THE ISOLATION OF METHYL DEFICIENT PHENYLALANYL TRANSFER RNA FROM E. COLI

Michel Revel and Uriel Z. Littauer
Biochemistry Section, Weizmann Institute of Science, Rehovoth, Israel

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The study of the role of methylated bases in transfer RNA (t-RNA) has been made possible by the discovery by Mandel and Borek (1961) that methyl deficient s-RNA is synthesized by a "relaxed" methionine requiring mutant of E. coli during methionine starvation. Preparations of s-RNA from methionine starved cells and from normal cells have been compared but no significant differences in the ability of these RNA's to accept (Starr, 1963; Littauer et al., 1963; Peterkofsky et al. 1964) and to transfer amino acids (Littauer et al., 1963; Peterkofsky et al. 1964) have been detected. Although this may indicate that the methylated bases do not participate in these functions of t-RNA, it should be recalled that "methionine starved" RNA preparations consist of a mixture of equal amounts of methyl deficient and normal s-RNA. Therefore, small differences in the activities of these RNA species in vitro, which could be very critical in vivo, might not have been detected with such crude RNA preparations. These considerations prompted us to develop a method for the isolation of methyl deficient t-RNA. Phenylalanyl transfer RNA (phe-t-RNA) from E. coli G-15, a relaxed double mutant which can be starved for methionine or for histidine, was selected for these studies. The present report describes the isolation of methyl deficient phe-t-RNA by chromatography on methylated albumin kieselguhr (MAK) columns.

METHODS: E. coli G-15 RC^{rel} $Meth^-Hist^-Biot^-$ was kindly supplied by Dr. G. Stent. Normal cultures were grown in minimal medium (Hershey and Chase, 1952) supplemented with 20 µg per ml L-methionine, 20 µg per ml L-histidine, 0.01 µg per ml biotin and 0.01 M phosphate. Cells were harvested in the middle of the logarithmic growth phase and the s-RNA isolated immediately according to Zubay (1962). The RNA was then incubated at 37° in Tris-HCl pH 8.8, 0.2 M for 5 hours. Methionine starved cultures were grown under the same conditions but only 4 µg per ml methionine was added. After growth had stopped, the culture was incubated with continued

aeration for an additional 3.5 hours. Histidine starved cells were given a normal amount of methionine but only 3 μg per ml histidine. Starved cultures yielded 50-100% more s-RNA than normal cultures.

The s-RNA preparations were charged (Berg. et al. 1961) with C^{14}phenylalanine (330 μc per μm) or H^{3}phenylalanine (1500 μc per μm) using a streptomycin supernatant (Daniel and Littauer, 1963) from E. coli $K_{12}W6$ or a purified phenylalanyl s-RNA synthetase. The chromatographic patterns obtained with both enzyme preparations were identical. For the MAK chromatography the method of Yamane and Sueoka (1963) was slightly modified. The height of the column was increased by 1/3 and the volume of the buffers used for elution was doubled. Two ml fractions were collected and aliquots were removed, precipitated with 5% TCA and filtered onto Millipore membranes. The radioactivity was measured in a liquid scintillation counter. RNA fractions from the MAK column were pooled, dialyzed against water for 3 hours and precipitated with 2.5 volume ethanol in 0.1 M NaCl. Ribosomal RNA which did not accept amino acids or methyl groups was added as carrier. The RNA samples were incubated in Tris-HCl, pH 7.4, 0.1 M at 37°C for 5 hours with 1 mg per ml pronase (Calbiochem), (to eliminate possible traces of RNase the pronase solution was pre-incubated for 90 minutes in Tris-HCl pH 7.4, 0.1 M at 37°C) treated 3 times with phenol and precipitated from the aqueous phase with ethanol. Recovery of RNA was about 85%.

RESULTS: 1. Chromatography of normal phenylalanyl-t-RNA

As reported by Sueoka and Yamane (1962) phe-t-RNA can be separated from the bulk of the s-RNA by chromatography on MAK columns. Figure 1A shows that in the case of s-RNA from bacteria grown under normal conditions, phe-t-RNA is eluted from the column at higher salt concentration than most of the s-RNA and is resolved into a major (tube # 67) and a minor (tube # 63) component. The identity of the minor component is unclear. Its amount varies from one preparation of s-RNA to another and increases consistently when the s-RNA is stored for prolonged periods of time. The biological activities of the two fractions are similar with respect to both acceptance and polyU directed transfer of amino acids. The phe-t-RNA eluted from the MAK column is contaminated by some proteinaceous material which can be removed by treating the RNA with pronase. The RNA obtained by this procedure is about 3 - 4 times more active for acceptance and transfer of phenylalanine than the unfractionated s-RNA.

2. Chromatography of a mixture of normal and methyl deficient phe-t-RNA

The s-RNA obtained from the methionine starved culture was charged with C^{14}phenylalanine and mixed with normal s-RNA charged with H^3phenylalanine. The chromatographic pattern obtained is shown in Figure 1A. In addition to the

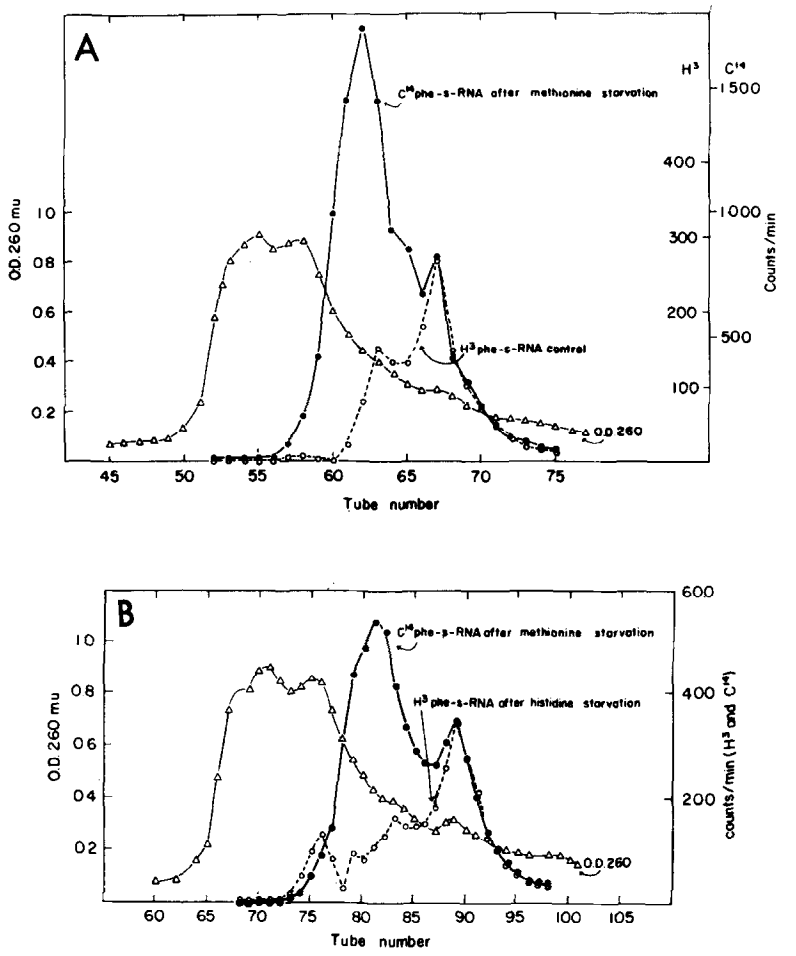

Figure 1. MAK chromatography of E. coli G-15 phe-t-RNA

1A. Normal and methionine starved RNA. 170 μg H^3phenylalanyl s-RNA from normal culture and 150 μg of C^{14}phenylalanyl s-RNA from a methionine starved culture were mixed with 700 μg cold normal s-RNA and submitted to MAK chromatography. Δ - Δ, adsorbancy at 260 mμ. Two ml aliquots were taken to measure TCA-precipitable radioactivity ●-● C^{14}, O--O H^3 radioactivity. 1B. Histidine and methionine starved RNA. 375 μg of H^3phenylalanyl s-RNA from a histidine starved culture, 100 μg C^{14}phenylalanyl s-RNA from a methionine starved culture and 500 μg cold normal s-RNA were mixed and analyzed as above. 1 ml was used to measure TCA precipitable radioactivity.

normal peaks of phe-t-RNA, the RNA after methionine starvation contains a new fraction of phe-t-RNA. The new component is eluted at a salt concentration lower than the normal phe-t-RNA and amounts to about 60% of the total phe-t-RNA in agreement with the amount of total methyl deficient s-RNA accumulating during methionine starvation (Mandel and Borek, 1961).

In order to establish that the appearance of the new phe-t-RNA is specific to methionine starvation a comparison with "histidine-starved" s-RNA was made. The new phe-t-RNA peak seen after methionine starvation does not appear when the bacteria are starved for histidine (Fig. 1B). The methyl content of each s-RNA preparation was measured. With a crude E. coli RNA methylase, methionine-starved s-RNA accepted 19.7 mμmoles of methyl residues per mg RNA. The corresponding value for both normal and histidine starved RNA was 0.1 mμmole per mg RNA.

From these studies it can be concluded that in methionine starved cells, a new phe-t-RNA appears which might correspond to methyl deficient phe-t-RNA. Similar results were obtained with RNA isolated from E. coli $K_{12}W6$ after methionine starvation.

3. Is the phe-t-RNA produced during methionine starvation, methyl deficient phe-t-RNA?

To answer this question the amount of methyl groups per phe-t-RNA chain present in this fraction was compared to that in normal phe-t-RNA. Since in the fractions obtained from the MAK chromatography phe-t-RNA represents less than 10% of the chains, additional purification of this particular t-RNA was required. This purification was achieved by using the property of phe-t-RNA to attach specifically to polyU-ribosome complexes, which can be separated from the other free s-RNA chains (Kaji and Kaji, 1963).

In a first series of experiments, s-RNA from methionine starved cultures was charged with H^3phenylalanine and submitted to chromatography on the MAK column. The fractions of phe-t-RNA were pooled as indicated in figure 2 and the RNA recovered as described in Methods. RNA fractions I and III were then methylated in vitro with C^{14}-CH_3-S adenosyl methionine and E. coli streptomycin supernatant. Under these conditions, the methyl deficient s-RNA only would be expected to be labeled (Srinivasan and Borek, 1963; Gold and Hurwitz, 1963). Since most of the radioactive phenylalanine had been lost from the s-RNA at this stage, the charging with H^3phenylalanine was repeated. The doubly labeled RNA was adsorbed on ribosomes previously supplemented with polyU and the complex was isolated by

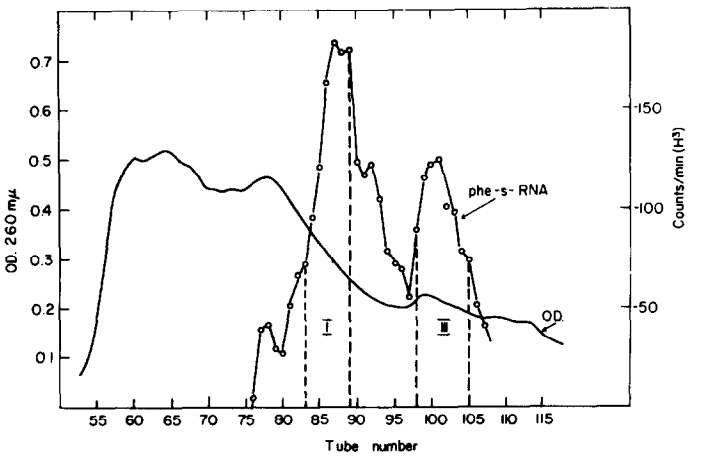

Figure 2. Separation by MAK chromatography of normal and methyl deficient phe-t-RNA

1 μg of methionine starved s-RNA was charged with H^3phenylalanine, and used for the chromatography. The tubes between the vertical lines were pooled and the RNA recovered as indicated in Methods. Δ-Δ, absorbancy at 260 mμ, O-O, H^3 TCA precipitable radioactivity in 0.4 ml aliquots.

filtration on Millipore membranes (Nirenberg and Leder, 1964). A significant amount of non-specific binding also took place under these conditions and was measured in the absence of polyU. The results presented in Table 1 indicate that per chain of phe-t-RNA adsorbed (column 5), fraction I which corresponds to the RNA appearing after methionine starvation is much richer in radioactive methyl groups than fraction III which contains normal phe-t-RNA[1]. When the results are expressed as per cent of C^{14}-CH_3 adsorbed (column 6 and 7) this value is 4 to 8 times higher in fraction I than in fraction III (e.g., 3.6/0.48 x 38/77). These data, therefore, strongly support the idea that fraction I is methyl deficient phe-t-RNA while fraction III is fully methylated.

To confirm this finding the inverse of the previous experiment was performed. The bacteria were first grown in the presence of C^{14}-CH_3-methionine and then starved for methionine during 3.5 hours. The normal phe-t-RNA should now be

[1] Although the method used gives satisfactory qualitative results it does not seem to be accurate enough to allow the calculation of the number of methyl groups per chain. Nevertheless, the data indicate, as found previously (Littauer et al., 1963), that phe-t-RNA contains relatively more methyl groups than the average of the other s-RNA chains.

Table 1

The ratio of C^{14}methyl to H^3phenylalanine in the two RNA fractions isolated from the MAK column and methylated in vitro

Expt. No.	RNA fraction*	RNA from MAK column		RNA adsorbed on polyU ribosomes			% adsorbed on polyU ribosomes**	
		1	2	3	4	5	6	7
		H^3phe	$C^{14}CH_3$	H^3phe	$C^{14}CH_3$	$\frac{CH_3 \times 10}{phe}$	H^3phe	$C^{14}CH_3$
		cpm		cpm			per cent	
1	I	650	3500	235	135	5.75	36	3.85
	III	450	3100	245	21	0.87	55	0.69
2	I	200	4360	154	156	10.0	77	3.60
	III	610	3300	232	16	0.69	38	0.48

*The numbers refer to the fractions indicated on Figure 2.
**Column 6 = column 3/column 1 x 100; column 7 = column 4/column 2 x 100.
The experimental procedure is described in the text. Adsorption on ribosomes was measured as Nirenberg and Leder (1964). The data presented were obtained after deduction of the control values in the absence of polyU (cpm; exp. 1 H^3 : I, 25; III, 25; C^{14} : I, 250; III, 250. Exp. 2 H^3 : I, 8; III, 22; C^{14} : I, 378; III, 426). One µmole of phenylalanine and of methyl residue correspond to 350 and 35 cpm respectively.

labeled while the methyl deficient species should not contain any radioactivity. The s-RNA prepared from such a culture was charged with H^3phenylalanine and fractions I and III were isolated after MAK chromatography as above. The chromatographic pattern indicated that the C^{14}-CH_3 label followed the distribution of the optical density. The ratio of C^{14} to H^3 in fraction I was, therefore, much higher than in fraction III, due to the higher amount of other t-RNA chains contaminating fraction I. Tp purify phe-t-RNA, the doubly labeled RNA fractions were incubated with ribosome and a limiting amount of polyU and the complex formed was examined by sucrose gradient centrifugation (Spyrides, 1964). The C^{14}/H^3 ratio was measured in the polyribosome region where most of the ribosomes have combined with the polyU and where a minimal binding of other t-RNA chains would be expected. The results of this experiment are presented in table 2. With both RNA fractions

Table 2

The ratio of C^{14}methyl to H^3phenylalanine in the two RNA fractions isolated from the MAK column after in vivo labeling with C^{14}-CH_3 methionine

RNA fraction	Distribution of label in the RNA from various portions of the sucrose gradient					
	> 100 S		70 - 100 S		< 70 S	
	H^3phe	$C^{14}CH_3$	H^3phe	$C^{14}CH_3$	H^3phe	$C^{14}CH_3$
	per cent of total label					
I	24	1.4	55	11	21	88
III	25	5.0	40	17	35	79
	C^{14}/H^3 ratio x 100					
I	5.8		20		420	
III	20.0		43		230	

The experimental procedure is described in the text. Incubation and sucrose gradient as Spyrides (1964). Total radioactivity introduced was in cpm: I : H^3, 1,000; C^{14}, 4,000; III : H^3, 2,000; C^{14}, 1,950. 20 fractions were collected and analyzed for adsorbancy at 260 mμ and TCA precipitable C^{14} and H^3 radioactivity.

about 25% of the phe-t-RNA is bound to ribosomal aggregates heavier than 100 S. However, the amount of C^{14}-CH_3 in this region is 3.5 times higher in the case of RNA III than with RNA I (20/5.8). A two fold difference is still observed in the 70 to 100 S region of the gradient where about one half of the total phenylalanine input is bound. These results are again in agreement with the conclusion that fraction I contains a methyl deficient phe-t-RNA.

DISCUSSION: Recently Lazzarini and Peterkofsky (1965) reported that during methionine starvation of a relaxed E. coli mutant a new peak of leucyl-t-RNA appears in the MAK chromatography. In the experiments reported here, MAK chromatography of methionine starved s-RNA reveals the existence of a new phe-t-RNA fraction distinct from the normal species. However, the altered chromatographic behaviour alone does not necessarily imply that the new peak is methyl deficient. In our studies we were able to demonstrate that the phe-t-RNA fraction appearing after methionine starvation is indeed methyl deficient. But it is not yet known if this methyl deficient phe-t-RNA is identical in all other respects, apart from methyla-

tion, with the normal species.

Previous work has shown that methyl deficient phe-t-RNA can accept phenylalanine and participate in the transfer of this amino acid to polypeptide. The present experiments, in addition, indicate that this RNA species binds to the polyU-ribosome complex with an efficiency similar to that of normal phe-t-RNA. A more detailed comparison of the two species is yet needed to elucidate the function of methylation in t-RNA. Efforts are underway to purify further the methyl deficient phe-t-RNA from other t-RNA chains.

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