A Factor Converting Monoribosomes into Polyribosomes during Protein Synthesis *in vitro*

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An extract was prepared from rabbit reticulocyte ribosomes after treatment with potassium chloride as described previously (Miller, Hamada, Yang, Cohen & Schweet, 1967). The participation of the extract in cell-free protein synthesis was studied. Purified polyribosomes were isolated and converted into monoribosomes by incubation in the cell-free protein-synthesis system. The monoribosomes were isolated and found to be unable to synthesize protein in the cell-free system. The addition of the ribosomal extract to the system stimulated protein synthesis. This was accompanied by the conversion of some of the monoribosomes into polyribosomes. The active component or components of the extract were shown to be protein.

In two systems, fractions that will stimulate cell-free protein synthesis have been prepared from ribosomes after treatment with a relatively high concentration of chloride ion. First, two different factors have been isolated from *Escherichia coli* ribosomes (Stanley, Salas, Wahba & Ochoa, 1966). These factors have been partially purified and were reported to be proteins that stimulate polypeptide chain initiation. Secondly, a protein-containing fraction has been isolated from rabbit reticulocyte ribosomes (Miller, Hamada, Yang, Cohen & Schweet, 1967). This fraction stimulates protein synthesis in systems containing ribosomes that have been inactivated by chloride treatment or by preincubation in the cell-free protein-synthesizing system. In the reticulocyte system it was shown that the fraction in question does not stimulate by contributing m-RNA* to the system. Thus it follows that messenger must still be present on at least some of the inactivated ribosomes.

This paper describes experiments with a fraction prepared from reticulocyte ribosomes by the method described by Miller *et al.* (1967). The results support the conclusions of Miller *et al.* (1967) that the activating component or components is not m-RNA but protein.

Results are also presented that show the participation of the fraction in cell-free protein synthesis. These results show that during cell-free protein synthesis polyribosomes break down into monoribosomes, which are inactive in protein synthesis. These can then be reactivated by the addition of the

fraction obtained from ribosomes after treatment with potassium chloride. Further, some of these monoribosomes are converted back into polyribosomes during protein synthesis.

MATERIALS AND METHODS

Trypsin (3 × crystallized) and soya-bean trypsin inhibitor (crystalline) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Ribonuclease A (bovine pancreas; 5 × crystallized) was obtained from Sigma (London) Chemical Co. Ltd., London, S.W. 6. Before use, these chemicals were dissolved in 10 mM-tris-HCl buffer, pH 7.5 at 22°.

All of the operations were carried out at or near 0° except where stated.

Preparation of polyribosomes. A clarified lysate was prepared from rabbit reticulocytes as described by Allen & Schweet (1962). The lysate was centrifuged at 78000g_{av} for 90 min. in the no. 30 rotor of the Spinco preparative ultracentrifuge; the supernatant was removed and used later to prepare an enzyme fraction. The ribosomal pellets were rinsed with 0.1 M-sucrose and resuspended in the same solution to give a final ribosome concentration of 30–50 mg./ml. The solution was adjusted to 0.07 M-KCl, 3.5 mM-MgCl₂ and 35 mM-tris-HCl buffer, pH 7.5 at 22°, i.e. the same salts concentration as the cell-free protein-synthesis reaction mixture. Three 1.5 ml. samples were applied to 28 ml. 10–23% (w/v) sucrose gradients adjusted to the same salts concentration, and centrifuged at 63500g_{av} for 5 hr. 40 min. in the SW25 rotor of the Spinco preparative ultracentrifuge. The pellets together with the bottom 1.5 ml. of each gradient were pooled and gently homogenized. This yielded 5 ml. of polyribosomes containing 14 mg./ml. from an initial volume of 200 ml. of clarified lysate.

Preparation of ribosomal extract and washed ribosomes. The resuspended 90 min. pellet described above was

* Abbreviations: m-RNA, messenger RNA; s-RNA, soluble RNA.

adjusted to 0.6 M-KCl by the addition of 2 M-KCl and kept for 30 min. at 0°. The mixture was centrifuged at 150 000 g_{av} . for 2 hr. to remove the ribosomes and the supernatant retained (designated 'ribosomal extract'). The protein concentration of the extract varied considerably (15–40 mg./ml.) among preparations, because of contamination with haemoglobin. The ribosomal pellet was rinsed and resuspended in 0.15 M-sucrose and the ribosomal concentration adjusted to about 20 mg./ml. (designated 'washed ribosomes').

Amino acid incorporation. The reaction mixture used was essentially that described by Allen & Schweet (1962) with the following differences. The preparation of the enzyme fraction was slightly modified in that the supernatant obtained after 90 min. centrifugation of the clarified cell lysate was centrifuged again for 4 hr. and the protamine sulphate treatment was altered to 0.07 mg./ml. of lysate. s-RNA was prepared from yeast (Monier, Stephenson & Zamecnik, 1960) instead of from reticulocytes.

The reaction mixture finally contained (per ml.): ATP, 0.71 μ mole; GTP, 0.18 μ mole; creatine phosphate, 7.1 μ moles; tris-HCl buffer, pH 7.5 at 22°, 35.7 μ moles; GSH, 14.3 μ moles; $MgCl_2$, 3.6 μ moles; KCl, 69 μ moles; 20 L-amino acids, 0.036 μ mole of each; s-RNA, 71 μ g.; creatine kinase, 21 μ g.; enzyme fraction, 5 mg. Ribosomes, ribosomal factors and L-[^{14}C]valine were added where indicated. The mixture was incubated at 37° and synthesis was terminated by chilling in ice. The incorporation of L-[^{14}C]valine into polypeptide was determined by the method of Bollum (1966). The counting efficiency obtained was 65–70%. A background of about 65 counts/min. was subtracted from all samples counted.

Degradation of polyribosomes. Polyribosomes (56 mg.) were incubated in 11.2 ml. of the above incubation mixture for 90 min. Samples (0.4 ml.) were withdrawn after 0, 10 and 90 min. for analysis in sucrose gradients. The rest was centrifuged at 150 000 g_{av} . for 90 min. and the supernatant discarded. The pellet was rinsed and resuspended in 0.15 M-sucrose and the ribosomal concentration adjusted to about 20 mg./ml.

Measurement of ribosomal concentrations. All ribosomal concentrations were estimated by taking $E_{1cm}^{1\%}$ 120 at 260 m μ .

Measurement of protein concentrations. Protein concentrations were measured by the method of Warburg & Christian (1941).

Determination of sedimentation coefficients. $S_{20,w}$ values of ribosomes in sucrose gradients were determined by the method of McEwen (1967), assuming a particle density of 1.4 (Noll, 1967).

RESULTS

Preincubation of ribosomal extract with ribonuclease. Ribosomal extracts were preincubated with low concentrations of ribonuclease and then assayed in the cell-free system with washed ribosomes (Fig. 1). The addition of high concentrations of ribonuclease (over 0.3 μ g./ml.) to a cell-free system containing ribosomal extract and washed ribosomes stopped synthesis completely. At lower concentrations the amount of synthesis was only

Fig. 1. Effect of ribonuclease on ribosomal extract. Samples (0.03 ml.) of ribosomal extract were incubated with 0.002 μ g. (○) or 0.01 μ g. (△) of ribonuclease in a final volume of 0.05 ml. After the times indicated the tubes were chilled and the components of the cell-free incubation mixture added along with 0.25 mg. of washed ribosomes and L-[^{14}C]valine of specific radioactivity 12 c/mole to give a final volume of 0.35 ml. The tubes were reincubated at 37° for 1 hr. Samples (0.1 ml.) were removed and the incorporation was measured. Appropriate controls are indicated in the Figure: ●, ribonuclease omitted; ----, ribosomal extract and ribonuclease omitted. The highest point in the Figure corresponds to an incorporation of 6.6 μ moles of valine/mg. of ribosomes.

diminished. The amount of amino acid incorporation was not significantly decreased by preincubating the ribosomal extract with the ribonuclease. The simplest explanation of this result is that RNA is not an active component of the ribosomal extract.

Preincubation of ribosomal extract with trypsin. Ribosomal extracts were preincubated with trypsin and the tryptic activity was stopped by the addition of trypsin inhibitor. The extracts along with controls were then assayed in the cell-free system with washed ribosomes (Fig. 2). The activity of the ribosomal extract was destroyed by trypsin. Thus the active component (or components) in the ribosomal extract is protein or partly protein in nature.

Fig. 2. Effect of trypsin on ribosomal extract. Samples (0.03 ml.) of ribosomal extract and 0.1 μ g. of trypsin (●) in a final volume of 0.05 ml. were incubated at 37°. After the times indicated 0.05 ml. of soya-bean trypsin inhibitor at a concentration of 2 mg./ml. was added and the tubes were chilled. Washed ribosomes (0.25 mg.), L-[¹⁴C]valine of specific radioactivity 12 c/mole and the other components of the cell-free incubation mixture were added and the tubes reincubated at 37° for 1 hr. Samples (0.1 ml.) were removed and the incorporation was measured. Appropriate controls are indicated in the Figure: ●, trypsin omitted; ▲, trypsin and trypsin inhibitor omitted; ---, washed ribosomes alone present. The highest value in the Figure corresponds to an incorporation of 11 m μ moles of valine/mg. of ribosomes.

Fig. 3. Separation of polyribosomes by sucrose-density-gradient centrifugation. A 1.5 ml. sample of the resuspended 90 min. ribosomal pellet was sedimented on a 28 ml. 10–23% (w/v) sucrose gradient as described in the Materials and Methods section. After sedimentation, the gradient was eluted at a constant rate and monitored at 260 m μ in a Unicam SP.800 spectrophotometer fitted with an LKB 4711A–3 flow cell.

Fig. 4. Amino acid incorporation by polyribosomes. Ten 0.35 ml. samples of the standard amino acid-incorporation mixture were incubated at 37°. In addition, the mixture contained L-[¹⁴C]valine of specific radioactivity 6 c/mole and 0.72 mg. of polyribosomes/ml. At the times indicated the tubes were chilled and 0.1 ml. samples were removed for estimating the radioactive incorporation. The incorporation shown after 90 min. corresponds to 1.95 m μ moles of valine/mg. of ribosomes.

Fig. 5. Sedimentation of incubated polyribosomes through sucrose density gradients. Polyribosomes (56 mg.) were incubated in 11.2 ml. of the standard incubation mixture. After 0 min. (---), 10 min. (.....) and 90 min. (—) samples (0.4 ml.) were withdrawn and chilled. Then 0.1 ml. of each sample was placed on a 4.9 ml. 15–30% (w/v) sucrose gradient containing 0.07 M-KCl, 3.5 mM-MgCl₂ and 35 mM-tris-HCl buffer, pH 7.5 at 22°. The gradients were centrifuged at 125 000 g_{av} for 1 hr. in the 3 × 5 ml. swinging-bucket rotor of the MSE Superspeed 50 centrifuge. After centrifugation, 70% (w/v) sucrose (containing haemoglobin as a visual aid) was pumped at a constant rate through the bottom of the tube so that the lightest fractions were collected first. The gradient was monitored at 260 m μ as described in Fig. 1.

Table 1. *Effect of ribosomal factors on protein synthesis by degraded polyribosomes*

Samples (0.35 ml.) of the standard amino acid mixture were incubated at 37° for 1 hr. In addition, the reaction mixture contained L-[¹⁴C]valine of specific radioactivity 12c/mole. Where added, the reaction mixture contained 0.114 ml. of ribosomal factors and polyribosomes degraded by 90 min. preincubation as described in the Materials and Methods section. Samples (0.1 ml.) were removed and the incorporation was measured. Samples of the reaction mixtures with the addition of only ribosomal factors gave less than 10 counts/min. Additional data are given in Fig. 6.

	Incorporation (mμmoles of valine/mg. of ribosomes)
Preparation 1	
Degraded polyribosomes (1.6 mg./ml. of reaction mixture)	0.01
Degraded polyribosomes + ribosomal factors batch A	0.90
Degraded polyribosomes + ribosomal factors batch B	0.63
Preparation 2	
Degraded polyribosomes (1.0 mg./ml. of reaction mixture)	0.05
Degraded polyribosomes + ribosomal factors batch A	1.60
Degraded polyribosomes + ribosomal factors batch B	1.21

Polyribosome degradation. Fig. 3 shows the separation of polyribosomes from other ribosomal components after centrifugation through a sucrose gradient. After centrifugation, only the pellet and bottom 1.5 ml. of the gradient were collected, to minimize contamination by monoribosomes. Thus the polyribosome fraction taken immediately after separation contains virtually no monoribosomes.

Cell-free protein synthesis by the purified polyribosomes is shown in Fig. 4. Synthesis has virtually stopped after 90 min. The change in ribosomal pattern during such an incubation is shown in Fig. 5. There is a progressive breakdown of polyribosomes into monoribosomes and perhaps a small amount of smaller units. At 90 min., when synthesis is ended, there are essentially no polyribosomes.

Stimulation by ribosomal extract. The results in Table 1 and Fig. 6 show that ribosomal extract will stimulate systems containing polyribosomes that have been broken down and inactivated by preincubation. The classes of ribosomes present after incubation times of 0, 4 and 8 min. are shown in Fig. 7. Fig. 7 also shows those ribosomes that are active in synthesizing protein. Initially there are no polyribosomes present, nor is there any incorporation. After 4 min. amino acid incorporation was into the polyribosome region and into released protein. This was accompanied by an increase in the extinction in the polyribosome region of the gradient. Incorporation into monoribosomes was minimal by comparison. After 8 min. a similar result was obtained.

An experiment was carried out to confirm that monoribosomes were the active component in degraded polyribosomes. Degraded polyribosomes were sedimented through a sucrose gradient and

Fig. 6. Amino acid incorporation by degraded polyribosomes. Six 0.35 ml. samples of the standard amino acid-incorporation mixture were incubated at 37°. In addition, the mixture contained L-[¹⁴C]valine of specific radioactivity 100c/mole, 2.4 mg. of degraded polyribosomes/ml. and 0.115 ml. of ribosomal factors/ml. At the times indicated the tubes were chilled and 0.05 ml. samples removed for counting as described in Fig. 4. The incorporation shown after 90 min. corresponds to 1.17 mμmoles of valine/mg. of ribosomes.

the fractions assayed in the cell-free system in the presence of ribosomal factors (Fig. 8). Only the monoribosome fractions showed amino acid incorporation.

Fig. 7. Sedimentation through sucrose gradients of degraded polyribosomes after incubation with ribosomal factors in the amino acid-incorporation system. Three 0.22 ml. samples of the standard incorporation mixture were incubated at 37°. In addition, the mixture contained L-[^{14}C]valine of specific radioactivity 2110 c/mole, 2.2 mg. of degraded polyribosomes/ml. and 0.115 ml. of ribosomal factors/ml. After 0 min. (a), 4 min. (b) and 8 min. (c) the tubes were chilled and 0.15 ml. of each was applied to a 4.6 ml. salts-adjusted 10–23% (w/v) sucrose gradient as described in Fig. 5. After centrifugation at 125 000 g_{av} for 85 min., the gradients were monitored and fractionated as described in Fig. 5. Six-drop fractions were collected and the radioactivity incorporated into protein was measured. —, E_{260} ; ----, radioactivity.

DISCUSSION

It is apparent from these results that a proportion of the monoribosomes, derived *in vitro* from polyribosomes, can be converted back into polyribosomes under the influence of ribosomal extract. Further, it is principally only the reconstituted polyribosomes and not the unconverted monoribosomes that can participate in protein synthesis. In the experiments reported by Miller *et al.* (1967), unfractionated potassium chloride-washed ribo-

somes were used. Washed ribosomes are prepared from a very heterogeneous ribosomal population consisting of polyribosomes, monoribosomes with attached endogenous m-RNA, monoribosomes without m-RNA but capable of attaching to m-RNA, inert monoribosomes and a small amount of sub-units, which makes relationships between the different components difficult to investigate. The experiments reported here eliminate these complications since pure polyribosomes, degraded to monoribosomes under near-physiological condi-

Fig. 8. Amino acid incorporation with degraded polyribosomes after sedimentation through a sucrose density gradient. Degraded polyribosomes were adjusted to 0.07 M-KCl, 3.5 mM-MgCl₂ and 35 mM-tris-HCl buffer, pH 7.5 at 22°. A portion (0.6 ml.) was placed on a 19 ml. 10–23% (w/v) sucrose gradient of the same salts concentration. The gradient was centrifuged at 95000g_{av} for 1 hr. in the 3 × 23 ml. swinging-bucket rotor of the MSE Super-speed 65 centrifuge. After centrifugation, 23 samples (30 drops each) were collected. Samples of each fraction were diluted and the extinction was measured at 260 mμ. A portion (0.1 ml.) of each fraction was added to the cell-free incubation mixture to give a final volume of 0.35 ml. In addition the reaction mixture contained 0.03 ml. of ribosomal extract and L-[¹⁴C]valine of specific radioactivity 120/mole. The tubes were incubated at 37° for 60 min., samples (0.1 ml.) were removed and the incorporation was measured. Samples of reaction mixtures without the addition of ribosomal extract gave less than 10 counts/min. The highest point in the Figure corresponds to an incorporation of 0.48 mμmole of valine/mg. of ribosomes. ●, E₂₆₀; ○, radioactivity.

tions, as distinct from 0.6 M-potassium chloride treatment, were used.

The treatment of ribosomal extracts with ribonuclease or trypsin suggests that the activity is not RNA but protein in nature. Nevertheless,

explanations involving m-RNA-protein complexes might still be valid, although unlikely, since in experiments with ribosomal factors and washed ribosomes from different genera the type of haemoglobin synthesized was dependent on the type of ribosomes and not on the extract (Miller *et al.* 1967). This would confirm that m-RNA is not an active component of the ribosomal extract.

One obvious question that arises is why only some of the monoribosomes are converted. Likely explanations are that not enough monoribosomes have an active m-RNA attached to them, or that not enough ribosomal extract was added, or both.

With intact reticulocytes, it has been shown that the polyribosome/monoribosome ratio decreases as reticulocytes mature. This is accompanied by an overall loss in protein-synthetic ability (Rifkind, Danon & Marks, 1964; Rowley, 1965). However, the polyribosomes isolated from young and old cells synthesize protein at similar rates (Warner & Rich, 1964). Thus it may be that either ribosomal extract or m-RNA, or both, are being depleted during reticulocyte aging.

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