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Polypeptide Chain Initiation in Eukaryotes: Functional Identity of Supernatant Factor from Various Sources*

(*Artemia salina*/embryos/cysts/brine-shrimp eggs/eukaryotic ribosomes)

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ABSTRACT **Eukaryotic cells contain polypeptide chain initiation factors that, like the prokaryotic initiation factor IF-2, promote the AUG-dependent binding of fMet-$tRNA_f$ to the small ribosomal subunit. The bound aminoacyl-tRNA is directly convertible to fMet-puromycin upon addition of 60S subunit. The reaction is sensitive to initiation inhibitors such as aurintricarboxylic acid and edeine but, unlike its prokaryotic counterpart, it does not require GTP. Factors that catalyze the binding and fMet-puromycin reactions with ribosomal subunits from *Artemia salina* embryos are present in postribosomal supernatants of *Artemia*, mouse fibroblasts (L cells), and rat liver, as well as in salt washes of rabbit reticulocyte ribosomes. However, whereas all three supernatant factors, like *Escherichia coli* IF-2, are sensitive to SH-binding reagents, the reticulocyte factor is not. The rat liver and *Artemia* factors function indiscriminately with *Artemia* or rat liver ribosomes, but the *Artemia* factor and the *E. coli* initiation factor IF-2 are not interchangeable.**

In a previous paper (1), we reported the isolation of an initiation factor from the postribosomal supernatant of embryos of the brine shrimp, *Artemia salina*. This factor appears to be the eukaryotic counterpart of the bacterial initiation factor IF-2, for it promotes the AUG-dependent binding of fMet-$tRNA_f$ to the small ribosomal subunit. However, in contrast to the reaction catalyzed by IF-2, the eukaryotic reaction has no requirement for GTP. The binding reaction is sensitive to aurintricarboxylic acid and edeine, specific inhibitors of initiation, and the bound fMet-$tRNA_f$ reacts directly with puromycin upon addition of the 60S subunit. The factor, a basic protein of molecular weight about 100,000, is inactivated by *N*-ethylmaleimide, an SH-binding reagent, and is clearly distinct from the *A. salina* elongation factors EF-1 and EF-2.

Factors that catalyze reactions similar to the one studied by us with *Artemia* have been reported in high-speed supernatants of wheat germ (2), rat liver (3–5), ascites cells (4), and yeast (6), as well as in washes of rabbit reticulocyte ribosomes with 0.5 M KCl [initiation factor M_1 of Anderson and collaborators (7)]. In this paper, we report that the activity present in postribosomal supernatants of rat liver and in preparations of reticulocyte initiation factor M_1, as well as an activity found in supernatant fractions from mouse L cells, is identical in many respects to that of the *Artemia* factor. The rat liver and *Artemia* factors and ribosomes are fully interchangeable.

* This is paper No. II in the series, "Polypeptide Chain Initiation in Eukaryotes," The preceding paper in this series is ref. 1.

MATERIALS AND METHODS

Ribosomal Subunits. *A. salina* ribosomes and subunits were prepared as described (1). Rat liver ribosomal subunits were prepared as described by Martin *et al.* (8), with some modifications. All operations were conducted at 0–4° if not otherwise noted. The ribosomal pellet, obtained as described below, was suspended in a buffer containing 50 mM Tris·HCl, pH 7.75 (25°)–150 mM KCl–5 mM magnesium acetate–20 mM 2-mercaptoethanol, to a concentration of 200 A_{260} units/ml. Puromycin and GTP were added to concentrations of 0.23 and 0.6 mM, respectively, and the suspension was incubated for 30 min at 37°. Sodium deoxycholate was then added to a concentration of 0.5%, and the mixture was stirred for 5 min. Insoluble material was removed by centrifugation for 20 min at 4000 rpm in a Sorvall SS-34 rotor, and the ribosomes were sedimented by centrifugation for 90 min at 50,000 rpm in a Spinco No. 65 rotor. This pellet was used for preparation of subunits, as was described for *A. salina* (1). Subunits were stored in 50% glycerol (v/v) at −20°. *Escherichia coli* Q13 30S subunits, prepared (9) from ribosomes washed with 1.0 M NH_4Cl, were kindly provided by Dr. H. A. Klein of this department.

Supernatant Factor. The *A. salina* factor was purified from extracts of encysted embryos (1) by pH and ammonium sulfate fractionation, chromatography on phosphocellulose and carboxymethylcellulose, and gel filtration on Sephadex G-200. The preparation used in this work had a specific activity of 10,000 (pmol of fMet-tRNA bound/mg of protein in 20 min at 0°) in the standard assay, as compared to about 10 for the pH 5.0 supernatant fraction. At this stage of purification the factor was not homogeneous. Details of the purification procedure, which is being continued, will be described elsewhere.

Rat liver supernatant factor was obtained from the postribosomal supernatant, prepared essentially as described by Moldave *et al.* (10). All operations were performed at 0–4°. The livers from three 200-g (white male) Wistar rats were washed in an ice-cold buffer containing 20 mM K_2HPO_4–35 mM $KHCO_3$–350 mM sucrose–25 mM KCl–4 mM magnesium acetate–10 mM 2-mercaptoethanol–0.1 mM EDTA (pH 7.4), and minced. The mince was suspended in 30 ml of the same buffer, homogenized in a Sorvall omnimixer at full speed, and centrifuged 30 min at 20,000 rpm in a Sorvall SS-34 rotor. The supernatant was collected by suction, filtered through glass wool, and centrifuged 10 min at 30,000 rpm in a Spinco No. 30 rotor to remove membrane debris and glycogen. The

resulting supernatant was centrifuged 120 min at 50,000 rpm in a Spinco 60 Ti rotor to pellet the ribosomes. The supernatant was dialyzed overnight against 2 liters of a buffer containing 50 mM Tris·HCl pH 7.75 (25°)–100 mM KCl–10 mM 2-mercaptoethanol–0.1 mM EDTA–10% glycerol (buffer A), and a portion of the preparation (450 mg of protein) was applied to a column (10 × 0.9 cm) of phosphocellulose (Whatman P-11), which had been washed with saturated NaCl and water, and equilibrated with buffer A. The column was washed with buffer A until the A_{280} of the effluent became negligible. The proteins were then eluted with buffer A containing 300 mM KCl. The peak protein fractions were pooled and concentrated to 7 mg of protein/ml by dialysis against a saturated solution of Carbowax 6000 (Union Carbide) in buffer A. The specific activity of the phosphocellulose fraction was about 40, as compared to about 2 for the postribosomal supernatant.

Mouse L cells (a line of L_2 cells obtained from Dr. S. Dales and propagated by Dr. L. Gober) were grown at 37° in Spinner culture (800 ml) in Eagle's minimal essential medium supplemented with 10% fetal-calf serum and containing penicillin G (100 units/ml) and streptomycin (100 μg/ml). The cells were harvested at a density of 3 × 10^5 cells/ml by centrifugation in a Sorvall GSA rotor for 3 min at 3000 rpm. After the cells were washed twice in a buffer containing 30 mM Tris·HCl, pH 7.5 (25°)–125 mM KCl–5 mM magnesium acetate–200 mM sucrose–10 mM 2-mercaptoethanol, they were resuspended in 1 ml of the same buffer and homogenized in a Dounce homogenizer. The homogenate was centrifuged for 130 min at 48,000 rpm in a Spinco No. 50 rotor, and the clear supernatant was concentrated by dialysis against a saturated solution of Carbowax 6000 in buffer A, to 7 mg of protein/ml. The specific activity of this supernatant in the standard assay was 9–12.

Other Preparations. The reticulocyte initiation factor M_1 (IF-M_1) was a kind gift of Dr. W. F. Anderson, NIH. This factor was partially purified from a 0.5 M KCl wash of rabbit reticulocyte ribosomes by DEAE-cellulose chromatography, ammonium sulfate fractionation, and Sephadex G-200 filtration (11). The specific activity of this preparation in our standard binding assay was about 100. Crystalline *E. coli* initiation factor IF-1 (12) and partially purified *E. coli* initiation factor IF-2 (13) were kindly provided by Drs. S. Lee-Huang and R. Mazumder of this department. Other preparations have been described (1).

Assays. The standard assay for the binding factor is based on its requirement for the AUG-dependent binding of fMet-$tRNA_f$ to *A. salina* 40S ribosomal subunits. Since the assay used earlier (1) has been slightly modified, it is described here in detail. Samples contain, in a volume of 0.06 ml, 90 mM Tris·HCl buffer, pH 7.5 (25°), 150 mM KCl, 4 mM magnesium acetate, 1.5 mM dithiothreitol, 0.3 A_{260} unit of 40S *Artemia* ribosomal subunits, and 10 pmol of f[^{14}C]Met-tRNA (*E. coli*, crude) (400 cpm/pmol). After incubation for 20 min at 0°, the ribosomal-bound radioactivity was determined (1). Other assays, including the determination of protein and of aminoacyl-puromycin synthesis, were described (1).

RESULTS AND DISCUSSION

Factors that can replace the *Artemia* supernatant factor in the AUG-dependent binding of fMet-$tRNA_f$ to *Artemia* 40S ribosomal subunits are present in postribosomal supernatants of mouse L cells and rat liver, and in preparations of reticulocyte initiation factor M_1.

It may be seen in Table 1 that supernatant preparations of mouse L cells and rat liver, and a partially purified preparation of rabbit reticulocyte initiation factor M_1, catalyze the binding of *E. coli* fMet-$tRNA_f$ to *A. salina* 40S ribosomal subunits as effectively as does the *Artemia* factor. It will also be noted that the various factors promote in all cases the binding of fMet-$tRNA_f$ to a site on the small subunit that corresponds to an initiation site, since subsequent addition of the 60S subunit results in near stoichiometric synthesis of the dipeptide analog fMet-puromycin. Moreover (Table 1), the *A. salina* and rat liver factors promote the binding of fMet-$tRNA_f$ to an initiation site on either rat liver or *A. salina* 40S subunits. This observation shows that there is full functional interchangeability of initiation factors and ribosomes from two evolutionarily distant eukaryotic organisms.

The binding reaction (Table 2) and, as will be shown in a subsequent paper, the ensuing synthesis of fMet-puromycin, are GTP-independent events. This independence is observed whether the reaction is promoted by the *Artemia*, L cell, rat liver, or reticulocyte factor with the *A. salina* 40S subunit

TABLE 1. *Distribution of factor*

Ribosomes used for assay	Factor: Source	Factor: Amount (μg protein)	f[^{14}C]Met-$tRNA_f$ binding (pmol)	f[^{14}C]Met-puro synthesis (pmol)	f[^{14}C]Met-puro synthesis (% of bound)
A. salina			0.38	0.30	—
	A. salina	1	3.19	3.00	93
	Mouse L cells	140	1.58	1.74	100
	Rat liver	160	3.50	2.48	71
	Rabbit reticulocytes	70	4.18	3.28	78
Rat liver			0.21	0.07	—
	A. salina	1	3.00	3.00	100
	Rat liver	160	3.73	2.65	71

Duplicate samples containing 40S subunits and the other components of the standard assay, as described in *Methods*, were incubated 20 min at 0°. 60S subunits were then added and, after a further 10 min at 0°, one of the samples received puromycin (75 μg) and the other an equal volume of water. Incubation at 0° was then continued for a further 15 min. The puromycin-treated sample was analyzed for the synthesis of fMet-puromycin, and the other sample for the ribosomal binding of fMet-$tRNA_f$. Subunit additions: *A. salina*, 40S, 0.35, 60S, 1.0 A_{260} units; rat liver, 40S, 0.6, 60S, 1.4 A_{260} units.

TABLE 2. *Effect of various inhibitors in factor-promoted binding of fMet-tRNA$_f$ to 40S ribosomal subunits*

	Factor			f[^{14}C]Met-tRNA$_f$ binding			
					% Inhibition by		
Source of subunits	Source	Amount (μg protein)	GTP	(pmol)	ATA	Edeine	NEM
A. salina	*A. salina*	0.2	−	2.30	100	100	100
		0.2	+	1.88			
		1.0	−	3.62			
	Mouse L cells	140.0	−	1.30	80	100	100
		140.0	+	1.13			
	Rat liver	32.0	−	1.30	100	100	87
		32.0	+	1.10			
		160.0	−	3.98			
	Rabbit reticulocytes	15.0	−	1.49	100	100	0
		15.0	+	1.26			
		70.0	−	2.84			
Rat liver	Rat liver	32.0	−	1.72	95	100	82
		32.0	+	1.64			
		160.0	−	4.03			
	A. salina	0.2	−	1.54	100	100	100
		0.2	+	1.78			
		1.0	−	3.25			

Conditions were as described for the standard assay with the addition of either *A. salina* (0.35 A_{260} unit) or rat liver (0.6 A_{260} unit) 40S subunits. Other additions, where indicated: GTP, 0.18 mM; aurin tricarboxylic acid (*ATA*), 0.1 mM; edeine, 7 μM. *N*-ethylmaleimide, (*NEM*), 10 mM (and up to 20 mM in the case of the reticulocyte factor) was preincubated with factor for 10 min at 24°, and the unreacted compound was inactivated with excess (50 mM) dithiothreitol. Net binding values are given after subtraction of a blank without factor of 0.22 pmol for *A. salina* and 0.15 pmol for rat liver 40S subunits. Factor preparations were as in Table 1.

or by the *A. salina* and rat liver factors with the rat liver 40S subunit. GTP-independence of the AUG-directed binding of fMet-tRNA$_f$ to rabbit reticulocyte ribosomes, a reaction promoted by reticulocyte factor IF-M$_1$, was also noted by Shafritz *et al.* (7). It may further be seen in Table 2 that the 40S ribosomal binding reaction is strongly depressed by initiation inhibitors, such as aurin tricarboxylic acid and edeine, irrespective of the source of factor or ribosomes.

We have reported (1) that the *Artemia* supernatant factor shares with the bacterial initiation factor IF-2 sensitivity to the SH-binding reagent *N*-ethylmaleimide. The same is true, as seen in Table 2, of the other two factors derived from post-ribosomal supernatants, i.e., the L cell and rat liver factors with *Artemia* 40S subunits, and of the *Artemia* and rat liver factors with rat liver 40S subunits. On the other hand, the reticulocyte factor IF-M$_1$, as assayed with *Artemia* 40S subunits, is quite resistant to *N*-ethylmaleimide (Table 2). We have found that the reticulocyte factor is also resistant to *p*-hydroxymercuribenzoate, at concentrations as high as 0.2 mM. It is not clear whether this difference reflects functional differences between IF-M$_1$ and the supernatant factors, since IF-M$_1$ is derived from a 0.5 M KCl wash of rabbit reticulocyte ribosomes.

TABLE 3. *Noncrossreactivity of A. salina and E. coli initiation factors in ribosomal binding of fMet-tRNA$_f$*

Ribosomal subunits	Factors	GTP	f[^{14}C]Met-tRNA$_f$ binding (pmol)
E. coli 30S	None	−	0.07
	E. coli IF-1 + IF-2	−	1.13
	Ditto	+	3.33
	A. salina supernatant factor	−	0.05
	Ditto	+	0.01
A. salina 40S	None	−	0.19
	E. coli IF-1 + IF-2	−	0.30
	Ditto	+	0.32
	A. salina supernatant factor	−	3.88

Conditions were as in Table 2. In the samples containing GTP, its concentration was 0.18 mM. Subunit additions: *E. coli* 30S, 0.37 A_{260} unit; *A. salina* 40S, 0.35 A_{260} unit. Factor additions: *A. salina*, 1 μg; IF-1, 3 μg: IF-2, 24 μg.

The *A. salina* factor, though functionally interchangeable with other factors of eukaryotic origin, does not substitute for its prokaryotic counterpart, bacterial initiation factor IF-2, nor does IF-2 substitute for the *Artemia* factor. As shown in Table 3, the *Artemia* factor is unable to promote the binding of fMet-tRNA$_f$ to *E. coli* 30S subunits, whether in the absence or presence of GTP, and *E. coli* IF-2 is inactive with *A. salina* 40S subunits under the same conditions. Table 3 also emphasizes the contrast between the IF-2-promoted and the eukaryotic factor-promoted reaction with regard to the GTP-dependence of the former and the GTP-independence of the latter. The noninterchangeability of the *Artemia* factor and IF-2 is further proof that the *Artemia* factor does not originate from contaminating prokaryotic organisms or from cellular organelles, e.g., mitochondria, whose protein-synthesizing machinery is of a prokaryotic rather than eukaryotic type (14).

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