FACTOR AND GTP REQUIREMENTS IN A EUKARYOTIC PROTEIN INITIATION SYSTEM WITH REOVIRUS MESSENGER RNA, MET-tRNA$_F$, AND RIBOSOMAL SUBUNITS

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1. Introduction

A previous report described the formation of a eukaryotic initiation complex composed of Reovirus mRNA, rat liver Met-tRNA$_F$, and 40 S and 60 S ribosomal subunits from mouse L-929 cells [1]. All of the three size classes of Reovirus mRNA were able to induce the formation of 80 S initiation complexes at a puromycin-sensitive site on the ribosome. Complex assembly was mRNA-dependent and was specific for Met-tRNA$_F$ [1]. However, no requirement for GTP or ribosomal factors could be demonstrated. The 40 S and 60 S subunits retained their initiation factors and sufficient GTP to support complex formation even after treatment with detergent and 0.5 M KCl. This phenomenon restricted detailed mechanism studies in this system.

In the present investigation, we report that differential treatment of L-cell 40 S and 60 S subunits with 1 M KCl is essential in order to establish a GTP dependency and a requirement for exogenous ribosomal factors during initiation. Although the 40 S subunit is relatively stable during such treatment, the 60 S subunit is rapidly inactivated. Treatment of either subunit with 1 M KCl elicits both the GTP and factor dependencies. Utilization of the treated subunits has facilitated the stepwise analysis of the initiation process.

2. Methods

The growth of mouse fibroblast L-929 cells, the preparation of Reovirus RNA transcriptase, the synthesis *in vitro* of Reovirus mRNA, and the purification of rat liver tRNA$_F^{Met}$ have been described [1, 2].

2.1. *Treatment of ribosomal subunits with 1 M KCl*

The isolation and separation of L-cell 40 S and 60 S subunits in 0.5 M KCl have been described [1]. For treatment of these particles with 1 M KCl, 20 A_{260} units of each subunit were diluted to 1 ml in 50 mM triethanolamine-HCl (pH 7.6)–1 M KCl–1.8 mM $MgCl_2$ (increased to 3 mM for 60 S). The 40 S suspension was incubated at 27° for 40 min, the 60 S suspension at 18° for 5 min. Each suspension was then centrifuged at 18° through separate 30 ml 10–30% sucrose density gradients in 50 mM triethanolamine (pH 7.6)–500 mM KCl–5 mM $MgCl_2$ for 5 hr at 64 000 *g* [1, 3]. The subunit peaks were collected and stored as described for the 0.5 M KCl subunits [1].

2.2. *Preparation of ribosomal factors*

A 1 M KCl extract of L-cell ribosomes prepared as previously described [1] was diluted in RSB [2] to 0.3 M KCl and passed through DEAE-cellulose in the cold in the same buffer–salt solution. The effluent protein peak (monitored at A_{280}) was concentrated by ammonium sulfate precipitation, dissolved in 30 mM Tris-HCl (pH 7.5)–100 mM KCl–1 mM $MgCl_2$–2 mM dithiothreitol and dialyzed against the same buffer for 90 min. Debris was removed by low-speed centrifugation and the extract was stored at –80°.

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Table 1
Requirements for methionyl-puromycin synthesis.

Additions	Methionine released
Complete	0.28
–mRNA	0.04
–factors	0.11
–GTP	0.06
–GTP + GDPCP*	0.04

Reaction mixtures contained the following components as indicated in a final volume of 100 μl: 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 0.4 mM GTP, 0.6 mM GDPCP, 1 mM puromycin-HCl, 0.6 A_{260} units of treated 40 S subunits, 1.2 A_{260} units of treated 60 S subunits, 0.6 A_{260} units of formaldehyde treated Reovirus mRNA [1, 4], 20 pmoles of [^{35}S]Met-tRNA$_F$ (4500 cpm/pmole), and 26 μg of crude ribosome factors. Incubation was at 37° for 30 min. [^{35}S]Met-puromycin was extracted into ethylacetate and counted as previously described [1, 5]. Values are corrected for controls incubated and extracted in the absence of puromycin.

* β,γ-methylene guanosine triphosphate.

3. Results

As previously indicated, ribosomal subunits prepared from L-cells and separated in 0.5 M KCl do not require exogenous GTP or ribosomal factors for the formation of a protein initiation complex containing Reovirus mRNA. However, differential treatment of each subunit fraction with 1 M KCl (see Methods) yields particles which are GTP- and factor-dependent during the formation of the initiation complex as measured by Met-puromycin synthesis (table 1). The inability of GDPCP to replace GTP (table 1) suggests that GTP hydrolysis is an essential property of the system. The factors utilized in these experiments were extracted from crude L-cell ribosomes by a 1 M KCl wash (see Methods). Conditions for treatment of the subunits with 1 M KCl were determined empirically. Whereas, the 40 S subunit could withstand prolonged exposure to 1 M KCl without loss of activity, the 60 S subunit was relatively sensitive to 1 M KCl and rapidly gave rise to a 58 S subunit which in the presence of factors was active in initiation and a 47 S particle

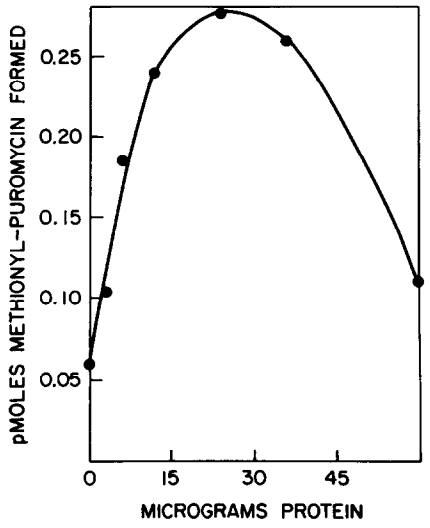

Fig. 1. Dependence of methionyl-puromycin synthesis on ribosome factor concentration. Incubation and assay descriptions for each point are as described in table 1. Each value is corrected for controls as described in table 1.

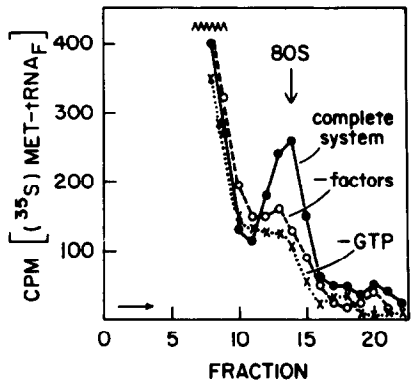

Fig. 2. Sucrose gradients of initiation complex with 1 M KCl-treated ribosomal subunits. Incubation components and conditions are as described in table 1 except that puromycin was omitted. After 30 min at 37°, the mixture was layered on 5 ml of a 10–30% sucrose gradient in 20 mM Tris-HCl (pH 7.5)–100 mM KCl–5 mM $MgCl_2$ and centrifuged for 75 min at 148 000 *g* at 0°. Gradients were monitored at A_{260} and collected and radioactivity in each fraction determined as previously described [1]. Complete system (●—●—●); minus factors (○- - -○- - -○); minus GTP (X··X··X). Arrow (lower left) denotes direction of sedimentation.

which was inactive. Treatment of either subunit with 1 M KCl created a requirement for exogenous ribosomal factors suggesting that each subunit contains at least one factor component essential for inititaion. As measured by Met-puromycin synthesis, the extent of complex formation was dependent upon ribosome factor concentrations (fig. 1). At optimal factor concentrations, Met-puromycin synthesis was 5-fold over background values obtained in the absence of factors. Initiation was relatively sensitive to factor concentration; high concentrations were inhibitory (fig. 1).

The GTP and ribosomal factor requirements during initiation could also be demonstrated by analysis in sucrose gradients (fig. 2). Utilizing 40 S and 60 S subunits treated with 1 M KCl, the binding of [^{35}S]Met-tRNA$_F$ in a complex at the 80 S region was dependent upon the addition of exogenous GTP and initiation factors. Omission of either component yielded little or no binding of the initiator Met-tRNA$_F$ (fig. 2). The absolute dependence of the formation of the 80 S complex on the presence of Reovirus mRNA (fig. 2) has also been previously demonstrated with subunits prepared in 0.5 M KCl which require no exogenous factors or GTP [1].

4. Discussion

We have examined the requirements for ribosomal factors and GTP in a specific eukaryotic initiation system utilizing Reovirus mRNA synthesized *in vitro* by the particulate RNA transcriptase of Reovirus cores, and purified L-cell 40 S and 60 S ribosomal subunits. Complex assembly is specific for Met-tRNA$_F$, and is dependent upon mRNA, ribosomal factors, and GTP. In the defined system, no requirement for any supernatant factor was detected. The data confirm a previous finding that L-cell 40 S and 60 S ribosomal subunits prepared and separated in 0.5 M KCl retain their initiation factors [1]. In that respect, the L-cell subunits differ, for example, from reticulocyte subunits prepared in 0.5 M KCl which manifest factor [6, 7] and GTP [8] requirements during initiation. In the present studies, factor-dependency was established only when the subunits were separately treated with 1 M KCl. This procedure also introduced a GTP-dependency which supports the hypothesis that some GTP remains bound to L-cell subunits prepared in 0.5 M KCl. The observed GTP-dependency is in agreement with results in other eukaryotic systems [9, 10]. Fractionation of the initiation factors in the crude ribosomal factor preparation has enabled us to separate some of the steps in the initiation process of this system. These studies will be reported subsequently.

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References

[1] D.H. Levin, D. Kyner and G. Acs, Proc. Natl. Acad. Sci. U.S. 69 (1972) 1234.
[2] D.H. Levin, N. Mendelsohn, M. Schonberg, H. Klett, S. Silverstein, A.M. Kapuler and G. Acs, Proc. Natl. Acad. Sci. U.S. 66 (1970) 890.
[3] G. Blobel and D. Sabatini, Proc. Natl. Acad. Sci. U.S. 68 (1971) 390.
[4] D.H. Levin, D. Kyner and G. Acs, Biochem. Biophys. Res. Commun. 42 (1971) 454.
[5] J.P. Leis and E.B. Keller, Biochem. Biophys. Res. Commun. 40 (1970) 416.
[6] D.A. Shafritz, D.G. Laycock and W.F. Anderson, Proc. Natl. Acad. Sci. U.S. 68 (1971) 496.
[7] C.L. Woodley, Y.C. Chen, K.K. Bose and N.K. Gupta, Biochem. Biophys. Res. Commun. 46 (1972) 839.
[8] R.G. Crystal and W.F. Anderson, Proc. Natl. Acad. Sci. U.S. 69 (1972) 706.
[9] S. Heywood and W.C. Thompson, Biochem. Biophys. Res. Commun. 43 (1971) 470.
[10] A. Burgess and B. Mach, Nature 233 (1971) 209.