POLYSOMES FROM *ESCHERICHIA COLI*: ESTIMATION BY PEPTIDYL-PUROMYCIN SYNTHESIS

Yair AHARONOWITZ and Eliora Z. RON
Department of Microbiology, The George S. Wise Center for Life Sciences, Tel Aviv University, Tel Aviv, Israel

Received 13 January 1975

1. Introduction

The level of polysomes in cells reflects their capacity to synthesize protein. The methods used for measuring polysomes are based on the size difference between polysomes and monosomes which permits their separation by sucrose gradient sedimentation or by gel electrophoresis [1,2]. The reliability of such measurements evidently depends on the integrity of the polysomes. Polysome breakdown during preparation (due to nucleases or to mechanical forces) will give an overestimation of the level of free ribosomes. Similarly, initiation complexes (ribosomes attached to mRNA and formylmethionyl-tRNA) would also be found with the free ribosome fraction, although they represent ribosomes which are actively involved in protein synthesis.

We describe here a different way of measuring the relative level of polysomes by estimating the level of 'polysomal' ribosomes. The method depends on the fact that 'polysomal' ribosomes are attached to mRNA and peptidyl-tRNA and can, therefore, react with puromycin to give peptidyl-puromycin. This reaction can be followed by the method of Pestka [3,4] in which the conversion of [^{3}H]-puromycin to peptidyl-[^{3}H]-puromycin is measured by determining TGA-precipitable ^{3}H counts.

In the following report evidence will be presented to show that the activity of cell extracts in the peptidyl–puromycin reaction reflects the protein synthesizing activity of the cells and is in direct correlation with the level of polysomes. The main advantage of this method is that the assay of ribosome activity does not depend on the integrity of the polysomes. An additional advantage of this method is its simplicity and convenience.

2. Materials and methods

E. coli K-12 strain 428 (pro$^-$, his$^-$, B$_1$ $^-$) was used in all experiments except the one summarized in table 3 (supplied by M. Meselson). Cells were grown at 37°C and aerated by shaking in Nutrient Broth (Difco) or in minimal medium A [5] containing 0.2% glucose. The minimal medium was supplemented with 50 μg/ml of each required amino acid and 1 μg/ml of thiamine.

Incorporation of radioactive puromycin into nascent peptides was measured as described by Pestka [3,4]. In a typical experiment 50 to 150 ml of a culture (about 5 × 10^8 cells per ml) was poured over an equal amount of ice and centrifuged immediately for 5 min at 10 000 *g*. The cells were washed once and resuspended in 1 ml cold buffer containing 10 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 100 mM ammonium chloride, 1 mM dithio-threitol (Sigma) ('TAM' buffer) with 10% glycerol. If not used immediately, cells were frozen at this stage at −20°C. Lysates were prepared by ultrasonic disintegration in a Branson Sonifier (model B-12; 10 seconds at intensity 3, unless otherwise stated). The lysates were then centrifuged at 30 000 *g* for 20 min at 4°C and the pellet discarded. The extract contained about 20–60 $A_{260\,nm}$ units per ml (1 $A_{260\,nm}$ unit of extract contains 60 μg of RNA as determined by the orcinol method [6]). Reaction mixture contained in 150 μl of TAM buffer, 100 pmol

of [^{3}H]-puromycin (Radiochemical Center, Amersham; specific activity 3.7 Ci/mmol) and cells extract (1–4 A_{260} units). Incubation was for 5 min at 37°C unless otherwise stated. Samples of 100 μl were placed onto Whatman 3 MM filter paper discs, precipitated with ice-cold 10% TCA, and washed with TCA, ethanol–ether (1:1) and ether. Radioactivity in the discs was measured in a Packard Tricarb scintillation counter in toluene.

Polysomes were extracted and assayed as previously described [7]. Rifampin (Rifadin, Lepetit) was a gift from Abic Ltd.

3. Results and discussion

3.1. *Effect of ribosome concentration and growth conditions on peptidyl–puromycin synthesis*

The level of peptidyl–puromycin which was produced by extracts obtained from growing cells was directly proportional to the concentration of RNA in the incubation mixture (fig.1). Moreover, the activity of extracts in the synthesis of peptidyl–puromycin was directly proportional to the level of ribosomes in the extracts. The results were obtained from a comparison of the activity of extracts made from cells grown in rich medium (Nutrient Broth) to that of extracts from cells grown in minimal medium (salt–glucose). Both cultures were harvested at the exponential phase of growth, at the same turbidity, and extracts were prepared from them. The activity of extracts made from cells grown in minimal medium was lower than this of extracts made from cells grown in Nutrient Broth when calculated per mg protein or per number of cells (table 1). However, when calculated per mg RNA in the extracts, the level of activity of both extracts was similar, because of the higher content of ribosomes in the faster growing cells.

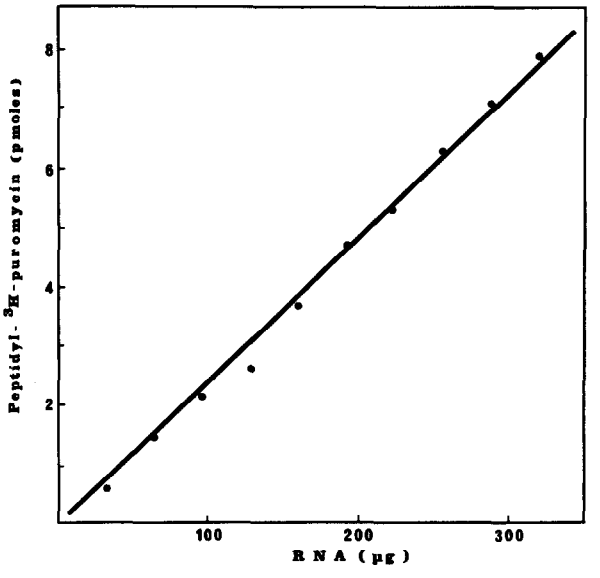

Fig.1. Effect of RNA concentration on the synthesis of peptidyl–puromycin. Samples of a bacterial extract were assayed for RNA content [6] and for activity in the synthesis of peptidyl–puromycin as described in Materials and methods. Incubation was for 1 min at 37°C.

3.2. *Effect of polysome level on activity in the peptidyl–puromycin reaction*

In order to determine whether the activity of extracts in the peptidyl–puromycin reaction is a reflection of the polysome level in the cells, extracts from cells containing varying levels of polysomes were assayed for synthesis of peptidyl–puromycin. Such extracts were obtained by treating exponentially growing cultures with rifampin [9], and removing samples at various intervals after the addition of rifampim. The addition of rifampin has been shown to result in polysome breakdown due to the inhibition on the synthesis of mRNA [10]. The results presented in fig.2 indicate that the fall in activity of extracts in the peptidyl–puromycin reaction parallels

Table 1
Synthesis of peptidyl–puromycin by cells grown in minimal medium and in Nutrient Broth

Growth medium	pmol of peptidyl–puromycin synthesized		
	per 10^{10} cells	per mg protein	per mg RNA
Salt–glucose	22.8	10.8	32.6
Nutrient broth	48	16	30

For details on the synthesis of peptidyl–puromycin see Materials and methods. Protein was determined by the method of Lowry et al. [8] and RNA by the orcinol method [6]. Cell counts were obtained using the Petroff–Hausser counting chamber.

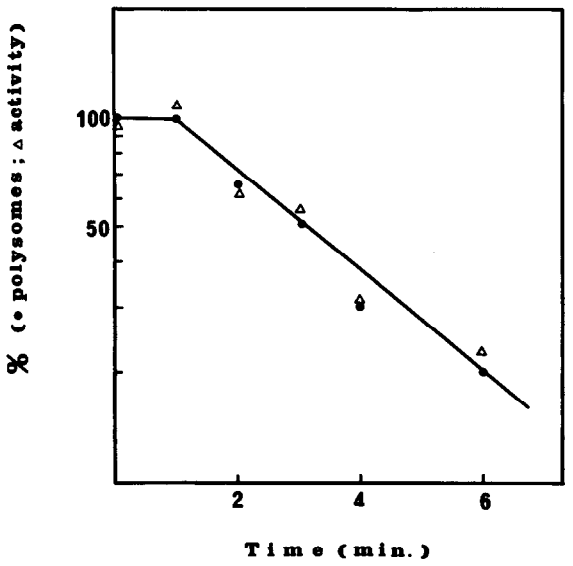

Fig.2. Polysome level and synthesis of peptidyl–puromycin in extracts from rifampin-treated cells. Rifampin (200 μg/ml) was added to a culture of about 5 × 10^8 cells/ml growing in A medium [5]. Aliquots were removed at various time intervals, harvested and assayed for activity in the synthesis of peptidyl–puromycin (△-△) and for polysome level (•-•) as described in Materials and methods.

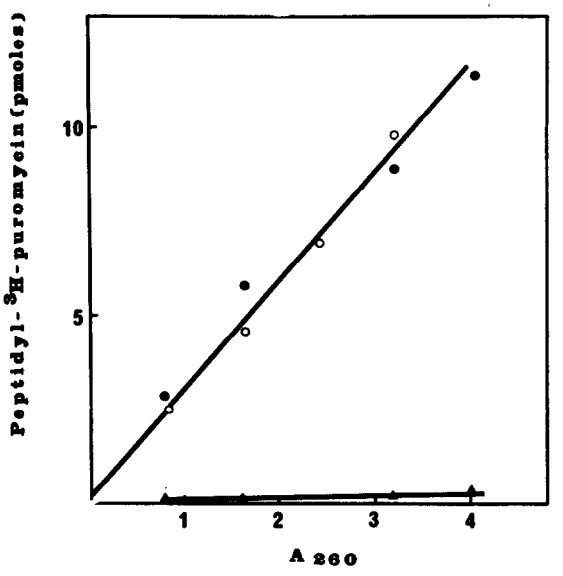

Fig.3. Synthesis of peptidyl–puromycin by extracts from rifampin-treated cells. Extracts were prepared from growing cells (•-•) and from cells treated with rifampin (see fig.2) for 15 min (△-△). Increasing amounts of each extract were incubated with puromycin. In addition, increasing amounts of untreated extract (○-○) were mixed with rifampin treated extract (to give 4 A_{260} units in total) and incubated with puromycin.

the drop in level of polysomes in the correponding extracts. Extracts prepared from cells treated with rifampin for over 10 min were completely inactive (fig.3). The inactivity of rifampin treated extracts could be due to inhibition of peptidyl–puromycin synthesis by residual rifampin in the extracts. This possibility was rendered unlikely by the finding that the presence of rifampin-treated ribosomes did not lower the activity of untreated ribosomes when the formation of peptidyl–puromycin was measured in mixed extracts (fig.3).

Table 2
Effect of sonication on polysome level and on activity of ribosomes in the synthesis of peptidyl–puromycin

Sonication intensity	% polysomes	RNA mg/ml	pmoles peptidyl–puromycin synthesized	
			per ml extract	per mg RNA
–	66	0.790	16.3	20.5
1	0	1.2	25.3	21.1
2	0	1.2	29.9	24.9
3	0	1.69	39	23.1
4	0	1.49	37.3	25

An extract containing polysomes was prepared as previously described [7] and aliquots were sonicated for 10 sec at the intensity specified. Samples were assayed for polysomes and for peptidyl–puromycin synthesis as described in Materials and methods.

Table 3
Synthesis of peptidyl–puromycin and polysome level in extracts of amino acid starved cells

Amino acid	Fraction of polysomes retained in its absence (%)	% activity in peptidyl–PM synthesis
Arginine	20	40
Leucine	20	55
Threonine	40	63
Histidine	100	90
Tryptophan	100	90

A culture of *E. coli* K-12 strain PA 309 which requires arginine, leucine, threonine, histidine and tryptophan was grown to 3×10^8 cells/ml. It was then washed and resuspended in minimal medium with all the required amino acids or lacking one amino acid (as specified). The cultures were harvested after 30 min and assayed for polysome level and activity in the peptidyl–puromycin reaction as described in Materials and methods.

3.3. *Effect of polysome integrity on ribosome activity in peptidyl–puromycin synthesis*

A major difficulty in measuring polysome level arises from the need to conserve the physical integrity of the polysomes. In order to find out whether the integrity of the polysomes affects the activity of polysomal ribosomes in the peptidyl–puromycin reaction, this activity was measured in extracts containing polysomes and in extracts subjected to polysome fragmentation. When extracts containing polysomes [7] were sonicated, the polysomes were broken down, but the activity of the ribosomes remained unaffected (table 2). It should be noted that although the level of peptidyl–puromycin synthesized per ribosome remained constant with increasing intensity of sonication, the total activity of the extracts increased, due to a better recovery of ribosomes. These results indicate that it is not necessary to preserve the integrity of polysomes in order to assay for polysomal ribosomes. Therefore, it is possible to lyse cells by methods which recover most of the cellular ribosomes. This allows more representative measurements since they are performed on a larger fraction of the cellular ribosomes.

3.4. *Peptidyl–puromycin synthesis by extracts from amino acid-starved cells*

During amino acid starvation the polysomes are broken down and resynthesized continuously, forming polypeptides of various sizes [10,11]. The level of polysomes extracted during deprival of a particular amino acid is a function of the relative frequency with which that amino acid appears in cellular proteins. Thus, deprival of a commonly occurring amino acid results in a more extensive breakdown of polysomes than deprival of a rare amino acid. We studied the peptidyl–puromycin reaction by ribosomes extracted from amino acid-starved cells in a polyauxotrophic strain which was starved for one amino acid at a time. Extracts were assayed for polysomes and for peptidyl–puromycin synthesis. The results presented in table 3 indicate that the activity of extracts from cells starved for various amino acids was proportional to the cellular level of polysomes. However, the activity in the peptidyl–puromycin reaction was generally higher than the corresponding level of polysomes. This discrepency can be explained by assuming that the fraction of 'monosomes' from amino acid-starved cells contains many ribosomes at the stage of initiation or carrying very short peptides. Such ribosomes, although sedimenting with the monosomes could react with puromycin. Active ribosomes which are not found with the large polysomes usually consist of only a small fraction of the cellular ribosomes. However, under certain growth conditions, such as amino acid starvation, this fraction increases considerably. The use of the peptidyl–puromycin method might serve as a tool to study this fraction of

ribosomes and allow for better understanding of polysome turnover.

4. Conclusion

This paper describes a method for estimating the fraction of ribosomes bound in polysomes by the use of radioactive puromycin. This method is rapid and convenient and allows for the assay of many extracts simultaneously. It is based on the finding that the synthesis of peptidyl–puromycin by extracts from *E. coli* is proportional to the cellular level of ribosomes which are actively synthesizing protein. The major advantage of this method is that it is an accurate assay of the fraction of active ribosomes, since it includes two classes of ribosomes which are physiologically active but are not found in the polysome fraction: ribosomes attached to mRNA and formylmethionyl-tRNA (or tRNA carrying a short peptide), and polysomal fragments.

Acknowledgements

We thank Z. Spector for excellent technical assistance and Dr E. Rosenberg for helpful comments on the manuscript.

References

[1] Modolell, J. (1971) in: Methods in Molecular Biology. (Last, J. A. and Laskin, A. I., eds.), Vol. 1, pp. 32–57, Marcel Dekker, Inc. N.Y.
[2] Dahlberg, A. E., Dingman, C. W. and Peacock, A. C. (1969) J. Mol. Biol. 41, 139–147.
[3] Pestka, S. (1972) Proc. Natl. Acad. Sci., U.S.A. 69, 624–628.
[4] Pestka, S. (1972) J. Biol. Chem. 247, 4669–4678.
[5] Davis, B. D. and Mingioli, E. S. (1950) J. Bact. 60, 17–28.
[6] Schneider, W. C. (1957) in: Methods of Enzymology (Colowick, S. P. and Kaplan, N. O. eds.), Vol. 3, pp 680–684, Academic Press Inc., N.Y.
[7] Ron, E. Z., Kohler, R. E. and Davis, B. D. (1966) Science 153, 1119.
[8] Lowry, O. H., Rosebrough, N. I., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[9] Haselkorn, R., Vogel, M. and Brown, R. D. (1969) Nature (London) 221, 836–838.
[10] Ron, E. Z. (1971) J. Bacteriol. 108, 263–268.
[11] Brunschede, H. and Bremer, H. (1971) J. Mol. Biol. 57, 35–57.