A STUDY OF THE ACCESSIBILITY OF THE 3'-ENDS OF rRNA WITHIN MAMMALIAN RIBOSOMES

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1. Introduction

Sequences at the 3'-ends of eukaryotic rRNA molecules have been implicated in various aspects of ribosome function. For instance, nucleotides near the 3'-end of 18 S rRNA have been proposed as being involved in interaction with mRNA as a preliminary to protein biosynthesis [1–4] and interaction with 5 S rRNA as a mechanism for ribosomal subunit binding [4]. Nucleotides near to the 3'-end of 28 S rRNA have been implicated in complex formation with 5.8 S rRNA [5]. However, knowledge of the locations of the 3'-ends of rRNA molecules within eukaryotic ribosomes is limited. We now describe the use of T4 RNA ligase and [5'-^{32}P]pCp to probe the accessibility of the 3'-ends of rRNA molecules within ribosomes and ribosomal subparticles of rabbit reticulocytes. Under the conditions used the ribosomes and subparticles remain functional in cell-free protein synthesis.

2. Methods

2.1. Materials

T4 RNA ligase was purchased from Miles Biochemicals; [5'-^{32}P]pCp (2000–3000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham.

2.2. Labelling of rRNA within ribosomes and subparticles

Polyribosomes were isolated from rabbit reticulocytes and ribosomal subparticles were separated as in [6]. Labelling of 3'-termini was done in a solution of 35 mM Hepes/15 mM Tris–HCl (pH 7.5)/30 mM NH$_4$Cl, 3.3 mM dithiothreitol/15 mM MgCl$_2$/7% dimethyl sulphoxide/0.01 mg serum albumin/ml. The

reaction mixture (100 μl) contained 500 μg polysomes (or equivalent mass of subparticles), 4 units T4 RNA ligase, 1.5 nmol ATP, 5 μCi pCp. Incubation was at 0°C for 16–24 h. Where appropriate, labelled subparticles were separated from the reaction mixture on sucrose gradients [8]. The pellets recovered from the peak fractions by centrifugation were resuspended in buffer and used without further treatment. RNA was isolated by phenol extraction and precipitated by the addition of 2.5 vol. ethanol. The RNA was dissolved in de-ionised sterile water and either purified on 10–25% (w/v) sucrose gradients in 0.1 M LiCl/0.01 M EDTA/0.01 M Tris–HCl (pH 7.4)/0.2% (w/v) SDS centrifuged at 23 000 rev./min at 20°C in a Beckman SW27 rotor, or freeze-dried and fractionated on polyacrylamide gels (fig.1).

2.3. Poly(U)-directed polyphenylalanine synthesis

The incorporation of [^{14}C]phenylalanine (spec. radioact. 50 mCi/mmol) into acid-insoluble material by 60 S subparticles in the presence of 40 S subparticles and supernatant factors at 37°C was measured as in [7]. Samples were generally 50 μl 2.0 A_{260} units/ml (i.e., 10 μg 60 S subparticles) in 0.5 ml assay mixture. The assay blank, the incorporated radioactivity equivalent to zero synthesis, was determined by omitting the energy source (pyruvate kinase, phosphoenolpyruvate, ATP and GTP) from a complete control assay mixture. Control 60 S subparticles incorporated 18–54 phenylalanine residues/ribosome during the assay.

2.4. Separation of core-particles and split-protein fractions by pelleting

The production of core-particles and split-proteins from rabbit 60 S subparticles by salt-shock treatment

was detailed in [8]. Effective conditions for producing extensively inactivated core-particles with good potential for reconstruction were taken from the earlier work. Thus, 60 S subparticles at 250 A_{260} units/ml in storage buffer were mixed at $0°C$ with 4 vol. salts solution such that the final composition was 2.75 M NH_4Cl/69 mM $MgCl_2$/3.5 mM EDTA/1 mM dithiothreitol/3% (w/v) glycerol/20 mM Tris—HCl (pH 7.6). After 15 min at $0°C$, the reaction mixture was centrifuged for 30 min at ~$0°C$ and 40 000 rev./min in the MSE 8 × 10 ml rotor (1.25 ml portions in 10 ml polycarbonate tubes, g_{max} = 145 000). The supernatant containing the split-proteins were pipetted off, and the pellets were homogenised in a volume of storage buffer equal to the volume of salt-shock mixture, to yield a cloudy suspension of core-particles.
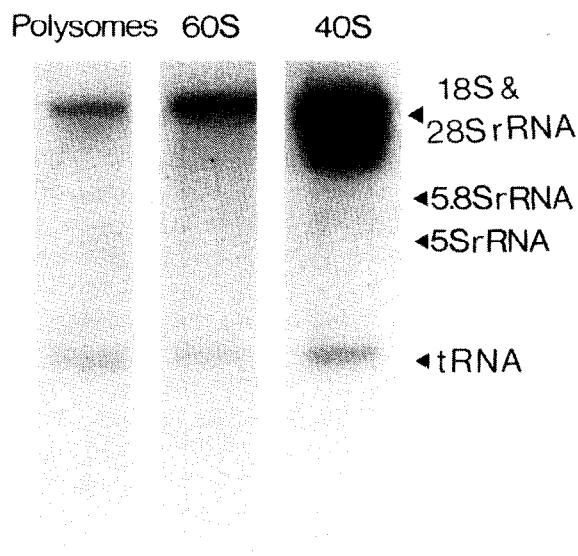

Fig.1. Autoradiograph of rRNA labelled at the 3'-end within intact subparticles or polysomes. RNA was labelled and isolated as in section 2. Freeze-dried samples were dissolved in 10 µl 8 M urea/20 mM Tris—HCl (pH 7.4)/1 mM EDTA/0.05% xylene cyanol/0.05% bromophenol blue, heated to $90°C$ for 30 s and chilled on ice prior to layering on a gel of 8% acrylamide/0.4% N,N'methylenebisacrylamide/7 M urea/50 mM Tris—borate (pH 8.3)/1 mM EDTA/0.08% ammonium persulphate. Electrophoresis proceeded at 200 V for 2—3 h, the gel was stained with toluidene blue dye, dried and autoradiographed. No bands were detected on the autoradiograph corresponding to the position on the gel of either 5 S or 5.8 S rRNA. The 18 S and 28 S rRNA components remained close to the origin.

3. Results

3.1. Extent of labelling of rRNA components in situ

The polyribosome fraction or subparticle fractions were incubated with [5'-^{32}P]pCp and T4 RNA ligase as in section 2. As far as possible, the reaction conditions were kept the same for each species. The amount of radioactivity incorporated into rRNA was measured by scintillation counting for both sucrose gradient fractions and bands cut from dried polyacrylamide gels. Both 18 S rRNA and 28 S rRNA became labelled but neither 5 S nor 5.8 S rRNA were radioactive (see fig.1). The relative amounts of radioactivity incorporated into the RNA moiety of 40 S and 60 S subparticles and ribosomes are compared in table 1. The principal result is that the modification of the 3'-end of 18 S rRNA is achieved far more readily on treatment of 40 S subparticles than on treatment of ribosomes.

A smaller RNA species, corresponding to tRNA was labelled to a variable extent in both subparticles and polysomes (fig.1). In the case of the subparticles, this tRNA is probably a contaminant since it could be removed by heating to $30-40°C$. It was noticed that some tRNA remained bound to the polysome fraction even after heating to $50°C$. This strongly bound, 3'-labelled RNA is probably peptidyl tRNA.

Table 1
3'-end labelling of rRNA molecules within intact subparticles or polysomes

Species labelled	rRNA species	Relative amount of label incorporated/mol rRNA
40 S subparticle	18 S	34
60 S subparticle	28 S	7
	5.8 S	n.d.
	5 S	n.d.
Polysomes	28 S	5
	18 S	1
	5.8 S	n.d.
	5 S	n.d.

n.d., not detectable

The amount of ^{32}P attached to the 3'-ends of rRNA molecules was measured by scintillation counting either of fractions from sucrose gradients or of bands cut from dried 25% denaturing polyacrylamide gels. The amount of RNA applied to gradients or gels was measured by absorbance at 260 nm. Up to 20% of the available [5'-^{32}P]pCp was incorporated into 18 S rRNA of isolated 40 S subparticles. The labelling was carried out in duplicate and the results of the two experiments were almost identical

3.2. Extent of labelling of rRNA components within protein-deficient 60 S subparticles

Treatment of the larger subparticle with high-salt (e.g., 2.75 M NH_4Cl/69 mM $MgCl_2$) yields a core-particle and split-protein fraction comprising ~8 proteins [8,9]. The core-particle fraction is inactive in both the puromycin reaction and in poly(U)-directed poly-phenylalanine synthesis but activity is recovered when core-particles and split-proteins recombine. The high-salt treatment could influence the accessibility of 5 S rRNA and 5.8 S rRNA to T4 RNA ligase because the peptidyl transferase centre of the ribosome is inactivated. However, the accessibility of the 3'-ends of rRNA molecules within these core-particles to end-group labelling was unchanged from that in intact 60 S subparticles, i.e., the removal of up to 8 proteins from the 60 S subparticles did not uncover the 3'-ends of 5 S and 5.8 S rRNA and the relative amount of pCp attached to 28 S rRNA was the same as for intact subparticles.

3.3. Activity of modified 40 S and 60 S subparticles in cell-free protein synthesis

The high specific activity of the [5'-^{32}P]pCp label leads to the incorporation of high levels of radioactivity (up to 20% of the available labelled pCp) although ⩽4–5 molecules/10 000 may be modified at the

Table 2
Activity in cell-free protein synthesis of polysomes and subparticles after treatment to label the 3'-ends of rRNA components

Species	Treatment	Activity in cell-free protein synthesis (% control)
Polysomes	None	100
	7% DMSO	75
	pCp labelling	77
40 S subparticle	None	100
	7% DMSO	82
	pCp labelling	86
60 S subparticle	None	100
	7% DMSO	101
	pCp labelling	107

The assay system in [7] was used. Samples were diluted with either 0.25 M sucrose/25 mM KCl/50 mM Tris–HCl (pH 7.6) or with 7% (v/v) dimethyl sulphoxide/0.25 M sucrose/25 mM KCl/50 mM Tris–HCl (pH 7.6) before addition to the standard assay. The control samples incorporated 3 μCi phenylalanine/assay, equivalent to the incorporation of 18 residues of phenylalanine/ribosome
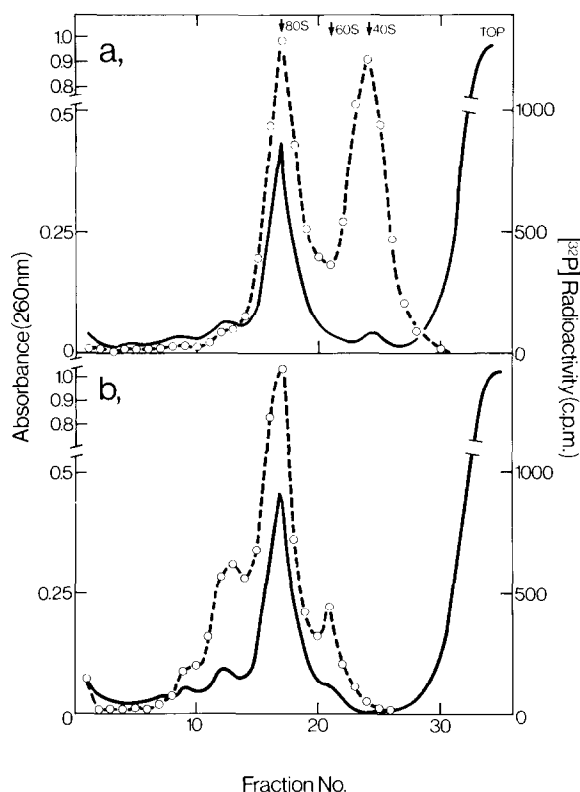

Fig.2. Interaction of labelled 40 S and 60 S subparticles to form ribosomes and polyribosomes in cell-free protein synthesis. Subparticles (8 A_{200} units in 50 μl) were labelled as above. A sample (0.16 A_{260} units in 1 μl) of labelled 40 S or 60 S subparticles was added to a system for cell-free rabbit globin synthesis [7] comprising a polyribosome fraction (5 A_{260} units/assay), pH 5 enzyme fraction and an energy source, and incubated at 37°C for 15 min. The reaction mixture was cooled in ice then layered onto a 15–45% linear sucrose gradient in 0.1 M NH_4Cl/5 mM $MgCl_2$/1 mM dithiothreitol/20 mM Tris–HCl (pH 7.6). The gradient was centrifuged for 16 h at 16 000 rev./min using a Beckman SW27 rotor. The gradients were pumped through a Uvicord II ultraviolet adsorptiometer and 35 fractions of ~1 ml were collected. Trichloroacetic acid solution (4 ml 10% (w/v)) was added to each fraction and left overnight at 4°C. The precipitate was collected by filtration through Nuflow cellulose-acetate filters (Oxoid Ltd.) and washed with hot 5% (w/v) trichloroacetic acid, cold 5% (w/v) trichloroacetic acid and 1:1 (v/v) ethanol/ether mixture. The radioactivity of the dried filters was measured by scintillation spectrometry (Beckman LS-150). Alone, labelled 40 S subparticles sedimented as a single peak with a maximum at fraction 24. Labelled 60 S subparticles sedimented a peak with a maximum at fraction 21. (a) Polyribosome fraction incubated with ^{32}P-labelled 40 S subparticles; (b) polyribosome incubated with ^{32}P-labelled 60 S subparticles. (——) absorbance at 260 nm; (– –o– –) radioactivity (cpm) above background.

3'-end. Although the level of modification is low, the available evidence suggests that the molecular species that are labelled are representative. The conditions used for 3'-end group labelling appeared to have no adverse effect on the activity of the subparticles in cell-free protein synthesis. Thus the level of dimethyl sulphoxide present in the reaction had little effect (table 2) and the activity of samples that had been labelled was close to that of the non-treated controls (table 2). However, these data do not directly reveal the activity of modified subparticles. Independent experiments showed that labelled 40 S and 60 S sub-particles retained their capacity to interact with their respective non-treated subparticles to form 80 S ribosomes. Labelled subparticles were also incorporated into ribosomes and polyribosomes in cell-free globin synthesis (see fig.2). Thus the capacity of subparticles to form ribosomes and to participate in protein synthesis was not apparently affected by modification of the 3'-ends of 18 S and 28 S rRNA.

From the results described in table 1 we have drawn the following conclusions:

(i) The 3'-end of 18 S rRNA is in an exposed position at the surface of 40 S subparticles. Ribosome formation greatly reduces the accessibility of the 3'-end in the labelling reaction, possibly as a result of a conformational change distant from the site of subparticle interaction. An alternative, and preferred, explanation is that the 3'-end of 18 S rRNA is situated close to the interface formed by the interaction of action of 40 S and 60 S sub-particles and so is no longer accessible to RNA ligase.

(ii) The 3'-end of 28 S rRNA is located at the surface of the 60 S subparticle, although it is less exposed than the 3'-end of 18 S rRNA in 40 S subparticles. Ribosome formation only slightly alters the accessibility of the 3'-end to labelling. Therefore, we have concluded that the 3'-end of 28 S rRNA is not immediately adjacent to the site of subparticle interaction.

(iii) The 3'-ends of 5 S rRNA and 5.8 S rRNA were unavailable for labelling in polysomes, in isolated 60 S subparticles and in protein-deficient core-particles. This suggests that the 3'-ends of both these molecules are either not in exposed positions at the surface of the 60 S ribosomal subparticle or are otherwise protected from the action of T4 RNA ligase.

These conclusions are similar to those based on studies of the accessibility of the 3'-ends of rRNA species within the bacterial ribosome. This similarity is to be expected if the functional roles of the 3'-ends of the corresponding rRNA species have been conserved during evolution.

Several studies have indicated that within bacterial 30 S ribosomal subparticles, nucleotides near to the 3'-end of 16 S rRNA lie exposed at the surface [10–13]. In 70 S ribosomes, however, nucleotides near to the 3'-end of 16 S rRNA have been shown to be no longer accessible to kethoxal modification [10] and the 3'-terminal residue does not bind fluorescent dye, although it is still accessible to periodate oxidation [12]. The 3'-terminal residue of 23 S rRNA had the same reactivity within both 50 S subparticles and 70 S ribosomes and was found to be accessible to both fluorescent dye and to periodate oxidation. By combining the techniques of immunology and electron microscopy [14], the 3'-end of 23 S rRNA was shown to be near to the surface of the 50 S subparticle but remote from the site of interaction with 30 S subparticles. It was further shown [12], that within both 50 S subparticles and 70 S ribosomes the 3'-end of 5 S rRNA is relatively inaccessible both to periodate and to fluorescent dyes. There is no separate 5.8 S rRNA species in bacterial ribosomes.

The ability to introduce radioactive label into the 3'-ends of the RNA moiety of subparticles and ribosomes may be of general use in topographical studies. For example, the presence of a specific label of high specific activity at the 3'-end makes it possible to map from the 3'-end the positions of RNA sequences within the ribosome and subparticles that are attacked by nucleases. The same strategy may also be of value in the study of other ribonucleoprotein particles.

Acknowledgements

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