1979) *Nature (London) 278*, 467–469.
Chinali, G., Sacchi, A., & Perrini, U. (1982) *Biochim. Biophys. Acta 697*, 193–201.
Collier, R. J., & Traugh, J. A. (1969) *Cold Spring Harbor Symp. Quant. Biol. 34*, 589–594.
Collins, J. F., Raeburn, S., & Maxwell, E. S. (1971) *J. Biol. Chem. 246*, 1049–1054.
Comstock, J. P., & Van, N. T. (1977) *Biochim. Biophys. Acta 477*, 199–220.
Danilchik, M. V., & Hille, M. B. (1981) *Dev. Biol. 84*, 291–298.
Davis, B. D. (1971) *Nature (London) 231*, 153–157.
Epel, D. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 899–906.
Epel, D. (1978) *Curr. Top. Dev. Biol. 12*, 185–246.

Felicetti, L., Metafora, S., Gambino, R., & Di Matteo, G. (1972) *Cell Differ. 1*, 265–277.
Fodor, E. G., Ako, H., & Walsh, K. A. (1975) *Biochemistry 14*, 4923–4927.
Gill, D. M., & Dinius, L. L. (1973) *J. Biol. Chem. 248*, 654–658.
Goor, R. S., & Maxwell, E. S. (1970) *J. Biol. Chem. 245*, 616–623.
Goustin, A. S., & Wilt, F. H. (1981) *Dev. Biol. 82*, 32–40.
Gross, P. R., Malkin, L. I., & Meyer, W. A. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 407–414.
Hellerman, J. G., & Shafritz, D. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1021–1025.
Hille, M. B. (1974) *Nature (London) 249*, 556–558.
Hille, M. B., & Albers, A. A. (1979) *Nature (London) 278*, 469–471.
Hille, M. B., Bechtold, M. A., Hall, D. C., & Yablonka-Reuveni, Z. (1980) *Am. Zool. 20*, 838.
Hille, M. B., Hall, D. C., Yablonka-Reuveni, Z., Danilchik, M. V., & Moon, R. T. (1981) *Dev. Biol. 86*, 241–249.
Hradec, J., & Dušek, Z. (1978) *Biochem. J. 172*, 1–7.
Ilan, J., & Ilan, J. (1978) *Dev. Biol. 66*, 375–385.
Infante, A. A., & Nemer, M. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 681–688.
Isono, N. (1963) *J. Fac. Sci., Univ. Tokyo 10*, 67–74.
Isono, N., Tsusaka, A., & Nakano, E. (1963) *J. Fac. Sci., Univ. Tokyo, Sect. 4 10*, 55–66.
Jenkins, N. A., Kaumeyer, J. F., Young, E. M., & Raff, R. A. (1978) *Dev. Biol. 63*, 279–298.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Laskowski, M., & Kato, I. (1974) *Bayer-Symp. 4*, 597–611.
Merrick, W. C., Kemper, W. M., Kantor, J. A., & Anderson, W. F. (1975) *J. Biol. Chem. 250*, 2620–2625.
Metafora, S., Felicetti, L., & Gambino, R. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 600–604.
Monroy, A., & Tyler, A. (1967) in *Fertilization* (Metz, C. B., & Monroy, A., Eds.) Vol. I, pp 369–408, Academic Press, New York.
Moon, R. T., Danilchik, M. V., & Hille, M. B. (1982) *Dev. Biol. 93*, 389–403.
Nakano, E., & Monroy, A. (1958) *Exp. Cell Res. 14*, 236–244.
Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem. 46*, 69–94.
Raeburn, S., Collins, J. F., Moon, H. M., & Maxwell, E. S. (1971) *J. Biol. Chem. 246*, 1041–1048.
Regier, J. C., & Kafatos, F. C. (1977) *Dev. Biol. 57*, 270–283.
Robinson, E. A., Henriksen, O., & Maxwell, E. S. (1974) *J. Biol. Chem. 249*, 5088–5093.
Rothschild, L., & Barnes, H. (1953) *J. Exp. Biol. 30*, 534–544.
Shen, S., & Steinhardt, R. A. (1978) *Nature (London) 272*, 253–254.
Showman, R. M., & Foerder, C. A. (1979) *Exp. Cell Res. 120*, 253–255.
Siler, J., & Moldave, K. (1969) *Biochim. Biophys. Acta 195*, 138–144.
Slobin, L. I., & Moller, W. (1975) *Nature (London) 258*, 452–454.
Smulson, M. E., & Rideau, C. (1970) *J. Biol. Chem. 245*, 5350–5353.
Smulson, M. E., Rideau, C., & Raebum, S. (1970) *Biochim. Biophys. Acta 224*, 268–271.
Traugh, J. A., & Collier, R. J. (1971) *FEBS Lett. 14*, 285–288.
Walsh, K. A. (1974) *Cold Spring Harbor Conf. Cell Proliferation 2*, 1–11.
Yablonka-Reuveni, Z., & Warner, A. H. (1979) in *Biochemistry of Artemia Development* (Bagshaw, J. C., & Warner, A. H., Eds.) pp 22–41, University Microfilms International, Ann Arbor, MI.
Yablonka-Reuveni, Z., Fontaine, J. J., & Warner, A. H. (1983) *Can. J. Biochem. Cell Biol. 61*, 833–839.