ISOLATION OF TRANSLATING RIBOSOMES WITH A RESIN-BOUND POLY-U COLUMN

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1. Introduction

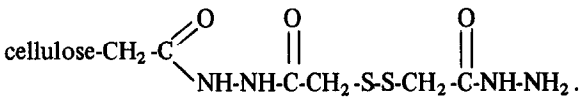
It has been shown earlier that polyuridylic acid (poly-U), covalently bound by its 3′-end to a solid-phase carrier (cellulose) through the hydrazide group can be translated by *Escherichia coli* ribosomes in a cell-free polypeptide-synthesizing system [1,2]. Such a solid-phase translation system opened a way for fractionating ribosomes and, in particular, for the selective isolation of translating ribosomes from a total ribosomal preparation. To achieve the latter, however, one needed a type of covalent coupling between a template and a carrier which could be subsequently uncoupled under mild condition innocuous for the translating ribosomes.

The present paper reports the preparation of poly-U coupled through its 3′-end to a cellulose carrier by an S–S bridge, and the use of poly-U-(S–S)-cellulose columns for isolation of the fraction of poly-U-bound translating ribosomes.

2. Materials and methods

Poly-U with an average chain length of about 100 to 120 residues was prepared from a polynucleotide phosphorylase-synthesized high-polymer poly-U by Dr. V. K. Rait, Novosibirsk State University, using a cobra venom endonuclease fragmentation technique [3]. Periodate oxidation of the 3′-ends of poly-U was performed as described previously [1].

Carboxymethylcellulose (CM-32, Whatman) was used as a solid-phase carrier for covalent coupling of the oxidized poly-U. The carboxymethylcellulose hydrazide was first prepared as described earlier [1] and then changed into azide by KNO_2 treatment in the presence of HCl. Dihydrazide of dithioglycolic acid was added to the carboxymethylcellulose azide for the formation of the following cellulose derivative:



The 3′-end dialdehyde group of the periodate-oxidized poly-U was then allowed to react with the hydrazide group of this cellulose derivative. The yield of the poly-U coupling reaction was 40–50%. The capacity of the poly-U-(S–S)-cellulose preparations obtained was 6.3–9.5 mg of poly-U per 1 g of the resin. Details of the procedure will be published elsewhere.

The ribosomal 30 S and 50 S particles were obtained from *Escherichia coli* MRE-600 by zonal sucrose gradient centrifugation of ribosomes in the presence of 0.5 M NH_4Cl with 1 mM $MgCl_2$ [4] using the B-XV rotor of the MSE SS-65 centrifuge.

To obtain the ^{14}C-labelled 30 S and 50 S particles, *E. coli* cells were grown in the mineral medium M-9 containing glucose and [^{14}C] protein hydrolyzate (57 μCi/μatom of carbon, Amersham). [^{14}C] 30 S subparticles with a specific activity of 180 dpm per pmole and [^{14}C] 50 S subparticles with a specific

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activity of 330 dpm per pmole were used in different experiments.

Translation of the poly-U-(S–S)-cellulose was performed in the standard buffer containing 10 mM Tris–HCl, 100 mM KCl and 10 mM $MgCl_2$, $pH_{25°C}$ 7.1-7.2. 25 μg of 30 S subparticles, 50 μg of 50 S subparticles, 40 nmoles of GTP, 100 μg of the protein fraction containing elongation factors [5], 150–300 μg of total tRNA containing 95–190 pmoles of [^{14}C] phenylalanyl–tRNA ([^{14}C] phenylalanine, 513 mCi/mmole, Amersham) or 160-180 pmoles of [^{3}H] phenylalanyl–tRNA ([^{3}H] phenylalanine, 5 Ci/mmole, Amersham) in 0.1 ml of the standard buffer were taken per each 14 μg of resin-bound poly-U. Incubation was at 25°C.

After a period of translation the poly-U-(S–S)-cellulose was placed into a column and successively washed: (1) with the standard buffer to remove all the components not bound to the poly-U-(S–S)-cellulose, (2) then with 1 M NaCl to dissociate the ribosomes translating the solid-phase poly-U and remove the polyphenylalanine released and rendered soluble under these conditions, (3) finally with 0.5 M KOH in ethanol to extract the polyphenylalanine which was insoluble in an aqueous medium and adsorbed on the resin. The amount of hot trichloroacetic acid insoluble [^{14}C]- or [^{3}H] polyphenylalanine was measured in all the fractions. The radioactivity of the trichloroacetic acid insoluble precipitates was determined on nitrocellulose filters in the toluene-PPO-POPOP system (when the ^{14}C-label was used), or after dissolving them in 0.5 ml Hyamine 10-X (Packard) (when the double label ^{3}H and ^{14}C was used). To measure the radioactivity of [^{14}C] phenylalanyl–tRNA a mixture of toluene-PPO-POPOP and Triton X-100 (Serva) was taken in a 2:1 ratio.

3. Results

3.1. *Translation of poly-U-(S–S)-cellulose*

Fig.1 shows the progress of [^{14}C] polyphenylalanine synthesis by ribosomes on this cellulose-bound poly-U. It is seen that a noticeable synthesis of polyphenylalanine occurs and that the spontaneous detachment of poly-U from the cellulose during incubation and polypeptide synthesis on the released template is relatively low.

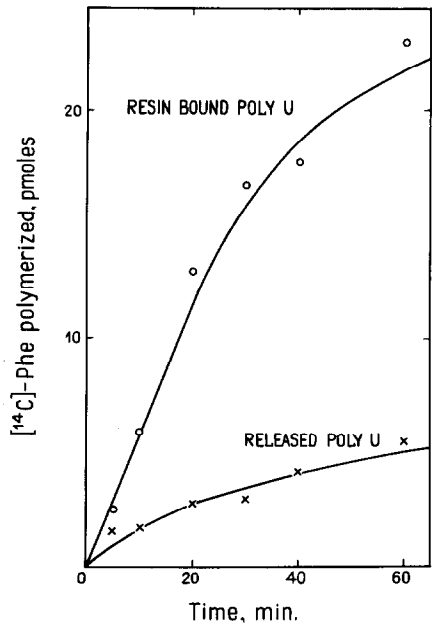

Fig.1. Kinetics of [^{14}C] polyphenylalanine synthesis in the cell-free system with poly-U-(S–S)-cellulose. Each time point corresponds to an aliquot with 14 μg of the resin-bound poly-U. (X——X——X) [^{14}C] polyphenylalanine removed from the poly-U-(S–S)-cellulose column by washing with the standard buffer; (o——o——o) [^{14}C] polyphenylalanine extracted from the column by washing with 1 M NaCl and ethanolic KOH.

Figure 2 shows the elution profile of translating ribosomes washed out from the poly-U-cellulose column by a buffer with an exponentially decreasing Mg^{2+} concentration. Elution was recorded by the appearance of ribosomal particles with newly-synthesized [^{14}C] peptide. Practically all the ribosomes translating resin-bound poly-U (containing [^{14}C] polyphenylalanine) were found to dissociate from the template (and, apparently, into their component 30 S and 50 S subparticles) in the region of 1 mM $MgCl_2$ and to come out of the column as one symmetrical peak. Thus, in the case studied, the stability of the ribosomes translating cellulose-bound poly-U coincides with that of ribosomes translating free poly-U as was already noted for the poly-U-cellulose system described earlier [2].

3.2. *Isolation of poly-U-translating ribosomes*

Usually a varying and often small fraction of particles of standard ribosome preparations can partici-

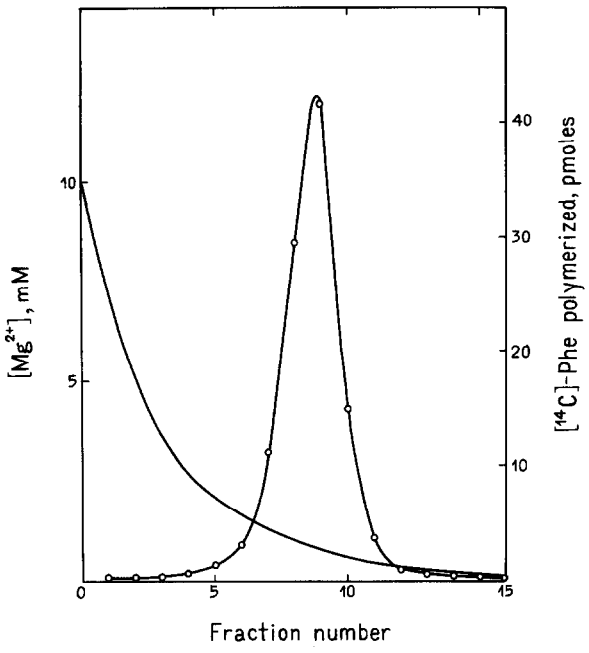

Fig.2. Elution of ribosomes containing [^{14}C]polyphenylalanine from the poly-U-(S–S)-cellulose column by a decreasing Mg^{2+} concentration. Translation was performed with 175 μg of resin-bound poly-U for 10 min at 25°C. (o——o——o) [^{14}C]polyphenylalanine; (————) Mg^{2+}.

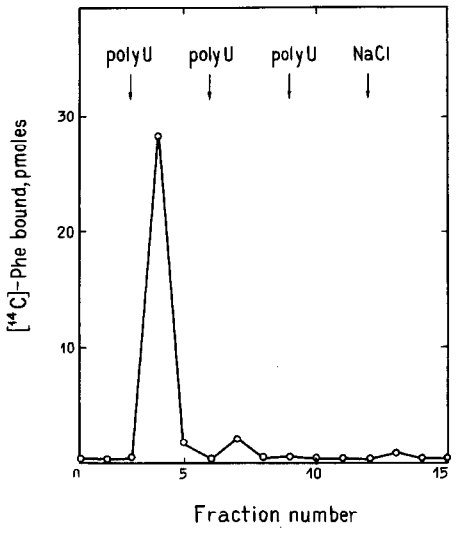

Fig.3. Dissociation of non-translating ribosome · [^{14}C]phenylalanyl–tRNA complexes from the poly-U-(S–S)-cellulose column by passing exogenous free poly-U. 175 μg of resin-bound poly-U was incubated with 260 μg of 30 S subparticles and 1 mg of [^{14}C]phenylalanyl–tRNA in 0.4 ml of the standard buffer for 30 min at 25°C. The resin was washed in the column with standard buffer and then 500 μg of 50 S subparticles in the same buffer was passed through the column for 10 min. After washing with buffer the column with the resin was incubated at 37°C for 20 min with 1.7 mg of exogenous free poly-U in standard buffer but with 20 mM $MgCl_2$; the procedure was repeated 3 times.

pate in in vitro translation. The solid-phase system automatically discriminates ribosomes capable of binding to the template from those incapable of doing so. The latter are simply removed during column washing. However, a special procedure is necessary to eliminate the ribosomes bound to the template, but not participating in translation. As seen in fig.3, the ribosome complexes containing aminoacyl–tRNA, but not translating the template, can be dissociated and thus removed from the resin by a competitive excess of exogenous free poly-U. Three washings with a 10-fold excess of exogenous free poly-U at 37°C leads

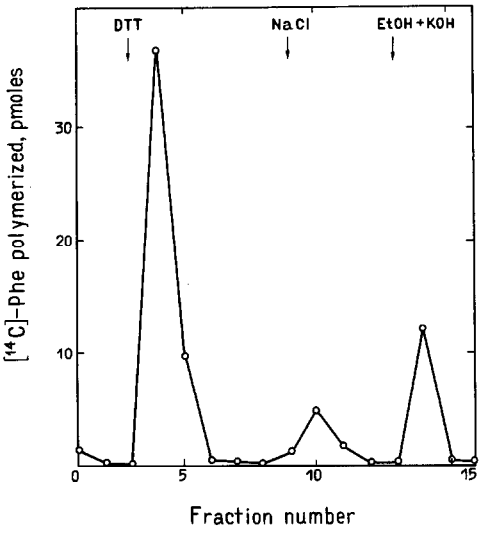

Fig.4. Release of poly-U · [^{14}C]polyphenylalanine-containing ribosome complexes from the poly-U-(S–S)-cellulose column by DTT. Translation was performed with 87 μg of resin-bound poly-U for 10 min at 25°C. Column washing was done by 0.02 M DTT in standard buffer for 20 min at 25°C.

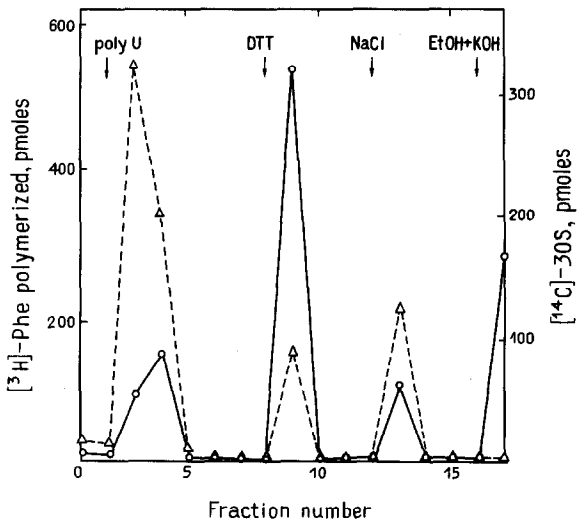

Fig.5. Isolation of translating ribosomes from the poly-U-(S–S)-cellulose column. Translation was performed with 4.9 mg of resin-bound poly-U for 20 min at 25°C. After washing the column with standard buffer, the resin was incubated with 42 mg of free exogenous poly-U for 30 min at 37°C; the procedure was repeated twice. Then the column was washed again with standard buffer. After this the resin was washed with 0.01 M DTT in standard buffer for 60 min at 25°C. Further washings were as described in Materials and methods. (△–△–△) [^{14}C] ribosomes (the labelled 30 S subparticles were used); (○–○–○) [^{3}H] polyphenylalanine.

to the removal of 97% non-translating ribosome · aminoacyl–tRNA complexes from the poly-U-(S–S)-cellulose column.

The detachment of translated poly-U coupled to the cellulose through S–S bridges can be seen in fig.4. The poly-U-cellulose column with the translating ribosomes was washed with 0.02 M dithiothreitol (DTT) in the presence of a high Mg^{2+} concn. 70% of the synthesized [^{14}C] peptide was found in the DTT eluate. Thus, the reduction of the S–S-bridge releases the greater part of poly-U with the translating ribosomes from the column.

The results of the final experiment on the selective isolation of translating ribosomes are shown in fig.5. The column with the poly-U-(S–S)-cellulose translated by the ribosomes was successively washed free from non-template-bound ribosomes with a buffer containing a high Mg^{2+} concn., then free from template-bound non-translating ribosomes by a buffer with free poly-U, and, finally, the poly-U with the translating ribosomes was released from the resin by a DTT buffer.

It is seen from fig.5 that the fraction of poly-U-bound non-translating ribosomes is indeed quite large: judging from the elution profile of ^{14}C-labelled subparticles it is about 60% of all the poly-U-bound ribosomes. It should be noted, however, that washing the column with a buffer with free poly-U also removes some part of the [^{14}C] peptide-containing particles; the reason for such a dissociation of part of the translating ribosomes under the action of exogenous poly-U is not yet clear.

3.3. *Some characteristics of the poly-U-translating ribosome preparation*

Figure 6 illustrates the result of sucrose-gradient sedimentation analysis of the DTT-eluted translating ribosomes (DTT peak from fig.5) under the same ionic conditions which are used in the translation

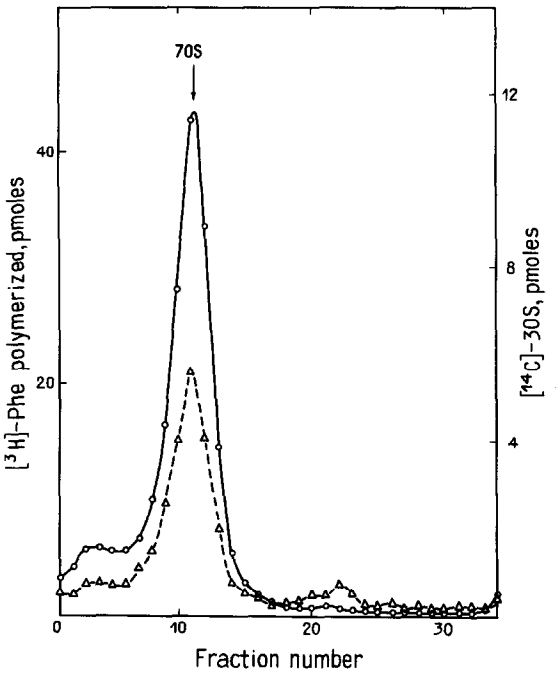

Fig.6. Sucrose gradient centrifugation of translating ribosomes isolated from the poly-U-(S–S)-cellulose column. The 5–20% sucrose gradient was prepared in a buffer containing 10 mM Tris–HCl, 100 mM NH_4Cl and 10 mM $MgCl_2$, $pH_{25°C}$ 7.2 SW-25 rotor, 19,500 rev/min 10 hr. (△–△–△) [^{14}C] ribosomes (the labelled 30 S subparticles were used); (○–○–○) [^{3}H] polyphenylalanine.

system (10 mM Mg^{2+}). It is seen that the isolated preparation of translating ribosomes practically contains no free subparticles; all the synthesized peptide is found in the 70 S ribosome fraction. An estimation of the average length of synthesized peptides from the ratio of the amounts of ribosomes (^{14}C-labelled 30 S subparticles) and phenylalanine residues (^{3}H-labelled polyphenylalanine) in the translating ribosome fraction eluted by DTT (figs. 5 and 6) gives 6 to 7.5 residues per ribosome for 20 min at 25°C.

To check the ability of the DTT-eluted translating ribosomes to continue the translation of their own poly-U template, an experiment analogous to the one described above (fig.5) was made, where [^{14}C] polyphenylalanine synthesis on the cellulose-bound poly-U in the column and then the continuation of the synthesis after DTT detachment were recorded. As seen from table 1, the isolated translating ribosomes are capable of continuing the translation of their template if [^{14}C] phenylalanyl–tRNA, GTP and elongation factors are again added to them.

The amount of polymerized phenylalanine per ribosome both in the fraction of the translating ribosomes after exhaustive translation of the resin-bound poly-U and in the initial total ribosome preparation after translation of free poly-U of the same length (incubation 2 hr at 25°C) was determined using [^{3}H] phenylalanyl–tRNA and [^{14}C] ribosomes (the labelled 50 S subparticles were used). It was found that the translating ribosome fraction contained 25.6 phenylalanine residues per ribosome while the initial total preparation had 2.2 residues per ribosome. Hence, more than a 10-fold purification was achieved. This means that the content of active ribosomes (capable of translating) in the initial preparation was about 10% or less.

Table 1
Continuation of translation by ribosome complexes eluted with DTT from the Poly-U-(S–S)-cellulose column

Conditions of translation:	[^{14}C] phenylalanine polymerized, pmoles:
Translation in resin-bound poly-U · ribosome complexes for 10 min	2.9
Subsequent translation in DTT-released poly-U · ribosome complexes for the next 10 min	6.7

Translation was performed with 175 μg resin-bound poly-U for 10 min at 25°C. After washing the column with standard buffer, the resin was incubated with 1.7 mg exogenous free poly-U in standard buffer, but with 20 mM $MgCl_2$, for 30 min at 37°C; the procedure was repeated three times. Then the column was washed with standard buffer. The translating ribosome complexes were eluted from the column by 0.02 M DTT prepared in standard buffer for 10 min at 25°C. The yield was 52% of all the [^{14}C] polyphenylalanine synthesized. To 0.1 ml of the isolated ribosomes (1/10 of the DDT eluate) containing 2.9 pmoles of the synthesized [^{14}C] polyphenylalanine were added 100 μg of the total tRNA with 60 pmoles of [^{14}C] phenylalanyl-tRNA, 75 μg of the protein fraction with the elongation factors and 48 nmoles of GTP, and incubation was continued for 10 min at 25°C.

Acknowledgements

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