SPECIFIC CLEAVAGE OF RIBOSOMAL RNA IN *DICTYOSTELIUM DISCOIDEUM* RIBOSOMES

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1. Introduction

Recently, evidence has been presented that in vivo, membrane-bound ribosomes of reticulocytes contain ribosomal RNA (rRNA) which exists in several pieces as a result of specific endonucleolytic cleavages and that this results in impaired function of the ribosomes in an in vitro protein synthesis system [1]. Using the cellular slime mould *Dictyostelium discoideum* we report here that similar specific cleavages may be generated artefactually during isolation of polyribosomes. We suggest that a criterion for the existence of such cleavages in vivo should be the demonstration that the fragments are present under conditions when intact messenger RNA (mRNA) may also be isolated.

2. Methods

All chemicals, unless otherwise stated, were obtained from Fisons (Loughborough, England) or BDH (Poole, England) and were of Analar quality or higher.

2.1. *Growth and labelling of cells*

D. discoideum (strain AX2) was grown axenically in glucose-supplemented HL5 medium [2]. RNA was labelled with $NaH_2\ ^{32}PO_4$ (Amersham) in MES (Sigma)-buffered HL5 in the presence or absence of 5 μg/ml of actinomycin D (Sigma) which suppresses the synthesis of rRNA but not of mRNA in this organism [3].

2.2. *Preparation of polysomes*

2.2.1. Method A

This method is essentially as described by Coccuci and Sussman [4]. Cells were harvested at $3–5 \times 10^6$ cells/ml and resuspended in lysis buffer A (0.05 M Hepes, 0.04 M $MgCl_2$, 0.025 M KCl, 1 mg/ml heparin, 5% sucrose, pH 7.5, with NH_4OH) and lysed by addition of Cemusol NPT12 (Progil) and Triton TX-100 (BDH), each to 2% final concentration, and agitation for 30 s. The lysate was centrifuged at 10 000 rev/min for 10 min in the Sorval HB4 rotor at 0–4°C (RC5 centrifuge) and the supernatant layered over 15–30% linear sucrose gradients made in buffer A (500 μg/ml Heparin) and spun for 3 h at 25 000 rev/min in the MSE 6×38 rotor at 1°C.

2.2.2. Method B

This method is identical to method A except that lysis buffer B contains 0.02 M Tris/Cl, pH 7.5, 0.01 M $MgCl_2$, 0.025 M KCl, 1 mg/ml heparin. The cells were lysed with 1% Triton TX-100 final concentration.

All operations were performed in ice, all solutions were autoclaved at 121°C for 20 min, all glassware was heated at 200°C for 5 h and all tubing etc. used in fractionation was rinsed with 0.01 M NaOH, 0.01% diethylpyrocarbonate and sterile distilled water, immediately prior to use.

Gradients were fractionated by puncturing the tube and pumping the gradient through a Uvicord II flow cell (LKB). Trisomes and larger were pooled as polysomes.

2.3. *Deproteinisation of RNA*

RNA was deproteinised using 'chloropane' [5] and precipitated by addition of 2.5 vol. ethanol. After standing at −25°C overnight the RNA was pelleted, washed twice with 50% ethanol/ether and dried with dry N_2. RNA was redissolved in SDS buffer (0.02 M Tris/Cl, pH 7.5, 0.1 M NaCl 0.001 M EDTA, 0.1% SDS (BDH specially purified) 250 μg/ml heparin) for analysis on gradients.

2.4. *SDS–sucrose gradients*

Linear sucrose gradients, 15–30%, were made in SDS buffer and spun 16 h at 30 000 rev/min in the MSE 6 X 14 titanium rotor. Gradients were fractionated by upward displacement using Fluorochemical FC43 and analysed by passage through a Uvicord II flow cell (LKB). Fractions were collected and assayed for polyadenylated mRNA content by passage through poly(U) filters [6].

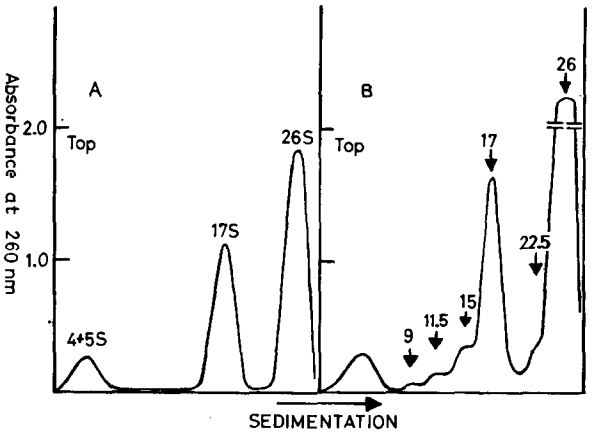

Fig.2. SDS–sucrose gradient analysis of polysomal RNA. Polysomes were deproteinised, the polysomal RNA centrifuged in 15–30% SDS–sucrose gradients and analysed by optical density measurements at 260 nm. Panel A: RNA from polysomes prepared by method A. Panel B: RNA from polysomes prepared by method B.

3. Results

The sedimentation values of polyribosomes prepared by method A and method B clearly show that whilst method A yields intact polysomes, predominantly 8–12 mers, method B results in smaller polysomes (fig.1). That the polysome profile obtained using method B is indicative of degradation rather than ribosome run-off is shown by the accumulation of small polysomes at the expense of larger ones without a concomitant accumulation of monosomes [7].

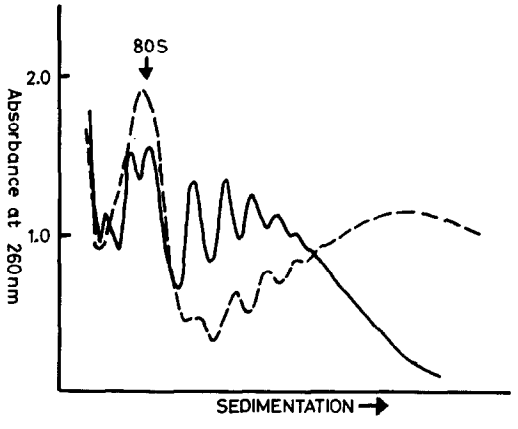

Fig.1. Sedimentation profiles of polyribosomes prepared by method A and method B. Polysomes were prepared, centrifuged in 15–30% linear sucrose gradients and analysed as described in Methods. Polysomes prepared by method A (- - -) or method B (——).

Analysis of the RNA obtained from these polysomes shows that the use of method A, giving mainly large polysomes, results in undegraded 17 S and 26 S rRNA but method B although still giving polysomes at least up to 7 mers, results in some of the rRNA being specifically cleaved to give 9 S, 11.5 S, 15 S and 22.5 S RNA products (fig.2). These values are very reproducible and hence the cleavages specific, although the proportion of rRNA which is degraded to these fragments varies with the preparation. Evidence for the observed RNA species being rRNA fragments includes the observations that the specific radioactivity and sensitivity to actinomycin D are the same as intact rRNA. In extreme cases those RNA products may entirely replace the 17 S and 26 S rRNA species. The results of a large number of experiments of this kind suggest that both 17 S and 26 S rRNA are subject to cleavage.

The sedimentation profiles of polyadenylated mRNA derived from polysomes prepared by the two methods are shown in fig.3. Ribosomal RNA synthesis

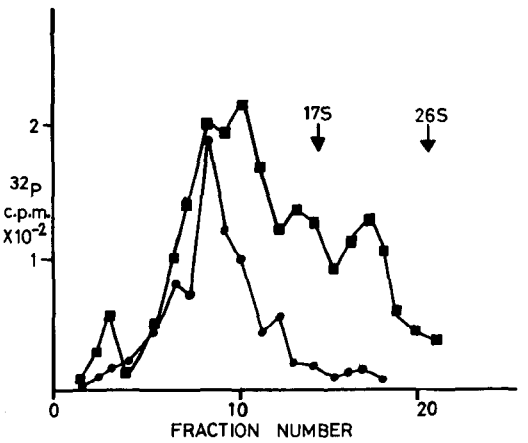

Fig.3. Polyadenylated RNA sedimentation profiles. Polysomes were prepared from cells labelled with NaH_2 $^{32}PO_4$, the RNA extracted and run on 15–30% SDS–sucrose gradients. Gradient fractions were assayed for polyadenylated mRNA by binding to poly(U) filters as described in Methods. Polyadenylated RNA from polysomes prepared by method A (■ - ■) or method B (● - ●).

was suppressed by the use of 5 μg/ml actinomycin D as described in Methods [3]. The average size of polyadenylated RNA is much reduced when the RNA is isolated from polysomes made by method B. It is interesting that there is not an accumulation of very small fragments suggesting that endonucleolytic cleavage is more likely to occur near the 5′ end of the mRNA. Total trichloroacetic acid precipitable radioactivity shows a greater bias to small fragments agreeing with this suggestion. In preparations of RNA made by method A the size distribution of RNA precipitable by trichloroacetic acid and polyadenylated RNA are the same.

4. Discussion

We have shown that, by inappropriate choice of salt and detergent conditions in lysis media, it is possible to obtain polyribosomes from *D. discoideum* which comprise intact ribosomes but which contain the rRNA in discrete fragments, analagous to the situation in membrane-bound ribosomes of reticulocytes [1]. However, we have demonstrated that such specific cleavage products of *D. discoideum* rRNA are not present in vivo but represent artefacts of the method of preparation, since conditions which maintain the integrity of polysomal mRNA result in the isolation of intact rRNA molecules. Wreschner et al. [1,8] working with reticulocyte membrane-bound polysomes, propose that a membrane fraction referred to as 'ghost factor' is responsible for the nicking of the rRNA. Other reports have demonstrated that treatment of ribosomes in vitro, with ribonucleases which cleave RNA at random (i.e., not at specific sequences), nevertheless causes specific breakage of the rRNA [9–11]. This is due to protection of most of the rRNA in intact ribosomes. Since ribonuclease is predominantly membrane associated in reticulocytes [12], degraded rRNA might be produced preferentially in membrane-bound ribosomes during prolonged co-purification procedures. Further, any treatment which disturbs the conformation of the ribosome increases its susceptibility to ribonuclease attack, EDTA or low Mg^{2+} concentrations being particularly implicated [10,11]. We have found that low temperature alone is not sufficient to inhibit the cleavage of the rRNA and conditions which confer greater ribonuclease resistance must be chosen (unpublished observations).

Since in reticulocytes the mRNA from membrane-bound polysomes has been shown to be less active than expected [13] and most of reticulocyte ribonuclease is membrane associated [12] we suggest that the observation of specifically-cleaved rRNA in membrane-bound ribosomes from this source is possibly due more to preparation artefacts than to their actual existence in vivo. In general, we propose that assertions of the in vivo relevance of rRNA cleavages should be accompanied by the demonstration that they occur under conditions when intact mRNA may be isolated.

Acknowledgements

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