ACTIVE COMPLEX FORMATION IN CELL-FREE EXTRACTS PROGRAMMED BY POLYURIDYLIC ACID SAMPLES OF DIFFERENT AVERAGE CHAIN LENGTH

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1. Introduction

In cell-free extracts synthesising protein, polyuridylic acid (poly U) acts as a synthetic messenger capable of directing the production of polyphenylalanine from phenylalanine [1]. Although previous workers have observed that highly polymerised poly U is more efficient than short chain poly U in directing phenylalanine incorporation [2, 3] this effect has never been explained satisfactorily. It is of particular interest that with excess ribosomes equal weights of poly U samples with different average chain lengths fail to stimulate total phenylalanine incorporation equally.

In the present work we report our observations on the active complexes formed in *Escherichia coli* cell-free systems programmed by commercial poly U samples of increasing molecular weight form increasingly heavy complexes in the cell-free system. These heavy complexes are directly responsible for the efficiency of polyphenylalanine formation as directed by highly polymerised poly U.

2. Materials and methods

E. coli strain B 163 was grown and harvested and the cells were stored frozen at -20° until use [4]. Crude extracts were prepared as described previously [5] and were adjusted to contain 10 mg/ml of ribosomes.

The reaction mixture for the incorporation of phenylalanine contained 1 mM ATP, 5 mM phosphoenolpyruvate, 40 μg pyruvate kinase, 17.5 mM magnesium acetate, 86 mM KCl, 6 mM mercaptoethanol, 5 mM tris-Cl buffer, pH 7.4 and 0.5 ml of crude extract per ml total of reaction mixture. After 10 min preincubation at 30° to degrade the natural messenger RNA the mixture was supplemented with further amounts of ATP and phosphoenolpyruvate as above, and poly U was added along with ^{14}C-phenylalanine. Incubation was continued at 30°. Samples were precipitated with 3 ml of ice-cold trichloroacetic acid (10%). The precipitates were filtered on to oxoid membranes and washed 3 times with 3 ml portions of ice-cold 10% trichloroacetic acid. The membranes were glued to aluminium planchets and counted at 20–30% efficiency in a gas flow counter for at least 1000 counts.

Sedimentation coefficients were measured in a Spinco Model E ultracentrifuge [6]. Analysis was in a solution containing 0.15 M NaCl and 0.015 M sodium citrate titrated to pH 6.0 with 0.2 M HCl. Analysis of poly U was also carried out in the above buffer on 5 ml linear sucrose gradients (5–20%) using the Spinco Model L2 SW50 rotor. 250 μg of poly U was layered and centrifugation was for 18 hr at 37,000 rpm.

Incorporation mixtures were analysed on 24 ml linear sucrose gradients (5–20%). 0.5 ml samples were layered and after centrifugation for 105 min at 25,000 rpm on the Spinco Model L2 SW25-1 rotor 10 drop samples were collected and analysed as described previously [5].

Poly U sample A was from P.L. Biochemicals, Inc., control number 201–13. Sample B was from Sigma (London) Chemical Company, control number 88B-1490. Samples C, D and F were from Miles Laboratories,

Inc., control numbers 46752, 115390 and 411647 respectively. Samples E and G were from the Boehringer Corporation (London), Ltd., control numbers 6098412 and 6227208 respectively. Samples C, D and F were as the ammonium salt. All other samples were as the potassium salt. Samples were stored at −20° on receipt from the suppliers. Solutions of poly U were freshly prepared at the start of each experiment at a concentration of 5 mg/ml in sterile distilled water.

^{14}C-Phenylalanine (468 mCi/mmole) was obtained from the Radiochemical Centre (Amersham, England).

3. Results

7 Different commercial samples of poly U were compared in relation to their ability to direct the incorporation of phenylalanine into polyphenylalanine. The reaction mixtures contained approximately 2.5 mg of ribosomes and were supplemented with 50 μg of the various poly U samples. The results are shown in table 1. Samples A and B gave the maximum stimulation of phenylalanine incorporation. Samples C–G gave respectively 86%, 85%, 50%, 20% and 11% relative to 100% for sample A. These levels of incorporation were consistent on a relative basis. Furthermore, poly U solutions prepared freshly from samples stored at −20° gave similar levels of stimulation over several months experimentation. Samples also retained their activity (90–95% of the initial level) - tested up to 2 months - while stored frozen in solution in water at concentrations of 5 mg/ml. With sample A phenylalanine incorporation was very efficient and represented approximately 60% of the phenylalanine present in the extract. This level is almost two fold higher than that reported previously for this system [7].

Samples A–G were analysed in the analytical ultracentrifuge and were found to have gross sedimentation values respectively of 7.35 S, 7.35 S, 5.66 S, 5.66 S, 4.52 S, 3.66 S and 3.13 S. Analysis on sucrose gradients confirmed this trend. The samples were all polydisperse and all absorbance profiles were similar except for the differences in sedimentation rates. Clearly, the sedimentation values of the samples increase in parallel with increasing ability to direct phenylalanine incorporation. However, if gradient fractions of the same number from different poly U samples were assayed, incorporation levels were the same.

Table 1
Phenylalanine incorporation directed by different samples of poly U.

Poly U Sample	cpm	cpm minus control	% Incorporation Sample A=100%
No poly U	560	–	–
A	6037	5477	100
B	5895	5335	97
C	5294	4734	86
D	5250	4690	85
E	3320	2760	50
F	1651	1091	20
G	1156	596	11

A reaction mixture containing crude extract (2.5 ml) was prepared and preincubated as described in Materials and methods. 0.5 ml portions were mixed with 0.15 μCi of ^{14}C-phenylalanine and 50 μg of the various poly U samples. After incubation at 30° for 30 min, 50 μl samples were removed and assayed for phenylalanine incorporation. Radioactivity is expressed as cpm precipitated by ice-cold 10% trichloroacetic acid.

Further experiments were carried out using samples B, E and G. In the cell-free system sample B saturates in amounts in excess of 1% (wt. of poly U/wt. of ribosomes), and sample E in excess of 2%. Sample G fails to saturate the system at low concentration. Instead phenylalanine incorporation increases slowly over a wide concentration range. For any given concentration of poly U, however, there is a wide difference between the 3 samples in their ability to direct phenylalanine incorporation. Even at saturation levels for each poly U sample, the incorporations never approach each other. Relative to sample B, sample E has an incorporation efficiency of 52% and sample G only 30%, after 30 min incorporation.

It has been known for some time that low molecular weight poly U is inefficient in directing phenylalanine incorporation relative to highly polymerised poly U. However, there has been no satisfactory explanation of this phenomenon. To elucidate this effect, poly U samples B, E and G have been used to study the active complexes formed in *E. coli* cell-free extracts utilising these poly U samples as synthetic messengers. Complexes of poly U and ribosomes

sedimenting in the range from 140–200 S have been described previously [7].

After preincubation for 15 min reaction mixtures were supplemented with poly U and ^{14}C-phenylalanine and incubated for a further 10 min. The samples were chilled quickly and then analysed directly on sucrose gradients. Fig. 1(a) illustrates the result of an incorporation directed by sample B. Practically all the counts on the gradient sediment in the heavy polysome region and only a very small amount of label is associated with the monosomes. Furthermore, of the total counts recovered on the gradient including the pellet approximately 22% are found in the pellet itself. This fraction represents extremely heavy polysomes. Fig. 1(b) shows the incorporation directed by sample E. Again most of the counts are in the polysome region but the complexes are much lighter than those in fig. 1(a). Furthermore, the pellet comprises only about 7% of the total gradient counts. Fig. 1(c) shows the incorporation directed by sample G. A large proportion of the counts sediments only very slightly heavier than the monosome peak and there is no marked polysome profile. The percentage of counts in the pellet (3%) is correspondingly very low. The results of the gradients shown in fig. 1 are summarised in table 2. For the above experiment saturating levels have been used for each of the poly U samples. An incubation time of 10 min was chosen as longer times result in the loss of polysome profile.

4. Discussion

Previous workers have described a relationship between the chain length of poly U and its messenger activity [2, 3] and this effect has been confirmed for seven distinct commercial poly U samples. There has hitherto been no satisfactory explanation for this phenomenon, but our results now enable an original interpretation to be made.

Table 2
Analysis of incorporation mixtures.

Gradient	Total counts	Pellet counts	% Counts in pellet
Fig. 1(a)	38,615	8350	22
Fig. 1(b)	18,555	1381	7
Fig. 1(c)	11,906	360	3

The experimental conditions are described in the legend to fig. 1. The counts quoted in this table are corrected for 10 drop samples.

Poly U samples B, E and G have respectively weight average molecular weights of approximately 390,000, 104,000 and 51,000 [6]. According to Martin and Ames [2] these molecular weights correspond to average chain lengths of 600, 160 and 78 nucleotides. Since poly U has no termination codon [8] the polyphenylalanine chains synthesised under its direction remain ribosome-bound [7]. Hence, the amount of polyphenylalanine synthesised relates directly to the length of poly U travelled by the ribosome before it reaches the end of the poly U chain. Thus, average single chains of sample B would direct the synthesis of approximately 8 times more polyphenylalanine than average single chains of sample G, each chain being translated by one ribosome only. With excess ribosomes and assuming all poly U chains to be translatable it might be argued that equal weights of poly U would direct the synthesis of the same total amount of polyphenylalanine. This, however, is clearly not the case as shown conclusively in table 1.

We have shown that poly U samples B, E and G differ greatly in their ability to form polysomes while directing polyphenylalanine synthesis. The precise length of poly U required to accommodate one ribosome is not known but it has been proposed that approximately 75 nucleotides are involved [9, 10]. On this basis poly U sample G could hold on average one ribosome only, sample E two ribosomes, and sample B eight ribosomes.

The active complexes shown in fig. 1 support the above interpretation. Clearly poly U sample B utilises 6–8 ribosomes and sample E 2–3 ribosomes. Sample G directs phenylalanine incorporation using single ribosomes only.

If poly U is read sequentially by ribosomes each starting at the beginning of the chain the polysome would form gradually. After translating the complete chain the first ribosome would remain fixed terminally, and other ribosomes would close up until the poly U was saturated, when synthesis would cease. If sample B bound 8 ribosomes at saturation and each occupied 1 unit of poly U, the first ribosome would translate 8

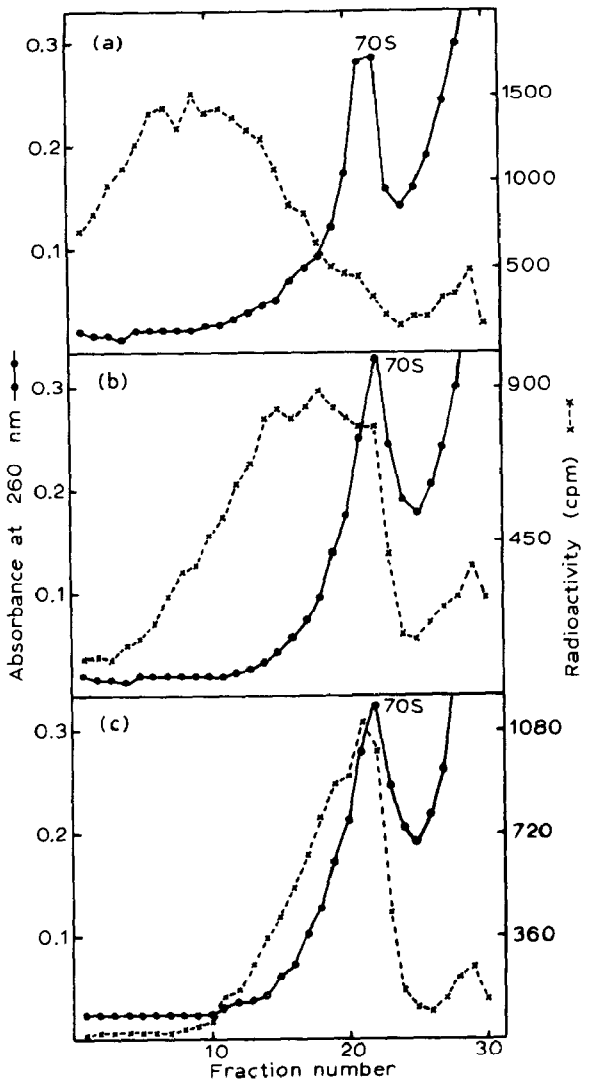

Fig. 1. Sucrose gradient analysis of poly U-directed ^{14}C-phenylalanine incorporation. A reaction mixture was prepared containing crude extract (1 ml) and was preincubated for 15 min. 0.5 ml portions were mixed with 0.125 μCi ^{14}C-phenylalanine and poly U and incubated for 10 min. The samples were chilled and analysed on sucrose gradients in 5 mM tris-Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 86 mM KCl. (a) 25 μg poly U sample B added. (b) 50 μg poly U sample E added. (c) 250 μg poly U sample G added.

units, the second 7 units and so on to give a total translation of 36 units. For sample E the same weight of poly U with 4 chains of 2 units would allow translation of 12 units For sample G with 8 chains of 1 unit the total would be 8 units. However, since Gilbert [7] observed polysome formation in the cold in the absence of protein synthesis, ribosomes may attach simultaneously at several points along the poly U chains. For both models the calculations would be valid on a relative basis and would give incorporation ratios. With simultaneous attachment the final incorporation levels would be lowered. The figures must be approximate but they fit the data of table 2 reasonably well from which the incorporation ratios may be calculated as 3.3:1.6:1.

Theoretically our model predicts that with excess ribosomes increasing chain lengths of poly U should always increase phenylalanine incorporation. It suggests that the original estimate of 8 S for optimal chain length [2] is an artefact resulting from the limitations of cell-free systems.

Finally, the reported variations in messenger activity with a random selection of commercial poly U samples is disconcerting. However, routine fractionation of poly U samples on Sephadex G-200 [3] to eliminate material with sedimentation values below 5 S would greatly improve the efficiency of all poly U samples in the cell-free system.

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