Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3435–3439, September 1974

Use of Eukaryotic Native Small Ribosomal Subunits for the Translation of Globin Messenger RNA

(rabbit reticulocytes/rat liver polysomes/Krebs ascites pH 5 supernatant)

CHRISTOPH FREIENSTEIN AND GÜNTER BLOBEL

The Rockefeller University, New York, N.Y. 10021

Communicated by George E. Palade, June 24, 1974

ABSTRACT **A highly active *in vitro* system for the translation of globin mRNA, resulting in more than 10 rounds of translation, is described. The reconstituted system consists of native small ribosomal subunits of rabbit reticulocytes (as a source of initiation factors as well as small ribosomal subunits), large subunits derived from rat liver polysomes by the puromycin–KCl procedure, and a pH 5 fraction obtained from a Krebs ascites cell high speed supernatant. In this system no differences were found between globin messenger ribonucleoprotein and globin mRNA.**

The special role which native ribosomal subunits play in the initiation of polypeptide synthesis *in vitro* was first recognized by Bishop (1). He showed that native ribosomal subunits present in reticulocyte lysates enhance the capacity of isolated reticulocyte polysomes to initiate globin chain synthesis. Subsequently it was shown that native subunits isolated from *Escherichia coli* were more active in viral RNA-dependent polypeptide synthesis than the 70S monomeric ribosomes, and that the high activity of the native ribosomal subunits was due to the localization of initiation factors on the small subunits (2, 3). In the case of eukaryotic native small subunits, they were found to be associated with additional non-ribosomal protein, up to 7×10^5 daltons per subunit (4), Met-$tRNA_f$ (5) and mRNA (6). Recently, salt extracts from native small subunits of reticulocytes have been shown to result in initiation of polypeptide synthesis when added to a polypeptide-synthesizing system containing purified reticulocyte polysomes (7).

In this paper we show that the native small ribosomal subunit isolated from rabbit reticulocytes can be used as a convenient source of initiation factors; together with large ribosomal subunits obtained by dissociation of rat liver polysomes by the puromycin–KCl procedure (8) and a pH 5 fraction obtained from a Krebs ascites cell high speed supernatant, they constitute a system that can translate globin messenger ribonucleoprotein (mRNP) more than 10 times.

METHODS

Translation of Reticulocytes from Anemic Rabbits. Rabbits were made anemic by consecutive injections of neutralized phenylhydrazine (9). A few minutes before the blood was collected by heart puncture the rabbit was injected intravenously with 2 ml of cycloheximide (10 mg/ml). The same amount of cycloheximide was also present in the beaker into which up to 100 ml of blood was collected. All further operations were performed in the cold (2–4°). Washed reticulocytes were resuspended at a concentration of 1 ml of packed cells per 3.0 ml of lysis buffer: 10 mM Tris·HCl (pH 7.5 at 20°)–10 mM KCl–1.5 mM $MgCl_2$–2 mM dithiothreitol, and homogenized with one stroke in a Potter-Elvehjem homogenizer. The homogenate was adjusted to 100 mM KCl, 20 mM Tris·HCl (pH 7.5), 3 mM $MgCl_2$, and 2 mM dithiothreitol with a 10-fold concentrated solution and centrifuged for 10 min at 20,000 × g_{av}. Aliquots (8 ml) of the resulting postmitochondrial supernatant were centrifuged two or three times in a Spinco no. 40 rotor at 40,000 rpm. First there was a 30-min centrifugation, yielding a 30-min pellet and a 30-min S100, followed by a 2.5-hr centrifugation of the 30-min S100 to give a 2.5-hr pellet and a 2.5-hr S100; occasionally the 2.5-hr S100 was centrifuged once more for 12 hr to yield a 12-hr pellet and a 12-hr S100, or the 8-ml aliquot of the postmitochondrial supernatant was centrifuged only once for 3 hr to sediment the bulk of the ribosomes (referred to as total ribosomes). The pellets were kept frozen at −80° until used. The rationale for this centrifugation scheme was to separate the bulk of the polysomes, subsequently used for the preparation of mRNP from the bulk of the native subunits (see Fig. 1).

Preparation of Native Small Subunits (S^N). Pellets (2.5-hr) were resuspended in double distilled water to a concentration of 100–200 A_{260} units per ml; 50–100 A_{260} units were layered on 12.5 ml of 10–30% sucrose gradients in 100 mM KCl–20 mM triethanolamine·HCl (pH 7.5 at 20°)–3 mM $MgCl_2$–2 mM dithiothreitol. The gradients were centrifuged in an SB283 rotor of an IEC centrifuge for 3 hr at 39,000 rpm (190,000 × g_{av}). The S^N peak was collected and subsequently sedimented in a Spinco no. 40 rotor for 12 hr at 40,000 rpm. The yield from 100 A_{260} units of a 2.5-hr pellet was approximately 5 A_{260} units of S^N. The pellets could be stored frozen at −80° for at least a month without loss in activity.

Preparation of a pH 5 Fraction from a Krebs Ascites Cell High-Speed Supernatant. Fractionation of Krebs ascites cells grown in the peritoneal cavity of mice was identical to that described above for reticulocytes. A pH 5 fraction was prepared as described by Falvey and Staehelin (10). In brief, a 2.5-hr S100 (see above) was diluted with 2 volumes of cold double-distilled water. Acetic acid (1.0 M) was added dropwise with constant stirring until the pH decreased to 5.1. The cloudy solution was centrifuged for 10 min at 10,000 × g_{av} and the pellet was resuspended in a solution of 100 mM KCl–20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; mRNP, messenger ribonucleoprotein.

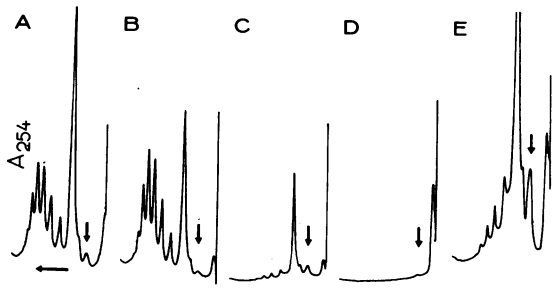

FIG. 1. Sedimentation profiles of fractions isolated by several consecutive differential centrifugations from a postmitochondrial supernatant. All pellet fractions were derived from 8 ml of postmitochondrial supernatant (see *Methods*). The pellets were resuspended in 2.0 ml of ice-cold double-distilled water. Aliquots were layered on 12.5 ml of 10–40% sucrose gradients in 100 mM KCl–20 mM triethanolamine·HCl (pH 7.5)–3 mM $MgCl_2$, and 2 mM dithiothreitol. The gradients were centrifuged at 4° in an SB283 rotor of an IEC centrifuge for 100 min at 39,000 rpm. Fractionation of the sucrose gradients and recording of the optical density at 254 nm were as described (8). For comparison of the resuspended pellets, either equal volumes (A, B, C, D) or equal A_{260} (B, E) were layered. (A) Total ribosomes (50 μl = 3.5 A_{260}); (B) 30-min pellet (50 μl = 2.5 A_{260}); (C) 2.5-hr pellet (50 μl = 0.5 A_{260}); (D) 12-hr pellet (50 μl = 0.2 A_{260}); (E) 2.5-hr pellet (250 μl = 2.5 A_{260}). Vertical arrows point to native small ribosomal subunits and the horizontal arrow indicates direction of sedimentation.

(Hepes)·KOH (pH 7.3)–3 mM $MgCl_2$–2 mM dithiothreitol in a volume corresponding to about $^1/_5$ of the original 2.5-hr S100. The pH was adjusted to 7.3 by the addition of 1.0 M KOH. Insoluble material was removed by centrifugation for 10 min at 1000 × g_{av}. One-milliliter portions of this pH 5 fraction were stored frozen at −80° until used. The protein concentration in the pH 5 fraction varied from preparation to preparation between 6–8 mg/ml.

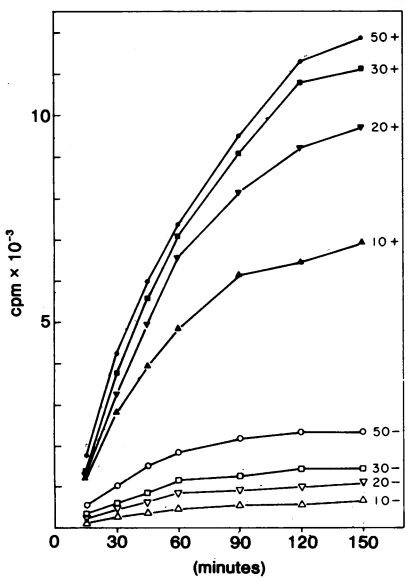

FIG. 2. Time course of polypeptide synthesis at various concentrations of S^N in the absence (−) and presence (+) of added globin mRNP. Numbers refer to μl of S^N (13.36 A_{260} unit/ml) present in 250 μl of reaction mixture; the latter also contained: pH 5 fraction (90 μl) and globin mRNP (10 μl = 0.013 A_{260} units) or water; cpm shown here and in following figures are those in 25-μl aliquots; background (time course of incorporation in the absence of S^N and globin mRNP) was subtracted; at 150-min time point, e.g., it amounted to less than 300 cpm.

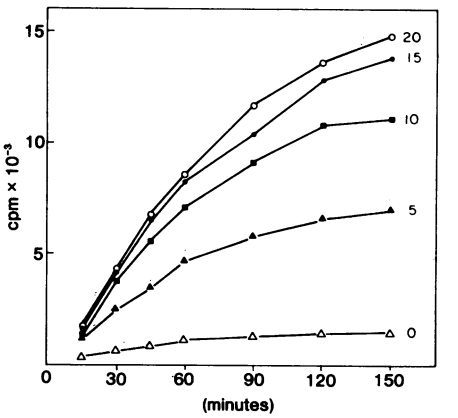

FIG. 3. Time course of polypeptide synthesis in the absence or presence of various amounts of added globin mRNP. Numbers refer to μl of globin mRNP (1.3 A_{260} unit/ml) present in 250 μl of reaction mixture; the latter also contained pH 5 fraction (90 μl) and S^N (30 μl = 0.402 A_{260} unit).

Preparation of large subunits (L°) from rat liver polysomes as well as globin mRNP from salt washed 30-min pellets was by the puromycin-KCl method as described previously (8, 11, 12). Globin mRNA was prepared from globin mRNP by treatment with sodium dodecyl sulfate and subsequent gradient centrifugation (12).

In Vitro Polypeptide Synthesis. The reaction mixture (250 μl) contained: 25 μmol of KCl, 5 μmol of Hepes·KOH (pH 7.3 at 20°), 0.75 μmol of $MgCl_2$, 0.5 μmol of dithiothreitol, 0.25 μmol of ATP, 0.05 μmol of GTP, 1.5 μmol of creatine phosphate, a few crystals of creatine phosphokinase, 7.5 nmol of each L-amino acid except for leucine, 0.5 μCi of [^{14}C]leucine [specific activity 312 Ci/mol (Fig. 4) or 161 Ci/mol (Figs. 2, 3, and 5)] and S^N, pH 5 fraction and globin mRNP or globin mRNA as specified in figure legends. All assays contained L° in three times the amount (A_{260}) of S^N. Globin mRNP and mRNA pellets stored at −80° were resuspended

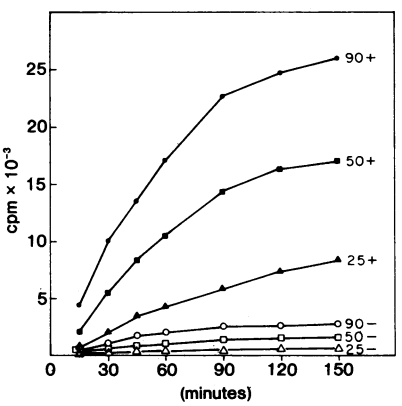

FIG. 4. Time course of polypeptide synthesis in the absence (−) and presence (+) of globin mRNP at various amounts of pH 5 fraction. Numbers refer to μl of pH 5 fraction present in 250 μl of reaction mixture; the latter also contained S^N (50 μl = 0.307 A_{260} unit) and globin mRNP (10 μl = 0.013 A_{260} unit) or water.

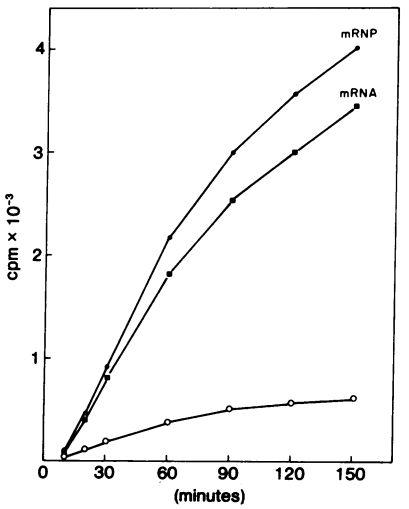

FIG. 5. Time course of polypeptide synthesis in the absence and in the presence of either globin mRNP or globin mRNA. Each 250-μl reaction mixture contained: 50 μl of pH 5 fraction, 10 μl of S^N (10.8 A_{260} units/ml), and either 10 μl of H_2O or 10 μl of globin mRNP = 0.0026 A_{260} unit) or 10 μl of globin mRNA = 0.0026 A_{260} unit).

in double-distilled water before use. The solutions of L°, globin mRNP and mRNA could be stored frozen at −80° and thawed for several times without loss of activity. No similar tests were performed on S^N solutions. Incubation was at 37°. Twenty-five microliter samples were removed at indicated time intervals and spotted on 3 MM Whatman filter paper disks which were processed according to Mans and Novelli (13). Radioactivity was determined in toluene–Liquifluor in a Beckman LS 350 liquid scintillation counter at about 75% efficiency.

Source of Materials. ATP, disodium salt (A3127); GTP, sodium salt (G8877); creatine phosphate, disodium salt (P6502), and creatine phosphokinase, salt-free powder (C-3755) from Sigma Chemical Co., St. Louis, Mo; L-[^{14}C]leucine (3122-41), specific activity 312 Ci/mol from Schwarz/Mann, Orangeburg, N.Y. Numbers in brackets refer to catalogue numbers.

RESULTS

A postmitochondrial supernatant from a reticulocyte lysate was fractionated by several consecutive differential centrifugations. Fig. 1 shows the sedimentation profiles of these fractions in sucrose gradients. The bulk of the polysomes can be found in the 30-min pellet (Fig. 1B), while the bulk of the native small subunit can be found in the 2.5-hr pellet (Fig. 1C and E). The 2.5-hr S100 is essentially free of ribosomes (Fig. 1D). The distribution between polysomes, monomers and native subunits in the total ribosome fraction is shown in Fig. 1A.

The isolated native small ribosomal subunits, together with the large ribosomal subunits derived from rat liver polysomes by the puromycin-KCl procedure and a pH 5 fraction prepared from a Krebs ascites cell high-speed supernatant are able to translate globin mRNP as well as globin mRNA (Figs. 2–5, and Table 1). The concentrations of KCl and of $MgCl_2$ were varied (data not shown) and found to be optimal as given in *Methods*. The following experiments were designed to investigate the response of this system to various concentrations of native small subunits, globin mRNP, or pH 5 fraction.

TABLE 1. *Rounds of translation per globin mRNP and rate of globin synthesis. Data from Figs. 2, 3, and 4.*

	S^N/mRNP (mol)	Leucine[a] (pmol)	Leucine/mRNP (mol)	Rounds of translation per mRNP[b]	pmol of leucine[c]/min	Time for synthesis of one globin chain[d] (min)
A. ΔS^N (Fig. 2)						
S^N (pmol)						
8	3.5	231	101	5.7	3.6	4.9
16	7.0	314	138	7.9	4.1	4.3
24	10.5	357	157	8.9	5.2	3.4
40	17.5	352	154	8.8	5.1	3.4
B. ΔmRNP (Fig. 3)						
mRNP (pmol)						
1.14	21.0	206	181	10.3	2.6	6.8
2.28	10.5	357	157	9.0	5.2	3.4
3.42	7.0	456	133	7.6	5.4	3.2
4.56	5.3	494	108	6.2	5.6	3.1
C. ΔpH 5 fraction (Fig. 4)						
pH 5 fraction (μl)						
25	8.1	128	56	3.2	1.5	11.7
50	8.1	297	130	7.4	3.9	4.5
90	8.1	446	196	11.2	6.3	2.8

[a] pmol of hot-acid-insoluble leucine in 250 μl of reaction mixture were calculated from cpm at the 150-min time point in the presence of globin mRNP, minus cpm at 150-min time point in the absence of globin mRNP. 1000 cpm = 3.7 pmol of leucine in Figs. 2 and 3, 1000 cpm = 1.93 pmol of leucine in Fig. 4 (see *Methods*).

[b] There are 17.5 mol of leucine per mol of globin chain.

[c] pmol leucine at the 30 min time point, minus pmol leucine at the 15-min time point, divided by 15.

[d] This calculation is based on the assumption that the increase in cpm between the 15-min and 30-min time points is due to radioactivity released from the ribosomes, i.e., represents finished globin chains.

Other numbers used in calculations were: 0.01 A_{260} unit of globin mRNP = 1.75 pmol; 1.0 A_{260} unit of S^N = 60 pmol.

Fig. 2 shows the time course of polypeptide synthesis when the concentration of native small subunits is varied in the presence of a constant amount of pH 5 fraction and in the absence or presence of a constant amount of globin mRNP. There was polypeptide synthesis in the absence of added globin mRNP. This synthesis is due to messenger activity present in the S^N fraction and increases linearly with increasing amounts of S^N (see open symbols in Fig. 2). Synthesis stimulated by globin mRNP also increases with increasing amounts of S^N but reaches a plateau at a molar ratio of about 10.5 S^N/globin mRNP (see Table 1). Replacement of native small subunits by small subunits derived from total reticulocyte ribosomes by puromycin–KCl (8) resulted in a loss of activity of the system (data not shown).

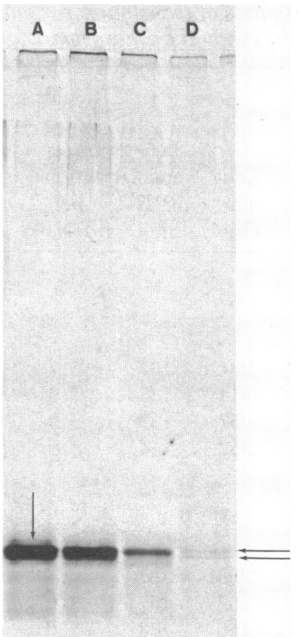

FIG. 6. Analysis of the product of *in vitro* polypeptide synthesis by polyacrylamide gradient gel electrophoresis in sodium dodecyl sulfate and subsequent radioautography of the dried gels. Fifty-microliter aliquots were removed after 150 min of incubation of the reaction mixture for *in vitro* polypeptide synthesis (see *Methods* and Fig. 4) and were precipitated with 1 ml of 10% trichloroacetic acid at 0°. The precipitate was resuspended by brief sonication in 25 μl of 30% sucrose containing traces of bromophenol blue. Sufficient 1.0 M Tris base was added until the solution turned blue; this was followed by the addition of 5 μl of a 25% solution of sodium dodecyl sulfate and 1 μl of a 1.0 M solution of dithiothreitol, boiling at 100° for 2 min, and cooling to room temperature. Two microliters of a 1.0 M solution of α-iodoacetamide was added; incubation at room temperature was for at least 2 hr, with maintenance of the blue color of the sample by further addition of Tris base, if necessary. All of the material was used for electrophoresis, which was performed as described by Maizel (15) with a gel slab apparatus and a 10–20% acrylamide resolving gel (1 mm thick). After electrophoresis the slabs were stained with 0.2% Coomassie blue in 50% methanol, 10% acetic acid, and destained in 20% methanol, 10% acetic acid. Gels were dried and exposed to medical x-ray film (Cronex $2_C{}^D$, E. I. Du Pont de Nemours & Co., Wilmington, Del.). Slots A to D: 50-μl aliquots derived from reaction mixtures described in Fig. 4; Slot A: 90 μl of pH 5 + 10 μl of mRNP; Slot B: 50 μl of pH 5 fraction + 10 μl of mRNP; Slot C: 25 μl of pH 5 fraction + 10 μl of mRNP; Slot D: 90 μl of pH 5 fraction without added mRNP. Horizontal arrows point to α and β globin chains; vertical arrow points to a band with a mobility slower than that of the β chain of globin (see *text*).

The time course of polypeptide synthesis at various concentrations of added globin mRNP but constant amounts of S^N and pH 5 fraction, is shown in Fig. 3. Increasing amounts of added globin mRNP resulted in increased polypeptide synthesis. However, there was a leveling-off at higher mRNP concentrations.

Finally, the concentration of pH 5 fraction was varied, while the amount of S^N and added globin mRNP was kept constant. It can be seen from Fig. 4 and Table 1 that both initial rates and final levels of synthesis stimulated by globin mRNP are linearly proportional to the amount of pH 5 fraction. Thus, the amount of pH 5 fraction is limiting in the performance of this system. At the highest concentration of pH 5 fraction, globin mRNP was translated more than 10 times with initial rates of less than three minutes for the synthesis of 1 globin chain.

Since mRNP obtained by the puromycin–KCl method has been shown to consist of mRNA with two proteins attached to it (12, 14), it was conceivable that the presence of protein on the globin mRNA has some modifying influence in our system. However, the results shown in Fig. 5 demonstrate that there was no significant difference between globin mRNP and globin mRNA. The slightly lower activity of the latter may have been the result of some degradation of the mRNA after the deproteinization of mRNP.

A product analysis from a few aliquots of the *in vitro* reaction mixture (see Fig. 4) was performed by electrophoresis in polyacrylamide gels and subsequent radioautography of the dried gels (Fig. 6). The translation products of the endogenous messenger activity present in S^N are composed of two bands of equal specific activity (Fig. 6, slot D, horizontal arrows). These two bands can be tentatively identified as the α and β chain of globin (the faster moving band corresponding to the α chain, the slower to the β chain), since authentic globin in the same gel also separates into two equally stained bands with identical mobilities. The translation products of added globin mRNP (Fig. 6, slots A–C) are somewhat more complex. At lower translation rates (see Fig. 4) only the β chain band is visible (slot C) while at higher rates the α chain band is made also. In addition to the two globin bands there are some minor bands which, with the exception of one band (vertical arrow, slot A), move faster. The radioactivity distribution between the faster moving bands, the two globin bands and the slower moving band in slot B was determined and found to be 15%, 80%, and 5%, respectively. Since the faster moving bands may represent unfinished globin chains, as much as 95% of the synthesized products may represent completed globin chains and globin chain fragments.

DISCUSSION

Our system to translate globin mRNP, consisting of native small ribosomal subunits (S^N) from reticulocytes, rat liver large ribosomal subunits, and a pH 5 fraction from Krebs ascites cell high-speed supernatant, is similar to the reconstituted heterologous system described by Schreier and Staehelin (16). We also obtained a similar efficiency of mRNA translation with respect to rate and rounds of translation. The main difference of our system is the use of *native* small ribosomal subunits from reticulocytes serving both as a source of initiation factors and as small ribosomal subunits, while Schreier and Staehelin (16) used *derived* small ribosomal subunits obtained by salt dissociation of ribosomes and an initiation factor fraction extracted by salt from total reticulocyte ribosomes. While a separation of individual initiation factors is absolutely essential for the study of the function of each factor, for the mere purpose of translation of mRNA the use of a complex of small ribosomal subunits and initiation factors is a less time consuming and a more practical procedure. Beyond that, our results indicate that the initiation factors usually extracted from total reticulocyte ribosomes by salt are in fact localized exclusively in the small amount of native small ribosomal subunits contained in the total crude ribosome fraction. Since crude ribosomes are known to contain substantial amounts of nonspecifically adsorbed cellular

proteins, our results suggest that a separation of native small subunits from crude ribosomes as a first step may facilitate subsequent purification of initiation factors by eliminating most of the cellular proteins adsorbed to the bulk of the ribosomes. In support of this contention are our preliminary experiments in which polyacrylamide gel electrophoresis in sodium dodecyl sulfate of salt extracts from native small ribosomal subunits resulted in about ten distinct nonribosomal bands as compared to many more bands obtained from salt extracts of crude ribosomes (in preparation).

The choice of components for our reconstituted heterologous system was arbitrary and based on the demonstration by Schreier and Staehelin (16) that interspecies reconstitution is possible. Thus it should be possible to use large ribosomal subunits from other species. The pH 5 fraction of ascites cells was chosen because of its known low endogenous mRNA activity. Preliminary experiments indicate that native small ribosomal subunits from rat liver as well as from ascites cells can replace those of reticulocytes, indicating that they too contain initiation factors. However, they also contain mRNA activity, which due to its heterogeneity results in the production of many polypeptide chains, while the endogenous activity of S^N from reticulocytes is restricted to two polypeptide chains. In the latter case the products of other exogenous mRNAs which may be translatable in this system could be distinguished more readily.

It should be noted that both the rate of synthesis and the number of translation cycles in our reconstituted system is still one order of magnitude less than those calculated for intact reticulocytes (17) or a reticulocyte lysate (18, 19). However, the observed linear increase in these parameters as a function of the amount of added pH 5 fraction indicated that some factors other than the initiation factors contained in the native small ribosomal subunit are rate-limiting.

1. Bishop, J. O. (1966) *Biochim. Biophys. Acta* **119**, 130–145.
2. Eisenstadt, J. M. & Brawerman, G. (1967) *Proc. Nat. Acad. Sci. USA* **58**, 1560–1565.
3. Parenti-Rosina, R., Eisenstadt, A. & Eisenstadt, J. M. (1969) *Nature* **221**, 363–365.
4. Hirsch, C. A., Cox, M. A., Venrooij, W. & Henshaw, E. C. (1973) *J. Biol. Chem.* **248**, 4377–4385.
5. Darnbrough, C., Hunt, T. & Jackson, R. J. (1972) *Biochem. Biophys. Res. Commun.* **48**, 1556–1564.
6. Darnbrough, C., Legon, S., Hunt, T. & Jackson, R. J. (1973) *J. Mol. Biol.* **76**, 369–403.
7. Lubsen, N. H. & Davis, B. D. (1974) *Proc. Nat. Acad. Sci. USA* **71**, 68–72.
8. Blobel, G. & Sabatini, D. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 390–394.
9. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1952) *J. Biol. Chem.* **196**, 669–694.
10. Falvey, A. K. & Staehelin, T. (1970) *J. Mol. Biol.* **53**, 1–19.
11. Blobel, G. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 832–835.
12. Blobel, G. (1972) *Biochem. Biophys. Res. Commun.* **47**, 88–95.
13. Mans, R. J. & Novelli, G. D. (1971) *Arch. Biochem. Biophys.* **94**, 48–53.
14. Blobel, G. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 924–928.
15. Maizel, J. V. (1969) in *Fundamental Techniques in Virology*, eds. Habel, K. & Salzman, N. P. (Academic Press, New York), Chap. 32, p. 334.
16. Schreier, M. H. & Staehelin, T. (1973) *J. Mol. Biol.* **73**, 329–349.
17. Knopf, P. M. & Lamfrom, H. (1965) *Biochim. Biophys. Acta* **95**, 398–407.
18. Adamson, S. D., Howard, G. A. & Herbert, E. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34**, 547–554.
19. Hunt, T., Vanderhoff, G. & London, O. M. (1972) *J. Mol. Biol.* **66**, 471–481.