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Met-tRNA$_f^{Met}$ Binding to 40S Ribosomal Subunits: A Site for the Regulation of Initiation of Protein Synthesis by Hemin

(translation control/initiation factors/buoyant density gradients)

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ABSTRACT **On incubation of reticulocyte lysates at 30° in the absence of added hemin, protein synthesis declines sharply within 4–6 min, due to the action of a translational inhibitor. Partially purified preparations of this inhibitor, in concentrations that inhibit protein synthesis in the lysate, cause reduced binding of Met-tRNA$_f^{Met}$ to derived 40S ribosomal subunits in a ribosomal-salt-wash-dependent assay system. Neither the association of salt wash proteins with the subunits nor the level of Met-tRNA$_f^{Met}$ bound in preformed 40S complexes is reduced by the inhibitor. No Met-tRNA$_f^{Met}$ deacylase activity could be detected in the inhibitor preparation. Protein synthesis in reticulocyte lysates lacking added hemin or containing exogenous inhibitor is maintained by addition of small amounts of an initiation preparation factor, "IF-MP," which may be involved in the binding of Met-tRNA$_f^{Met}$ to 40S subunits. This binding constitutes a site for control of protein synthesis by hemin in reticulocytes.**

The initiation of the synthesis of globin and other proteins in rabbit reticulocytes and lysates prepared from them is dependent on a supply of hemin (1–4). The cessation of protein synthesis and loss of polysomes that occur in hemin-deficient lysates are preceded by disappearance of [^{35}S]Met-labeled initiator tRNA, Met-tRNA$_f$, from the native 40S ribosomal subunits (5, 6) and have been attributed to the action of a translational inhibitor protein (7–9). The reduced labeling of native subunits with [^{35}S]Met-tRNA$_f$ has been ascribed both to impaired binding (5) and to deacylation of the subunit-associated tRNA (10). It is difficult to distinguish between these possibilities in a crude lysate, and we have therefore investigated the effect of partially purified inhibitor on the binding of Met-tRNA$_f$ to 40S subunits and on the stability of the complex formed, in a fractionated system.

When a ribosomal salt wash is incubated with purified 40S subunits, Met-tRNA$_f$, and GTP, proteins in the salt wash become associated with the subunits, giving rise to particles with buoyant densities on CsCl gradients of 1.40 and 1.49 g/cm^3 (11), and Met-tRNA$_f$ becomes bound to the subunits of density 1.40 g/cm^3 (K. E. Smith and E. C. Henshaw, manuscript in preparation). We show here that the binding of Met-tRNA$_f$ to these particles is inhibited by partially purified preparations of the translational inhibitor protein.

The rate of polypeptide chain initiation in hemin-deficient lysates can be maintained (12–14) or restored (15) by a ribosomal salt wash preparation, the active component of which (13–15) has chromatographic properties on DEAE-cellulose similar to those of initiation factor M$_3$ of Prichard *et al.* (16). This has now been subfractionated to yield an initiation factor "IF-MP," which mediates binding of Met-tRNA$_f$ to the 40S subunit, probably through the formation of a ternary complex of IF-MP·Met-tRNA$_f$·GTP (17). We have found this factor to be highly active in allowing continuation of protein synthesis *in vitro* in the absence of added hemin. It is therefore proposed that IF-MP-dependent binding of Met-tRNA$_f$ to the small ribosomal subunit is the step at which hemin regulates the rate of protein synthesis in reticulocytes.

MATERIALS AND METHODS

Preparation of Reticulocyte Lysates. Reticulocytosis was induced in 3-kg rabbits by daily injection of 25 mg of acetylphenylhydrazine for 4 days. Blood was obtained by cardiac puncture on the ninth day and the red cells were washed and lysed as previously described (3).

Protein Synthesis in the Reticulocyte Lysate. Freshly thawed lysates were assayed for their ability to incorporate [^{14}C]-leucine (273 Ci/mole) into protein by incubation at 30° under the conditions described by Hunt *et al.* (3). The lysate made up at least half the total incubation volume. When hemin was added, the relevant volume of a 1 mM solution in 90% ethylene glycol–10 mM Tris·HCl (pH 7.8) was used to give a final concentration of 30–40 μM hemin. An equal volume of ethylene glycol was added to the controls. Amino-acid incorporation into protein was assayed in 10-μl aliquots of the incubation mixtures on Whatman 3 MM filter discs, using the procedure of Mans and Novelli (18).

Preparation of Ribosomal Salt Washes and Initiation Factors. Reticulocyte ribosomal KCl washes were prepared as described by Shafritz and Anderson (19), except that 1.0 M KCl was used instead of 0.5 M. Purified initiation factors were a generous gift from Drs. W. C. Merrick and W. F. Anderson. IF-M$_3$ was obtained by DEAE-cellulose (Whatman DE 52) chromatography of a 0.5 M KCl ribosomal wash (16), with batch elution at 0.15 M KCl. IF-MP was further purified by

Abbreviation: IF, initiation factor.

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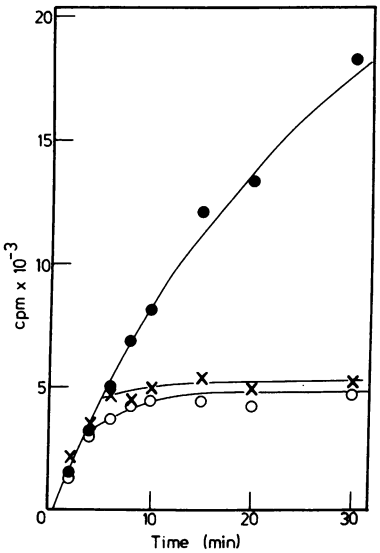

FIG. 1. Protein synthesis in the reticulocyte lysate in the absence of added hemin or in the presence of added inhibitor. Reaction mixtures (100 μl) were incubated at 30° in the presence or absence of hemin (30 μM) and inhibitor protein (25 μg). Aliquots (10 μl) were removed at the times indicated for assay of leucine incorporation into protein. ×, minus hemin; ●, plus hemin; ○, plus hemin and inhibitor.

phosphocellulose chromatography of the IF-M$_3$ and was eluted at 0.75 M KCl.

Preparation of Derived 40S Ribosomal Subunits. Ribosomal subunits were prepared using the procedure of Schreier and Staehelin (20). Briefly, this consisted of incubation of reticulocyte polysomes under conditions of protein synthesis to convert them to 80S monomers, followed by dissociation of the ribosomes into subunits with 0.5 M KCl. The derived subunits were separated on sucrose gradients containing 0.3 M KCl and stored in small aliquots under liquid nitrogen in 0.25 M sucrose, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetate. The 40S subunits used contained approximately 8% 60S subunits.

Preparation of Met-tRNA$_f^{Met}$. Deacylated rat liver tRNA was charged with [^{35}S]methionine by means of a preparation of aminoacyl-tRNA synthetases from *Escherichia coli* MRE 600 (21). The incubation conditions and procedure for isolation of the charged tRNA were as described by Takeishi *et al.* (22) and Gupta *et al.* (23). The specific activity of [^{35}S]-Met-tRNA$_f^{Met}$ used in these experiments was 152 Ci/mmole of methionine.

Preparation and Characterization of the Translational Inhibitor. Reticulocyte S-100 (100 ml), prepared by centrifuging lysates at 150,000 × *g* for 2 hr, was incubated in the absence of added hemin for 20 min at 37°. A pH 5 precipitate was prepared from this material (13) and dissolved in 20 ml of 36 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.2. After clarification of the solution by centrifugation at 27,000 × *g* for 15 min, solid ammonium sulfate was added to a final concentration of 30% saturation and the precipitated protein was sedimented at 27,000 × *g* for 15 min. The precipitate was dissolved in 4 ml of Hepes buffer and dialyzed against the same solution for 18 hr at 4°. The partially purified inhibitor preparation, containing 2.5 mg of protein per ml, was stored in small aliquots under liquid nitrogen.

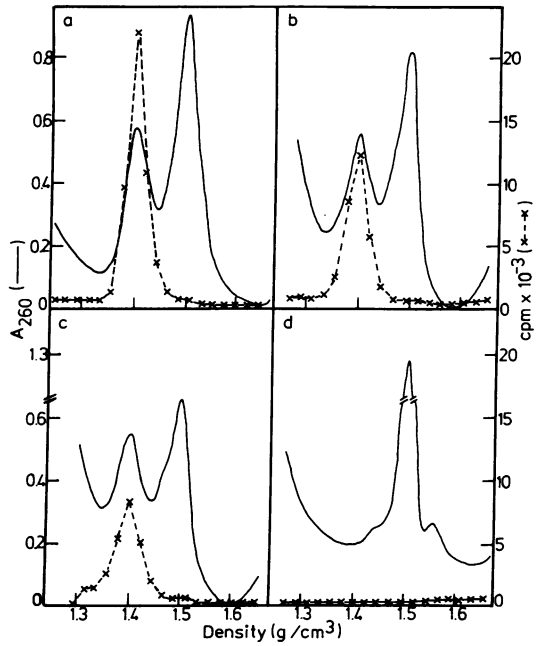

FIG. 2. Influence of the inhibitor on binding of salt wash proteins and Met-tRNA$_f^{Met}$ to 40S subunits. Reaction mixtures were incubated for 15 min at 30° with (*a*) no added inhibitor; (*b*) and (*d*) 250 μg of inhibitor protein; (*c*) 500 μg of inhibitor protein. Incubations (*a–c*) contained 235 μg of salt wash protein; (*d*) contained no salt wash. Other conditions and the method of preparation of samples for CsCl density gradient analysis were as described in *Materials and Methods. Solid line,* absorbance at 260 nm; ×, *broken line,* cpm per fraction.

The time-course of action of the inhibitor on protein synthesis in a reticulocyte lysate is shown in Fig. 1. The concentration dependence and specificity for the inhibition of amino-acid incorporation is given in Table 1. Whereas leucine incorporation into globin at 2 mM Mg^{2+} was sensitive to the inhibitor, polyphenylalanine synthesis at 10 mM Mg^{2+}, under

TABLE 1. *Concentration dependence for inhibition of protein synthesis in the reticulocyte lysate by partially purified inhibitor*

Inhibitor concentration (μg/10 μl assay)	Leucine incorporation (cpm × 10^{-3}/10 μl assay)	Poly(U)-directed phenylalanine incorporation (cpm × 10^{-3}/10 μl assay)
0	10.23	2.59
0.05	12.14	—
0.25	8.50	2.79
0.50	7.09	2.65
1.25	5.80	2.51
2.50	3.86	2.57

Incubation was for 30 min in the presence of hemin. Assays were performed with [^{14}C]leucine at 2 mM Mg^{2+} or [^{14}C]phenylalanine in the presence of 200 μg of poly(U) per ml at 10 mM Mg^{2+}. In the absence of both added hemin and added inhibitor, leucine incorporation into globin was 3.97 × 10^3 cpm and phenylalanine incorporation into poly(Phe) was 2.20 × 10^3 cpm/10 μl assay.

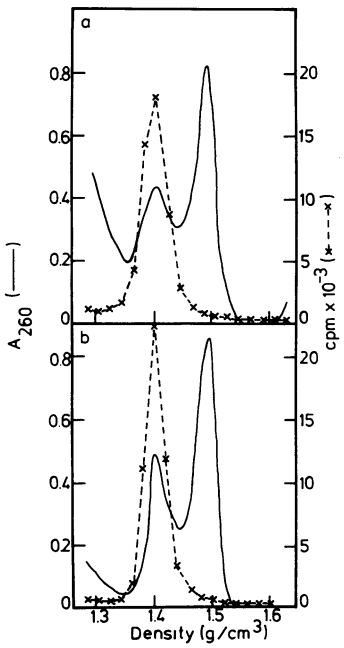

FIG. 3. Stability of preformed 40S subunit·Met-tRNA$_f^{Met}$ complexes in the presence of the inhibitor. Three identical reaction mixtures were incubated as described in *Materials and Methods* for 15 min at 30° with 235 μg of salt wash protein and no inhibitor. One incubation was then fixed for analysis (shown in Fig. 2*a*) and the others were incubated for a further 10 min at 30° in the presence of (*a*) 250 μg of inhibitor protein or (*b*) an equal volume of additional buffer only. *Solid line*, absorbance at 260 nm; ×, *broken line*, cpm per fraction. Total cpm associated with the subunits of density 1.40 g/cm^3 are in (*a*), 47725; in (*b*), 51813. (Compare with 48901 cpm in Fig. 2*a*.)

which conditions the natural mechanism of initiation is bypassed, was not sensitive.

Analysis of Met-tRNA$_f^{Met}$ Binding to 40S Ribosomal Subunits. One ml incubation mixtures contained approximately 1 A_{260} unit of derived 40S subunits, 5 μg of [^{35}S]Met-tRNA$_f^{Met}$ (4.6×10^5 dpm), 40 mM triethanolamine·HCl (pH 7.1), 80 mM KCl, 3mM Mg acetate, 1 mM dithiothreitol, and 0.2 mM GTP. Salt wash proteins and inhibitor were added as described in individual tables and figures. No exogenous mRNA was added. After incubation at 30° for the times stated, reactions were terminated by chilling on ice. In some experiments, 10-μl aliquots were removed at this stage for determination of total radioactivity in Met-tRNA$_f^{Met}$ (see Table 3). The subunits were immediately fixed by adding 6 ml of 9 mM morpholinopropanesulfonic acid, 1.3 mM Mg acetate, 22 mM KCl, 4% formaldehyde, pH 7.0, and the mixtures were kept at 0° for 30 min before overlaying onto 5 ml of CsCl (density 1.51 g/cm^3) in the same buffer plus 0.1 mg of Brij per ml. Gradients were formed by centrifugation at 37,000 rpm for 40 hr at 3° in a Beckman SW41 rotor. Absorbance at 260 nm was monitored by pumping the gradients through a flow cell (24). Approximately 30 fractions were collected from each gradient and the densities of selected parts of the gradient were determined by weighing aliquots of solution. One drop of 0.5% bovine serum albumin and 1 ml of cold 5% trichloroacetic acid were added to each fraction and precipitates were collected on Whatman GF/C glass fiber filters which were washed with 2 ml of cold 5% trichloroacetic acid. The filters were dried and radioactivity determined in a Beckman scintillation counter at 61% efficiency.

TABLE 2. *Relationships between amounts of salt wash protein and inhibitor and the binding of Met-RNA$_f^{Met}$ to 40S subunit*

Salt wash protein (μg per incubation)	Inhibitor protein (μg per incubation)	Met-tRNA$_f^{Met}$ bound to 40S (cpm × 10^{-3})	Inhibition of binding (%)
Experiment I			
—	—	2.1	—
235	—	44.7	—
470	—	84.4	—
470	125	61.8	26.8
470	250	53.9	36.1
Experiment II			
235	—	48.7	—
235	250	30.2	38.1
235	500	22.6	53.6

The incubation conditions and procedures used for analysis of Met-tRNA$_f^{Met}$ binding were as described in Fig. 2. The amounts of [^{35}S]Met-tRNA$_f^{Met}$ per 1 ml of incubation were 3.51×10^5 cpm (1.4 pmoles) in *Experiment I* and 3.16×10^5 cpm (1.2 pmoles) in *Experiment II*.

RESULTS

When derived 40S ribosomal subunits, of buoyant density 1.51 g/cm^3, are incubated at 30° with reticulocyte ribosomal salt wash in the presence of GTP and [^{35}S]Met-tRNA$_f^{Met}$, and the fixed material is then analyzed on CsCl gradients, two peaks of absorbance are observed, corresponding to subunits with buoyant densities of 1.40 and 1.49 g/cm^3 (Fig. 2*a*). These two forms of the 40S subunit contain approximately 750,000 and 100,000 daltons, respectively, of additional protein compared to the derived subunits (25). Radioactivity is associated solely with the 1.40 g/cm^3 particles, indicating that at least part of the extra protein occurring in this fraction is necessary for Met-tRNA$_f$ binding (K. E. Smith and E. C. Henshaw, manuscript in preparation). In the presence of added translational inhibitor, the amount of subunits of density 1.40 g/cm^3 is not diminished, but the associated Met-tRNA$_f$ radioactivity is reduced (Fig. 2*b* and *c*). In the

TABLE 3. *Deacylation of total Met-tRNA$_f^{Met}$ during incubation under conditions of the binding assay*

Inhibitor protein (μg per incubation)	Met-tRNA$_f^{Met}$ at start of incubation (cpm/10 μl)	Met-tRNA$_f^{Met}$ at end of incubation (cpm/10 μl)	Deacylation (%)
—	3070	2460	20.1
250	3070	2430	21.1
500	3150	2380	24.4

Incubations were as described in Fig. 2, with 235 μg of salt wash protein and 1.1 A_{260} units of 40S subunits per ml. At the start and end of the 15-min incubation, 10-μl aliquots of total reaction mixture were pipetted onto Whatman 3 MM filters for estimation of cold-acid-insoluble radioactivity (18). The remainder of each incubation mixture was analyzed for Met-tRNA$_f^{Met}$ binding to 40S subunits (*Experiment II* in *Table 2*).

TABLE 4. *Stimulation of protein synthesis by initiation factor preparations in the absence of hemin or in the presence of inhibitor protein*

IF concentration (μg/10-μl assay)	Lysate minus hemin		Lysate plus hemin and inhibitor	
	(cpm × 10^{-3})	(% stimulation)	(cpm × 10^{-3})	(% stimulation)
DEAE "IF-M_3"				
0	3.15	—	—	—
3.84	3.62	14.9	—	—
7.68	3.71	18.0	—	—
19.2	4.24	34.7	—	—
38.4	1.77	Inhibition	—	—
"IF-MP"				
0	4.02	—	3.05	—
0.13	4.30	6.9	3.06	0
0.32	4.71	17.3	3.04	0
0.64	5.78	43.8	3.68	20.6
1.28	6.05	50.5	4.28	40.4

Aliquots of a lysate were incubated for 30 min at 30° either in the absence of added hemin or in the presence of 40 μM hemin plus 2 μg of inhibitor protein per 10 μl of reaction mixture. The amounts indicated of "IF-M_3," prepared by DEAE-cellulose chromatography (16) or of "IF-MP," purified from DEAE "IF-M_3" by further chromatography on phosphocellulose (17), were added from the start of incubation. Duplicate 10-μl aliquots were assayed for leucine incorporation into protein as described in *Materials and Methods*.

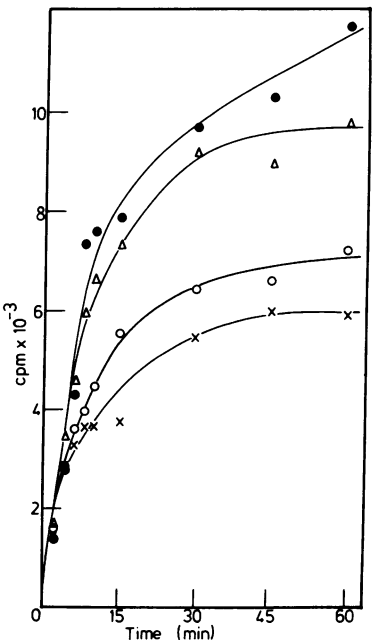

FIG. 4. Protein synthesis in the lysate in the presence and absence of hemin and added initiation factor preparations. Reaction mixtures (100 μl) were incubated at 30° and 10-μl aliquots were removed at the times indicated for assay of leucine incorporation into protein. ×, minus hemin; ○, minus hemin, plus DEAE "IF-M_3" (128 μg/100 μl); △, minus hemin, plus "IF-MP" (26 μg/100 μl); ●, plus hemin (40 μM).

absence of the salt wash the 40S subunits have a buoyant density of 1.51 g/cm³, even in the presence of the inhibitor protein, and no radioactivity from [^{35}S]Met-tRNA$_f^{Met}$ is associated either with the subunits or with proteins in the inhibitor preparation (Fig. 2*d*). The amount of initiator tRNA bound to the low-density subunits is a function of the amount of salt wash protein present in the incubation and the concentration of the inhibitor (Table 2). However, varying the ratio of inhibitor concentration to salt wash concentration over an 8-fold range produced only a 2-fold difference in the extent of inhibition of binding, a finding which suggests that some association of Met-tRNA$_f^{Met}$ with the subunits may be resistant to the inhibitor under our conditions.

The inhibitor does not act by deacylating the Met-tRNA$_f^{Met}$ available for binding during the incubation (Table 3). Furthermore, incubation of preformed subunit-Met-tRNA$_f^{Met}$ complexes with the inhibitor does not lead to loss of bound radioactivity (Fig. 3); this suggests that it is the formation of the complexes rather than their stability that is affected by the inhibitor.

The time-course of action of the inhibitor on protein synthesis in the reticulocyte lysate (Fig. 1 and ref. 3) suggests that a component that is essential for initiation becomes inactivated after a few minutes of incubation. This conclusion is also supported by the fact that a ribosomal salt wash, which contains the components necessary to support polypeptide initiation, will allow amino-acid incorporation to continue for longer in the absence of added hemin or in the presence of added inhibitor (12–15). The active component of a salt wash that can achieve this effect has chromatographic properties on DEAE-cellulose similar to those of IF-M_3 (13–15). Further purification of IF-M_3 has now revealed the presence of a constituent factor, "IF-MP," which forms a ternary complex with GTP and Met-tRNA$_f^{Met}$ and is required for synthesis of methionyl-puromycin on ribosomes (17). When this factor is added to hemin-deficient lysates, protein synthesis continues at almost the same rate as when hemin is present (Fig. 4). IF-M_3 prepared on DEAE-cellulose has a small stimulatory effect, but only when added at much higher concentrations of protein. IF-M_3 separated from IF-MP on phosphocellulose was slightly inhibitory for amino-acid incorporation at concentrations in the range of 50–170 μg/ml (unpublished observations). Very small amounts of IF-MP significantly stimulate leucine incorporation in the lysate, both in the absence of added hemin and in the presence of exogenous inhibitor (Table 4).

DISCUSSION

In the reticulocyte lysate protein-synthesizing system, the presence of the translational inhibitor reduces the level of Met-tRNA$_f$ associated with the native subunits (5, 6). This may be a consequence of impaired binding of the initiator tRNA or may result from deacylation of the Met-tRNA$_f$ on the 40S subunit (10). Our data favor a direct inhibition of binding, since the inhibitor had no effect on preformed 40S subunit·Met-tRNA$_f^{Met}$ complexes. Furthermore, although reticulocytes have a potent Met-tRNA$_f^{Met}$ specific deacylase activity (26, 27), this was not present in our preparations of the inhibitor (Table 3).

The initiation factor IF-MP, which maintains protein synthesis in the hemin-deficient reticulocyte lysate, has properties similar to those of a eukaryotic initiation factor, recently described by several laboratories (28–32), which forms a ternary complex with Met-tRNA$_f$ and GTP. The formation of

this complex appears to be a prerequisite for Met-tRNA$_f$ binding to 40S ribosomal subunits at an early step in the initiation mechanism (29, 33). Our data indicate that it is the function of IF-MP that is impaired when reticulocytes are deprived of hemin.

We do not yet know the precise mechanism whereby the translational inhibitor acts to prevent the normal functioning of IF-MP, but some possible modes of action can be excluded. Since the inhibitor, which is reported to have a molecular weight of approximately 3×10^5 (34), does not alter the buoyant density of derived 40S subunits, it probably does not bind to them. However, the binding of a small polypeptide to the subunits is not excluded. In addition, Met-tRNA$_f^{Met}$ itself is not bound to any protein in the inhibitor preparation in a form that is stable to CsCl gradient analysis (unpublished observations). It is also apparent that the association of salt wash proteins with the 40S subunits is not detectably reduced in the presence of the inhibitor. A factor recently described by Cashion and Stanley (35), which is almost certainly identical with IF-MP, has a molecular weight of 3.7×10^5 (at least as it exists in a crude salt wash). This is equivalent to one-half of the amount of extra protein associated with the subunits of buoyant density 1.40 (25). The results shown in Fig. 2 rule out any extensive inhibition of binding of such a large mass of protein as this to the subunits in the presence of the inhibitor, but again more subtle effects would not be seen on the CsCl gradients.

It has been suggested that hemin regulates protein synthesis by controlling the activity of a factor "IF-3$_{rr}$," which could function in a manner analogous to that of bacterial IF-3 (14). It is probable, however, that "IF-3$_{rr}$" contains more than one component, since it was prepared by DEAE-cellulose chromatography in a manner similar to that which was used for the earlier IF-M$_3$ (16). Our findings suggest that the effects described by Kaempfer and Kaufman (14) were due to the action of the factor IF-MP, which is a constituent of M$_3$ prepared in this manner (15, 17). It should be stressed, however, that until IF-MP is purified to homogeneity, the preparation cannot be assumed to contain only one initiation factor.

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