Use of Purified Polysomes from Rabbit Reticulocytes in a Specific Test for Initiation Factors
(protein synthesis/native subunits)

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ABSTRACT Initiation factors, extracted from ribosomal particles by high salt, are usually tested for with the extracted ribosomes. However, it is theoretically possible that such tests may respond not only to factors (i.e., proteins that join and leave a ribosome during its cycle), but also to true ribosomal proteins that may have been extracted. We have eliminated this circularity by tests with purified polysomes, which have not been extracted with high salt; the polysomes are deficient in initiation factors because they have been largely separated from the native subunits. Tests with both systems, applied to extracts of different classes of particles, suggest that high salt does extract both initiation factors and essential ribosomal proteins. Extracts of native subunits contain both, and stimulate both systems; while, in the presence of these extracts, the extracts of polysomes further stimulate only the extracted ribosomes.

Initiation factors are obtained by extraction of ribosomal particles with high salt (e.g., 0.5 M NH₄Cl or KCl) and are then generally detected by their ability to restore the capacity of the extracted ribosomes to initiate translation of natural messenger or to bind fMet-tRNAf. This approach is subject to the criticism that high-salt washing might remove essential ribosomal proteins as well as initiation factors. Indeed, at least one ribosomal protein in *Escherichia coli*, S12, is known to be required for physiological initiation [but not for translation of poly(U)] (1), and thus might mimic an initiation factor. Moreover, with extracted 30S subunits the addition of ribosomal protein S2, S3, or S14 stimulates fMet-tRNAf binding (2). It is therefore clear that a ribosome-bound protein is not adequately established as an initiation factor by showing that it is required for initiation by salt-washed ribosomes. A true factor, in contrast to a ribosomal protein, also joins and leaves the ribosome during its cycle (3), and it should be active with ribosomes that have not been subjected to high-salt extraction.

Both these additional criteria have been met for the three known initiation factors of *E. coli*: in cell lysates they are found only on the native small subunit (4, 5) and they have been shown to be released during its incorporation into a polysomal ribosome (6–8); moreover, they stimulate initiation by purified polysomes (9) that have not been extracted with salt. But with various eukaryotic cells, in which salt-extractable ribosome-bound proteins are also required for initiation (10–12), the evidence that these are true factors is incomplete. Their distribution among the various ribosomal particles has not been determined, and their requirement has been demonstrated only with extracted ribosomes. A ribosome dissociation factor in rabbit reticulocytes, which is found only on native subunits (13), is presumably an additional initiation factor, since it is distinct from initiation factors M1, M2A, M2B, and M3 (14); but its role in initiation cannot be tested until the other factors have been more completely purified.

It has been shown with bacteria that ribosomes can be freed of initiation factors not only by salt extraction but also by separating the native subunits from the polysomes, whether by gradient sedimentation or by gel filtration (9); and these procedures should be less threatening than high-salt extraction for the retention of ribosomal proteins. This paper compares such purified polysomes from rabbit reticulocytes with salt-extracted ribosomes, in tests for initiation factor activity. The results suggest that the active components detected with salt-washed ribosomes include one or more ribosomal proteins, as well as true initiation factors.

MATERIALS AND METHODS

The total ribosomes from rabbit reticulocyte lysates were extracted with 0.5 M KCl as described by Gilbert and Anderson (15). Extracts of polysomes, 80S ribosomes, the two native subunits were prepared as described (13), except that dialysis was omitted. The small subunit fraction was essentially pure, but the large subunits were visibly contaminated with small subunits.

To isolate polysomes for use in protein synthesis, we layered 1 ml of lysate on a 4-ml sucrose gradient (15–30%) in 10 mM Tris·HCl (pH 7.4)–10 mM KCl–1.5 mM MgCl₂–2 mM dithiothreitol. After centrifugation for 90 min at 45,000 rpm in the Spinco SW 50.1 rotor the pellets, containing most of the polysomes but virtually no subunits, were resuspended in standard sucrose (0.25 M sucrose–0.1 mM EDTA at pH 7.0–2 mM dithiothreitol) to 250 A₂₅₀ units/ml and were stored in small portions at –70°. The experiment of Fig. 6 also used polysomes that had been extracted with 0.5 M KCl (see legend).

For protein synthesis the source of supernatant enzymes and tRNA was the 40–70% ammonium sulfate cut (AS 40–70) of a post-ribosomal supernate, prepared as described by Allen and Schweet (16) except that the proteamine sulfate step was omitted. Amino-acid incorporation mixtures (25 μl) contained, unless otherwise indicated, 0.15 A₂₅₀ units of polysomes, 5 μl of AS 40-70, 1 mM dithiothreitol, 0.05 mM of each [¹⁴C]amino acid except valine, 0.02 mM [¹⁴C]valine (66 mCi/mmol), 1 mM ATP, 0.2 mM GTP, 3 mM phosphoenolpyruvate, 0.3 units pyruvate kinase, 80 mM KCl, 20 mM Tris·HCl (pH 7.4), 3 mM MgCl₂, and 4 μl of ribosomal
extract. After incubation for 1 hr at 37° (unless otherwise indicated) 1 mL of 6% trichloroacetic acid with 1% Casamino acids was added and the mixtures were heated for 15 min at 90°. The precipitates were collected on Millipore filters, which were washed twice with 1 mL of 5% trichloroacetic acid, dried, and counted in 5 mL of toluene-based scintillation fluid in a Nuclear Chicago Mark I scintillation counter at 70% efficiency, or, when indicated, in a Nuclear-Chicago gas flow counter at 11% efficiency.

[14C]Valine was obtained from New England Nuclear Corp. GTP and ATP Tris salt from Sigma; and phosphoenolpyruvate (potassium salt), dithiothreitol, and pyruvate kinase from Calbiochem. All other chemicals used were reagent grade.

RESULTS

Deficient Initiation by Isolated Polysomes. Fig. 1 shows a typical profile of polysomes isolated from reticulocyte lysates by pelleting through a sucrose gradient (see Materials and Methods). These preparations were not as devoid of reinitiation activity as those from bacteria (prepared by filtration through Sepharose, ref. 9), for 50% of their amino-acid incorporation was inhibited by aurintricarboxylate (Fig. 2), a specific inhibitor of physiological initiation (17). However, addition of a high-salt ribosomal extract (without aurintricarboxylate) approximately doubled the incorporation. This effect evidently depended on remedying a deficiency in initiation factors, for the incremental incorporation was entirely inhibitable by aurintricarboxylate (Fig. 2), the duration of incorporation was prolonged (Fig. 3), and the Mg++-dependency curve showed a sharp optimum at 3 mM Mg++ in the presence of the extract but was much broader in its absence (Fig. 4). [Both in reticulocytes (18) and in E. coli (9),

![Graph](image-url)

**Fig. 1.** Absorbance profile of isolated polysomes. Polysomes were isolated as described in Methods. 0.5 A254 units was layered on a 4-mL sucrose gradient (15–30%) above an 0.8-mL sucrose cushion (45%), both containing 10 mM Tris·HCl (pH 7.4), 100 mM KCl, and 3 mM MgCl2. Centrifugation was for 45 min. at 45,000 rpm in the Spinco SW 50.1 rotor. In all figures, the convention is that the experimental cpm have been multiplied by the factor in parentheses to give the numbers on the ordinates.

![Graph](image-url)

**Fig. 2.** The effect of aurintricarboxylate (ATA) on incorporation of amino acids by isolated polysomes in the presence or absence of an optimal concentration of high-salt extract. Reaction mixtures as in Methods (except with 0.5 A254 units of polysomes) were incubated with ATA at the concentration indicated. The Mg++ optimum for polypeptide elongation is higher than that for initiation.

Initiation Factor Content of Various Ribosomal Fractions. Since the isolated polysomes were deficient in (though not devoid of) initiation activity, it appears that the native subunits in reticulocytes do contain initiation factors that are released in the course of forming polysomes. To test this inference more directly we separated polysomes, 80S ribosomes, and a mixture of native subunits in a sucrose gradient (see Materials and Methods), and a high-salt extract of each fraction was tested for total initiation factor activity with purified polysomes.

The results showed that reticulocytes do indeed contain initiation factors specifically associated with native subunits.

![Graph](image-url)

**Fig. 3.** Time curve of amino-acid incorporation by isolated polysomes. Reaction mixtures (100 µl) as in Methods, with or without high-salt extract, were incubated at 37°. At the times indicated 15-µl samples were withdrawn, precipitated with trichloroacetic acid, and analyzed in a gas-flow counter as in Methods.
As Fig. 5 shows, the mixed native subunits yielded several times as much activity (per $A_{260}$ unit) as the 80S fraction, while the polysomal fraction was relatively inactive. Any mRNA that may have been present evidently did not contribute to the observed responses, since these were not reduced by treatment of the various extracts with DEAE-cellulose (a treatment described in Table 1).

The mixture of native subunits was further separated into two fractions, containing predominantly small or large subunits. Extracts of these fractions exhibited similar amounts of initiation factor activity (Table 1). To determine whether these extracts contained the same or different initiation factors, we tested them for additivity or synergism in mixtures. The results were not conclusive (about 20% synergism: Table 1), perhaps because different factors may have been limiting in different tests. However, further observations showed that the ratio of alpha to beta globin chain synthesis was much lower with the small subunit extract than with the large subunit extract (unpublished data), which suggests that the two subunits may carry different factors, with possible regulatory significance.

Polysomal Extracts Specifically Stimulate Synthesis by Extracted Ribosomes. If high salt can extract ribosomal proteins, as well as initiation factors, an extract of native subunits (or of the total pellet) should contain both, but an extract of polysomes should contain only the former. Unextracted ribosomes might therefore be expected to respond only to the subunit extract, while extracted ribosomes might well be further stimulated by the ribosomal proteins present in a polysomal extract.

To test for such selective stimulation, we added initiation factors as an extract of small subunits, which should have a high ratio of initiation factors to ribosomal protein. As Fig. 6 shows, in the presence of small amounts of this extract the polysome extract did stimulate the activity of the extracted ribosomes but not that of the purified polysomes. This stimulation is not seen in the presence of larger amounts of the subunit extract (Fig. 6), which evidently provide enough ribosomal protein to saturate the system. These findings support the theoretical prediction that ribosomal proteins, as well as true initiation factors, might be contributing to the response of extracted ribosomes to their own extract. However, definitive proof will require tests for the distribution of individual proteins among the ribosomal particles and tests for their activity with extracted and unextracted ribosomes.

The ribosomes used in this experiment had been exposed to high salt for only 10 min in order to preserve the bound hnRNA. Their response to the polysomal extract is unlikely to be due to its content of mRNA, since the response was eliminated by a large amount of 40S subunit extract, which should supply ribosomal proteins (as well as initiation factors) but not mRNA. Ribosomes that had been exposed to high salt for 30 min exhibited a greater dependence upon polysomal extract; perhaps this reflects more complete removal of either the loose ribosomal proteins or the mRNA.

**DISCUSSION**

In tests for the initiation factor activity of high-salt extracts, the salt-extracted ribosomes generally used might well be responding to extracted ribosomal proteins as well as to initiation factors. We have therefore tested for initiation factors with unextracted, purified endogenous polysomes. This system
is shown to be stimulated by high-salt extracts of the total ribosomes of rabbit reticulocytes, and the incremental synthesis results entirely from increased reinitiation, as shown by kinetics, Mg++, optimum, and inhibition by aurantricarboxylate (Figs. 2–4).

Since the test polysomes should not require ribosomal proteins, their response to the high-salt extracts supports the earlier conclusion that these extracts contain true initiation factors. Moreover, like the initiation factors of E. coli (4, 5), at least some of the stimulating factors are present only on the native subunits, for extracts of the subunit fraction were much more active than those of the 80S and the polysome fractions. [Our data cannot exclude the possibility that additional initiation factors are present in the post-ribosomal supernate in reticulocytes, as in supernates from certain other eukaryotes (wheat embryos, ref 20; Artemia, ref. 21).] The slight activity of the polysomes may be largely due to the presence of 40S initiation complexes, which are observed in polysomes (as 11/2-somes) under certain conditions (19). The activity of the 80S fraction (Fig. 5) may not be entirely due to contamination with 50S subunits, but a definitive explanation must await analyses for individual factors.

In contrast to E. coli, in which the initiation factors are associated only with small native subunits (4, 5), with reticulocytes the initiation factor activity (for purified polysomes) was recovered from both subunits. This distribution cannot be readily explained by contamination of either preparation with the complementary subunits, for the two extracts were about equally active (Table 1). Since overall initiation factor activity, in a system involving multiple factors, cannot be interpreted with certainty, the apparent activity of both subunits requires further study, with tests for individual factors. However, it seems likely that each kind of native subunit in mammalian cells carries specific initiation factor(s), for the two subunits of rabbit reticulocytes each appear to carry a specific dissociation factor (22). Moreover, if either the small or the large subunits of mouse L cells are washed with high salt and mixed with the complementary unwashed subunits, a requirement for an initiation factor is seen (12).

Though it is clear that high-salt extracts of the total ribosomes contain true initiation factors, they also appear to contain essential ribosomal proteins. Extracts of polysomes, which lack initiation factors, stimulated extracted ribosomes but not unextracted polysomes, in the presence of initiation factors from the small subunits (Fig. 6). The extracted ribosomal proteins evidently arise primarily from the small subunits, for an excess of extract from an essentially pure preparation of these subunits caused the same stimulation as the extract of polysomes.

Hirsch et al. have observed that the small subunits derived from Ehrlich ascites cell ribosomes at high salt have about 700,000 daltons less protein than the native small subunits (23), while the derived and native large subunits show no significant difference (24). However, the sum of the molecular masses of 1F-M1, 1F-M2A, 1F-M2B, and 1F-M3 from rabbit reticulocytes is 1.1 × 10^6 daltons (25); the dissociation factor removed by high salt from the subunits would add further

Table 1. Initiation factor activity of 40S and 60S extracts

<table>
<thead>
<tr>
<th>Extract added (μl)</th>
<th>Incorporation of amino acids</th>
<th>% of expected</th>
</tr>
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<tbody>
<tr>
<td>40S 60S</td>
<td>Observed (cpm)</td>
<td>Expected if additive (cpm)</td>
</tr>
<tr>
<td>2  —</td>
<td>671</td>
<td>—</td>
</tr>
<tr>
<td>4  —</td>
<td>1169</td>
<td>—</td>
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<td>—</td>
</tr>
<tr>
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<tr>
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<td>3965</td>
<td>3493</td>
</tr>
</tbody>
</table>

Extracts were prepared from the native 40S and 60S fractions as described in Methods, except that the fractions were separated by zonal centrifugation in a Spinco Ti 14 rotor. The extracts were batch-treated with DEAE-cellulose at 0.35 M KCl to remove nucleic acids. Incorporation of amino acids was measured as in Methods, except that the final volume of the mixtures was 50 μl instead of 25 μl. Because of this dilution of the polysomes and the added extract, the activities are not comparable to those in the Figures. 40S extract (100 μl) was obtained from 7 A_150 units, and 100 μl of 60S extract from 15 A_150 units. Incorporation in the absence of extract (2541 cpm) has been subtracted. The expected incorporation with each mixture was calculated from the curves for the incorporation by each of the separate components.

Fig. 6. Effect of extract of polysomes on amino-acid incorporation in the presence of initiation factors. Extracted and unextracted polysomes were prepared as described in Methods, except that the former were exposed for 10 min to 0.5 M KCl, while the control preparation was washed with standard sucrose. The polysomes were then pelleted, resuspended in standard sucrose, and stored in small portions at −70°C. Incorporation was measured as described in Methods. 40S extract (100 μl) was obtained from 1.13 A_150 units of native 40S particles, and 100 μl of polysome extract was obtained from 4.9 A_150 units of polysomes. λ = μl.
mass (13). [Schreier and Staehelin (11) have also separated four initiation proteins from rabbit reticulocyte ribosomes, and one of these (IF-Eq), like IF-M3, was reported to exceed 600,000 daltons.] This discrepancy in mass suggests that one or more of the reported reticulocyte initiation factors may be a ribosomal protein, and our findings on the stimulatory effect of extracts of polysome, in tests with salt-washed ribosomes but not with purified polysomes, support the same theoretical possibility.

It is hoped that this preliminary investigation will encourage tests with individual "factors" to see whether they are present only in native subunits or also in polysomes. The purified polysomes described here may also be useful in distinguishing true factors from extracted ribosomal proteins; but their value is limited, so far, by their high background activity, which is only doubled or tripled by an optimal supply of factors.

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