INITIATION FACTOR PROMOTED REASSOCIATION OF EUKARYOTIC RIBOSOMAL SUBUNITS

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SUMMARY

A factor present in rat liver cytosol caused 40S and 60S subunits to reassociate, forming 80S ribosomes. Reassociation required this factor, an RNA template (poly U), and aminoacyl-tRNA (Phe-tRNA); it was enhanced by GTP. Factor-promoted reassociation was not inhibited by puromycin.

Initiation of protein synthesis in bacteria requires the formation of an initiation complex containing the 30S ribosomal subunit, mRNA (with an AUG or GUG codon at or near the 5' end), fmet-tRNA, and initiation factors (1). Less is known about the process in animal cells, although there are indications that it is similar. The aminoacyl-tRNA required for initiation is met-$tRNA_f$, which, however, is not formylated (2-6); there are specific initiation factors (7, 8); and, most likely, a complex is formed between the factors, the initiator aminoacyl-tRNA, mRNA, and the 40S subunit of the ribosome (8).

Rat liver cytosol contains a factor (40S binding factor - 40S BF) which catalyzes poly U-dependent binding of Phe-tRNA to the smaller (40S) subunit of rat muscle (or liver) ribosomes in low concentrations of magnesium (9). The characteristics of the reaction suggest it is a paradigm for the initiation of cellular protein synthesis. This suggestion is supported by the finding that two of the three initiation factors required for de novo synthesis of hemoglobin by reticulocyte ribosomes in vitro are also required

for the synthesis of polyphenylalanine at low concentrations of magnesium (10). If the 40S BF is in fact an initiation factor, then it might catalyze the reassociation of ribosomal subunits. We have now tested this possibility.

MATERIALS AND METHODS

Rat liver ribosomal subunits were prepared by a modification (11) of a method described previously (12), dialyzed against Buffer A (10 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 80 mM KCl; 10 mM β-mercaptoethanol (MSH)), and concentrated by ultrafiltration. Ribosomal subunits were incubated for 15 min at 30° in 0.4 ml of Buffer B (10 mM Tris-HCl, pH 7.6; 3.5 mM $MgCl_2$; 120 mM KCl; 10 mM MSH), together with various substrates---the exact conditions are given in the legends to the figures. After incubation, 0.3 ml of the reaction mixture was layered on a 10-30% linear sucrose gradient in Buffer B (but with no MSH) and centrifuged at 4° for 90 min in a Spinco SW65 rotor at 60,000 rpm. The distribution of ribosomal material in the gradient was determined using an ISCO density gradient analyzer (12). In some experiments gradient fractions (0.25 ml) were collected in glass vials and the volume adjusted to 1 ml with water; 10 ml of Triton-toluene scintillation fluid (13) were added and the radioactivity was measured in a Packard TriCarb spectrometer; the counting efficiency was 18%.

RESULTS AND DISCUSSION

Our first task was to determine conditions in which ribosomal subunits alone did not reassociate, but in which the 40S BF was still active. We knew (12) that raising the concentration of potassium or lowering that of magnesium inhibited reassociation. There was considerable formation of 80S ribosomes when the concentrations of magnesium and potassium were 5 and 80 mM respectively (Buffer A)---results not shown, but there was no 80S formation when the concentrations were 3.5 and 120 mM (Buffer B)---Fig. 1a. Since appreciable activity of the 40S BF

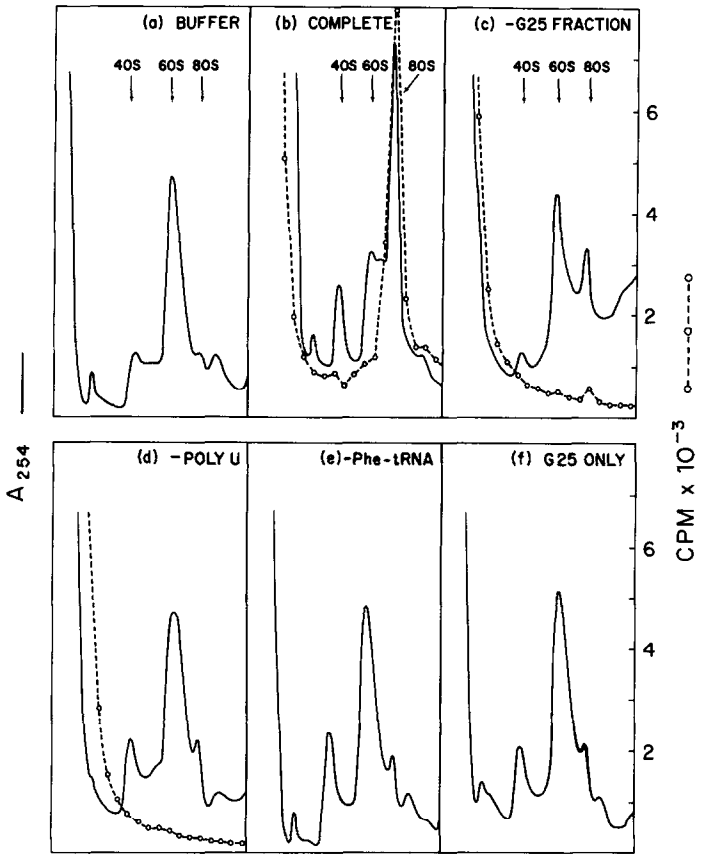

Figure 1. Reassociation of ribosomal subunits catalyzed by G-25 fraction. Ribosomal subunits (40S, 10.7 μg of RNA; 60S, 26.8 μg of RNA) were incubated for 15 min at 30° in 0.4 ml of Buffer B containing: 160 μg [^{3}H] Phe-tRNA (120,000 cpm); 40 μg poly U; 0.2 μmole GTP; 1.1 mg G-25 fraction (9). In some experiments one or another component of the mixture was omitted. After incubation, samples (0.3 ml) were analyzed by centrifugation on 10-30% linear sucrose gradients; in some cases fractions were collected and the radioactivity assayed.

occurs in Buffer B (80% of that in Buffer A - see reference 9), this is a suitable medium for investigating formation of an initiation complex and factor-catalyzed reassociation of ribosomal subunits.

The formation of 80S ribosomes took place when ribosomal subunits were incubated in Buffer B with poly U, Phe-tRNA*, GTP and a crude preparation of 40S BF (G-25 fraction)---

* Unfractionated E. coli B tRNA acylated with 20 amino acids including [^{3}H] phenylalanine (5 Ci/mmole).

Fig. 1b. Reassociation was dependent on the presence of template (poly U) and Phe-tRNA (Fig. 1d and 1e). A puzzling observation was that deacylated-tRNA could substitute for Phe-tRNA. It is worth noting here that Culp, et al. (14) have data which suggest deacylated-tRNA may be required to initiate globin peptide synthesis. In the absence of the G-25 fraction (Fig. 1c), or if that fraction was heated to 55°, subunit reassociation was considerably less extensive than in the complete system. Omission of GTP reduced reassociation by 40% (results not shown), just as it reduces binding of Phe-tRNA to the 40S subunit (9).

We should point out that exact quantitation of reassociation is difficult. Firstly, there is a tendency for subunits to aggregate in Buffer B (see Fig. 1a where a portion of the 40S subunits have formed 55S particles). Secondly, the G-25 fraction reduces aggregation of the 40S subunit (compare Fig. 1a and 1f).

In the complete system (Fig. 1b), the radioactivity from [^{3}H] phenylalanyl-tRNA co-sedimented with the 80S ribosome. In a separate experiment this radioactive material was shown

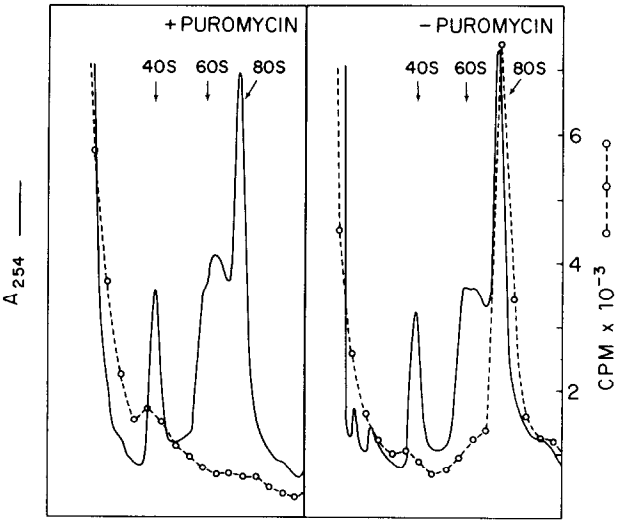

Figure 2. The effect of puromycin on reassociation of ribosomal subunits. The conditions of incubation and analysis were the same as in Fig. 1 except that in one experiment 0.05 μmole of puromycin was added to the reaction mixture.

to be insoluble in hot trichloroacetic acid, indicating that the synthesis of polyphenylalanine had occurred. (The G-25 fraction contains aminoacyltransferases I and II---T-I and T-II.) Nonetheless, polypeptide synthesis is not required for reassociation, since 80S ribosomes were formed in the reaction catalyzed by 40S BF even in the presence of sufficient puromycin to prevent the synthesis of protein (Fig. 2).

A purified preparation of 40S BF, freed from T-I by successive chromatography on hydroxylapatite and DEAE-cellulose, catalyzed reassociation (Fig. 3), whereas a purified preparation of T-I (15) did not (results not shown). Thus the reassociation activity in the G-25 fraction is not due to T-I, just as the 40S binding activity of G-25 can not be attributed to T-I (9). It is worth noting that when reassociation was catalyzed by purified 40S BF no radioactive material insoluble in hot trichloroacetic acid co-sedimented with the 80S ribosomes (Fig. 3).

In our analysis of the reassociation mixture on sucrose gradients we have noted a particle that sediments in a

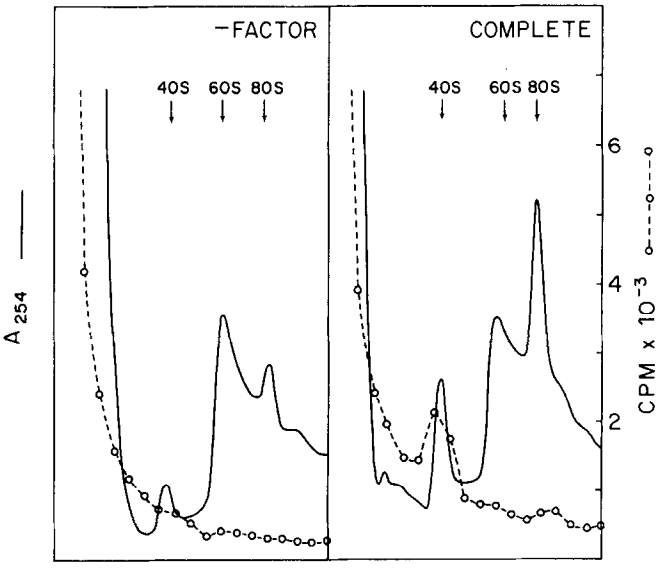

Figure 3. Reassociation of ribosomal subunits catalyzed by purified 40S BF. The conditions of incubation and analysis were the same as in Fig. 1 except that 0.18 mg of purified 40S BF was used instead of the G-25 fraction.

position between the 60S subunit and the 80S ribosome---best seen in figure 2. There are reports of ribosomes and ribosomal subunits whose conformations are altered during dissociation and reassociation (16-18). It is possible that the particle we observe is analogous to one of these.

The reassociation of ribosome subunits can be catalyzed by a factor present in rat liver cytosol. The reaction requires mRNA, aminoacyl-tRNA, 40S BF and to a limited degree GTP. These requirements are similar to those for the binding of aminoacyl-tRNA to the 40S subunit (9), and since both reactions are catalyzed by purified preparations of 40S BF it is likely they are causally related. The results accord with the suggestion (19, 20) that protein synthesis in animal cells is initiated on the 40S subunit.

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REFERENCES

1. Guthrie, C., and Nomura, M., Nature 219, 232 (1968).
2. Smith, A. E., and Marcker, K. A., Nature 226, 607 (1970).
3. Brown, J. C., and Smith, A. E., Nature 226, 610 (1970).
4. Jackson, R., and Hunter, T., Nature 227, 672 (1970).
5. Housman, D., Jacobs-Lorena, M., Rajbhandary, U. L., and Lodish, H. F., Nature 227, 913 (1970).
6. Wigle, D. T., and Dixon, G. H., Nature 227, 676 (1970).
7. Prichard, P. M., Gilbert, J. M., Shafritz, D. A., and Anderson, W. F., Nature 226, 511 (1970).
8. Heywood, S. M., Nature 225, 696 (1970).
9. Leader, D. P., Wool, I. G., and Castles, J. J., Proc. Natl. Acad. Sci. 67, 523 (1970).
10. Shafritz, D. A., and Anderson, W. F., J. Biol. Chem. 245, 5553 (1970).
11. Stirewalt, W. S., Castles, J. J., and Wool, I. G., Biochemistry, in press (1971).
12. Martin, T. E., Rolleston, F. S., Low, R. B., and Wool, I. G., J. Mol. Biol. 43, 135 (1969).
13. Patterson, H. S., and Greene, R. C., Anal. Chem. 37, 854 (1965).
14. Culp, W., Morrisey, J., and Hardesty, B., Biochem. Biophys. Res. Comm. 40, 777 (1970).

15. Schneir, M., and Moldave, K., Biochim. Biophys. Acta 166, 58 (1968).
16. Talens, J., Kalousek, F., and Bosch, L., FEBS Letters 12, 4 (1970).
17. van Diggelen, O. P., Heinsius, H. L., Kalousek, F., and Bosch, L., J. Mol. Biol. 55, 277 (1971).
18. Schreier, M. H., and Noll, H., Nature 227, 128 (1970).
19. Adamson, S. D., Howard, G. A., and Herbert, E., Cold Spring Harbor Symp. Quant. Biol. 34, 547 (1969).
20. Kaempfer, R., Nature 222, 950 (1969).