PROCESSING OF PRECURSOR PARTICLES CONTAINING 17S rRNA IN A CELL FREE SYSTEM

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Received 17 October 1974

1. Introduction

A number of studies have shown that 16S rRNA is not a primary transcription product (for review see [1], but rather that it is preceded by at least one precursor molecule (17S RNA). This molecule referred to as p16 RNA, is known to have about 200 more nucleotides than the mature 16S RNA (m16 RNA) found in the 30S ribosome [1–5].

In the cell p16 RNA is apparently found in precursor particles which contain a large proportion of the 30S ribosomal proteins [6,7]. Under normal growth conditions these precursor particles mature to 30S ribosomes.

Processing of p16 RNA involves cleavages at both ends of the RNA molecule [1–5] probably by ribonucleases. Previous studies with a strain, that contained a thermolabile ribonuclease II suggested that this enzyme is involved in the maturation of p16 to m16 RNA [8,9]. However, genetic analysis of this mutant strain indicated very clearly that the assignment of RNase II as the maturation enzyme was erroneous since strains which still carried the thermolabile RNase II matured p16 RNA normally [10]. Therefore we tried to reinvestigate this problem and to identify an activity(ies) in a cell free system which could process 17S RNA in a precursor particle to 16S RNA.

The experiments presented here provide evidence that such an activity can be identified in extracts of *E. coli* cells and that it is neither ribonuclease I, II nor III, but rather a different enzyme which is tentatively referred to as ribonuclease M (maturation).

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2. Materials and methods

Precursor particles were isolated from *E. coli* strain N7081, which is similar to strain N7082 [11]. Maturation activity was isolated from extracts of *E. coli* strain N7060 [10]. Both strains are devoid of ribonuclease I and are characterized by a thermolabile ribonuclease II; in addition N7060 has a modified polynucleotide phosphorylase.

Carrier free $H_3[^{32}P]O_4$ (50 mCi/ml) was purchased from New England Nuclear Corp. Lysozyme (egg white grade I) was purchased from Sigma Chemical Comp., DNase I (RNase free) from Worthington Biochemical Corp., sucrose for density gradients from Schwarz/Mann and polyethyleneglycol 20,000 from Fisher Scientific Products. Kodak X-ray film (NS2T, 12.5 X 17.5 cm) was used for autoradiography.

2.1. *Isolation of precursor particles*

Cells of strain N7081 were grown at 18°C in minimal medium (per liter: 14.5 g Tris, 100 mg $MgSO_4$, 500 mg NaCl, 150 mg KCl, 500 mg NH_4Cl, adjusted to pH 7.4 with HCl) containing 50 mg of L-methionine and 1 mg of thiamin–HCl per liter and 0.2% glucose. One liter of this medium was supplemented by 15 ml of a phosphate-deprived rich medium (100 g of Difco Nutrient Broth and 50 g of Difco Casamino Acids dissolved in 900 ml of water; inorganic phosphate was precipitated according to Hayes et al. [12]. Cells were labeled by the addition of carrier free $H_3[^{32}P]O_4$

(15 mCi/50 ml of culture) at an A_{560} of 0.6 for 6% of the generation time (18 min). The culture was chilled on ice, and all subsequent steps in the preparation of precursor particles were carried out at 4°C. Cells were pelleted by low speed centrifugation and washed once in 0.01 M Tris–HCl (pH 7.5) containing 0.1 mM magnesium acetate. The washed cells (approx. 5×10^{10}) were resuspended in 1 ml of the same buffer containing 0.1 M NH_4Cl. EDTA pH 7.2 (final concentration 0.01 M), lysozyme (final concentration 0.5 mg/ml) and DNase 1 (final concentration 20 μg/ml) were added and the cells lysed by five cycles of thawing and freezing in a mixture of methanol–dry ice. After cell lysis the magnesium concentration was raised to a final concentration of 0.02 M. The cell lysate was clarified by low speed centrifugation (20 000 *g*). The supernatant was used as a source for precursor particles.

Precursor particles containing 17S RNA were also isolated by centrifugation of such preparations on sucrose gradients (5–20% in Tris–HCl pH 7.5, 0.01 M magnesium acetate and 0.1 M NH_4Cl).

2.2. *Isolation of maturation activity*

Cells of strain N7060 were grown at 37°C in 50 liters of yeast extract-glucose medium (10 g Difco yeast extract, 34 g KH_2PO_4, 8.4 g KOH, 10 g glucose per liter) with aeration in a Biogen fermenter and stored at −70°C. 40 g of cells were ground with alumina [13] and extracted with Tris–HCl pH 7.5, containing 0.01 M Mg^{2+}, 0.03 M NH_4Cl and 1 mM dithioerythritol (1 ml of buffer/g wet cells). After the addition of DNase 1 (10 μg/g wet cells) the suspension was kept on ice for 15 min. The clarified extract (40 min at 20 000 *g*) was centrifuged in a fixed-angle rotor for 2 hr at 200 000 *g*. The ribosome pellet was washed once with extraction buffer containing 0.3 M NH_4Cl. The ribosomes were pelleted and resuspended in the same buffer. The solution was clarified by low speed centrifugation (20 000 *g* for 20 min) and the NH_4Cl concentration was raised to 0.6 M. After 2 hr of shaking slowly in the cold the ribosomes were pelleted again (2 hr at 200 000 *g*). The supernatant was stored on ice. It will be referred to as ribosome wash (0.3–0.6 M NH_4Cl).

Gel filtration on Sephadex G-150: The ribosome wash (0.3–0.6 M NH_4Cl) was concentrated in polyethyleneglycol 20 000 at about 0°C by a factor of 7 and then applied with dextrane blue to a Sephadex G-150 column (95 × 1.4 cm) equilibrated with 0.01 M Tris–HCl pH 7.5 and 1 mM dithioerythritol.

For inactivation of RNase II the ribosome wash was heated for 4 min at 50°C in a waterbath. The solution was clarified by low speed centrifugation and the supernatant concentrated and gel filtrated as above.

Assays for RNase II and III were carried out as described previously [14,15].

2.3. *Assay for maturation activity*

^{32}P-labeled precursor particles in cell lysates of strain N7081 (diluted by a factor of 20 to 100 with 0.01 M Tris–HCl pH 7.5, 0.01 M Mg^{2+}, according to the specific radioactivity of the RNA), or precursor particles from sucrose gradients, were incubated with enzyme fractions in the presence of 0.01 M Tris–HCl pH 7.5, 0.01 M Mg^{2+}, 0.016 M NH_4Cl and 1 mM dithioerythritol at 37°C in a total volume of 30 μl. The reaction was stopped by addition of a mixture containing sodium dodecyl sulfate (final concentration 0.1%), EDTA (2 mM), sucrose (20%), and Bromphenol Blue (dye marker). Samples were applied to 3% polyacrylamide slab gels and run in Tris–acetate buffer pH 7.6 with 0.2% sodium dodecyl sulfate for 4 hr at 110 V [14]. The slab gels were either dried and exposed for 16 hr to X-ray films or cut longitudinally to separate samples. The gel strips were frozen on dry ice and sliced into equal fractions. Each slice was incubated in a scintillation vial overnight at 65°C in the presence of 0.3 ml H_2O_2 (30%). Radioactivity was counted in 5 ml of Insta-gel (5.5 g PPO, 0.1 g POPOP, 667 ml toluene, 333 ml Triton X-100).

3. Results and discussion

For in vitro maturation experiments, precursor particles were incubated at 37°C with enzyme fractions of strain N7060 and analysed on polyacrylamide gels. Cell lysates (fig. 1) or sucrose gradient fractions were used as source for precursor particles. In order to prevent self digestion of the precursor particles they were incubated in presence of 0.01 M Mg^{2+} and 0.1 M NH_4Cl and the protein concentration of the substrate was reduced to 10 μg/ml or less. Under these conditions even after 2 hr of incubation at 37°C unspecific degradation of 23S and 17S RNA is no

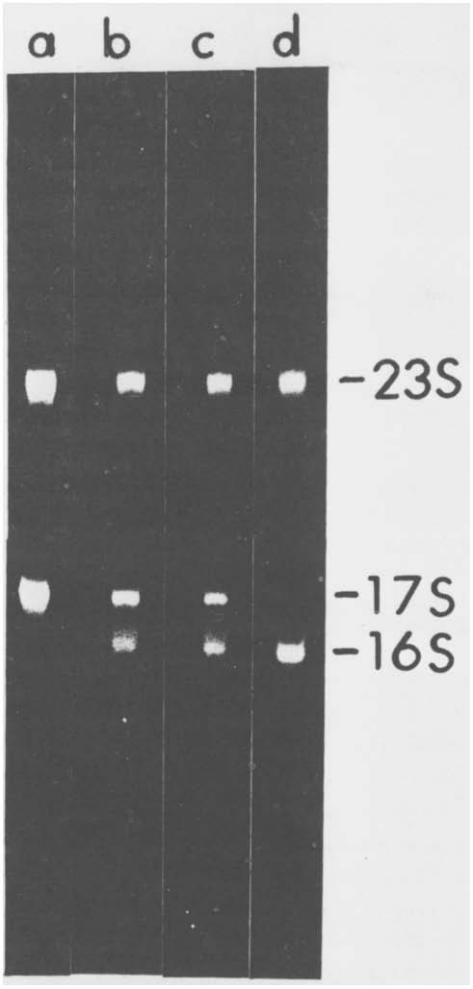

Fig. 1. Transformation of 17S precursor RNA to 16S RNA. Lysates of strain N7081 containing ^{32}P-labeled precursor particles with 17S RNA were diluted 1:20 and incubated with or without enzyme fractions, pool b (heated ribosome wash, 0.3–0.6 M NH_4Cl, fractionated on Sephadex G-150, as described in table 1). a) 60 min incubation without enzyme, b) 60 min with enzyme, c) 120 min with enzyme, d) marker for 23S and 16S RNA (^{32}P-long term label at 37°C). RNA molecules were analysed on polyacrylamide gels by autoradiography. For assay conditions see Materials and methods.

more than 20% and no appreciable amounts of 16S RNA are formed (fig. 1).

Fractionation of extracts of strain N7060 showed that the maturation activity was mainly attached to the ribosomes. Stepwise washing of ribosomes with increased NH_4Cl concentrations eluted the enzyme. Most of the activity was released between 0.3 and 0.6 M NH_4Cl. This wash fluid was concentrated to increase the protein content and assayed for known ribonucleases. As can be seen in table 1, the ribosome wash (0.3–0.6 M NH_4Cl) includes measurable RNase III and high RNase II activity. This was expected since most of the RNase III activity is eluted from the ribosome pellet by 0.3 M NH_4Cl [15], while RNase II is not eluted by such a treatment [16].

Strain N7060 contains a thermolabile RNase II [10]. The ribosome wash (0.3–0.6 M NH_4Cl) was therefore heated for 4 min at 50°C and clarified by centrifugation. RNase II activity in the supernatant was reduced to 0.4% by this treatment, RNase III to 20%, while maturation activity was not appreciably changed as judged by inspection of autoradiograms (table 1). These results rigorously rule out RNase II as the maturation enzyme and indicate rather strongly that it is not RNase III either. Heated ribosome wash (0.3–0.6 M NH_4Cl) was concentrated and applied to a Sephadex G-150 column. Fractions were pooled according to maturation activity. As shown in table 1 there is no RNase II activity left in these fractions and RNase III activity is as low as 1% of the original activity in the ribosome wash, which is at the limit of detection by this assay. On the other hand there is appreciable maturation activity converting 17S RNA to 16S RNA as can be judged by inspection of autoradiograms (fig. 1). Since the enzyme is not pure, there is some unspecific degradation of 23S and 17S RNA, increasing with incubation time and with the amount of enzyme added to the incubation mixture. The transformation of 17S to 16S RNA by the same enzyme fraction was also observed when precursor particles isolated from sucrose gradients were used, which contained only 17S RNA. This experiment rules out the possibility that the 16S RNA observed in the maturation reaction was derived from 23S RNA when crude cell lysates were used as the source of substrate. The experiments presented here demonstrate very clearly that a specific activity exists in *E. coli* cells which can transform 17S rRNA in a precursor particle to 16S rRNA. They also show that this activity cannot be either RNase I, II or III. The possibility that this activity might be RNase P [17] could not be ruled out by the experiments reported here. This is rather a remote possibility however, since a) RNase P is found mainly in a different fraction of the ribosome

Table 1
RNase II and RNase III activity of various fractions containing maturation activity

		Enzyme activity				
		RNase II		RNase III		Maturation
	Heating	cpm	%	cpm	%	
Ribosome wash	–	72 000	100	825	100	+
(0.3–0.6 M NH_4Cl)	+	320	0.4	165	20	+
Sephadex G-150 fractions						
pool a	+	24	0	7	1	±
pool b	+	10	0	8	1	+
pool c	+	0	0	0	0	±

Heated ribosome wash (0.3–0.6 M NH_4Cl) was fractionated on Sephadex G-150 (see Materials and methods). 64 Fractions with 1.7 ml each were collected and every second tube was assayed for maturation activity by analysis of reaction mixtures on polyacrylamide gels. Fractions were pooled according to maturation activity (pool a, nos. 23–31); b, nos. 32–42; c, nos. 43–52), concentrated and used to assay RNase II (input 2300 cpm/assay, blank 68 cpm), RNase III (input 1800 cpm/assay, blank 33 cpm). Pool b contained the highest maturation activity, an experiment with this fraction is shown in fig. 1.

wash than RNase M (17, 0.2 M NH_4Cl wash) and b) the enzyme RNase P showed thus far a very restricted specificity, for precursor tRNA molecules only.

It is also unclear at present to what extent the product of the RNase M reaction resembles m16S RNA. Unfortunately, comparison of finger prints of m16 and the RNase M product would not suffice for such an analysis, since obviously such a product would contain many oligonucleotide in common with m16S RNA. Therefore only comparison of sequences of the maturation product with those of m16S RNA would decide to what extent the process in vitro resembles that in vivo.

Since the product of the maturation reaction migrates on the gels in exactly the same place as m16 rRNA, and since p16 RNA differs from m16 RNA in nucleotides on both its ends (1–4), one should entertain the possibility that there are two enzymes in the maturation activity, or that a single enzyme introduces two endonucleolytic cuts one near the 5′ end and the other near the 3′ end of the p16 RNA molecule. A reaction of such a nature by a single enzyme was demonstrated in the in vitro maturation of the precursor molecule for 5S RNA in *Bacillus subtilis* [18].

Table 2
Transformation of 17S precursor RNA to 16S RNA

Time of incubation (hr)	Enzyme	Area of 17S RNA (%)	Area of 16S RNA (%)
2	–	85	15
1	+	69	31
2	+	32	68

Incubation of cell lysates with and without enzyme was as described in legend to fig. 1. The distribution of labeled RNA molecules was determined on polyacrylamide gels by counting the radioactivity of 30 gel slices (1.5 mm thick) collected from the top of the gel. The area under the radioactivity profile of 17S and 16S RNA was measured by weight (background subtracted). The distribution of both RNA species is given in percent of the total area.

Acknowledgements

Supported by grants from the NSF GB34158, and NIH GM 19821.

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