A SIMPLIFIED METHOD FOR PURIFICATION OF YEAST "RENATURABLE" LEUCINE TRANSFER RNA: THE NUCLEOTIDE SEQUENCE OF FRAGMENTS OBTAINED BY PANCREATIC RIBONUCLEASE DIGESTION

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1. Introduction

Leucine transfer ribonucleic acid ($tRNA^{Leu}$) from baker's yeast has been fractionated into three forms using counter current distribution technique [1]. One of these tRNAs ($tRNA_3^{Leu}$) can be denatured reversibly and further purified by gel filtration [2]. In this communication, we report a simplified purification method, namely, successive chromatography on benzoylated DEAE cellulose and Sephadex G-100. Sequence analysis of the fragments obtained by degradation of this tRNA with pancreatic ribonuclease showed that the chain consists of 85 nucleotides with pGpGpU---- at the 5′ terminus. This tRNA contains no modified adenosine, but contains a new minor nucleoside, N^6-acetyl-5-methylcytidine.

2. Materials and methods

Crude yeast tRNA was prepared according to Holley et al. [3]. Benzoylated DEAE cellulose (BD-cellulose) was prepared by the method of Gillam et al. [4]. Amino acid acceptor activities of tRNAs were assayed by the filter paper disc method of Bollum [5]. Crude aminoacyl tRNA synthetase from yeast was prepared according to Hoskinson and Khorana [6].

Pancreatic RNase, snake venom phosphodiesterase, and bacterial alkaline phosphatase were obtained from Worthington, T_1 RNase from Sankyo Chemical Company and polynucleotide phosphorylase was a gift from P-L Biochemicals. DEAE cellulose was obtained from Whatman, Biogel P-2 from Bio-Rad Laboratories and ^{14}C amino acids from Amersham.

Descending paper chromatography was carried out on Whatman No. 1 paper. The solvent systems used were: (A) isobutyric acid–ammonium hydroxide–water (66:1:33), (B) isopropanol–ammonium hydroxide–water (7:1:2), (C) propanol–ammonium hydroxide–water (55:10:35) and (D) ethylacetate–propanol–water (4:1:2, v/v, upper phase).

Ultraviolet absorption spectra were measured using a Cary 14 UV Recording Spectrophotometer.

3. Results and discussion

3.1. *Chromatography of crude yeast tRNA on BD-cellulose.*

In a preliminary experiment, 23 mg of crude yeast tRNA was applied to a BD-cellulose column (1.8 × 80 cm) and eluted with a linear gradient of 0.5 to 1.2 M NaCl in 0.05 M NaOAc, pH 5.0 at 4° [7]. Total volume of the eluent was 1600 ml. Fractions were collected at a rate of 11 ml per 30 min. Absorbance at 260 nm for every fraction was measured and acceptor activity for 18 amino acids, was assayed for every third fraction. Results for leucine and methionine are shown in fig. 1. Three peaks of $tRNA^{Leu}$ were observed, with the major species as the first peak ($tRNA_a^{Leu}$)*. $tRNA^{fMet}$ was eluted immediately after $tRNA_a^{Leu}$ with substantial overlap.

* This is the species earlier referred to as $tRNA_3^{Leu}$ [1, 2].

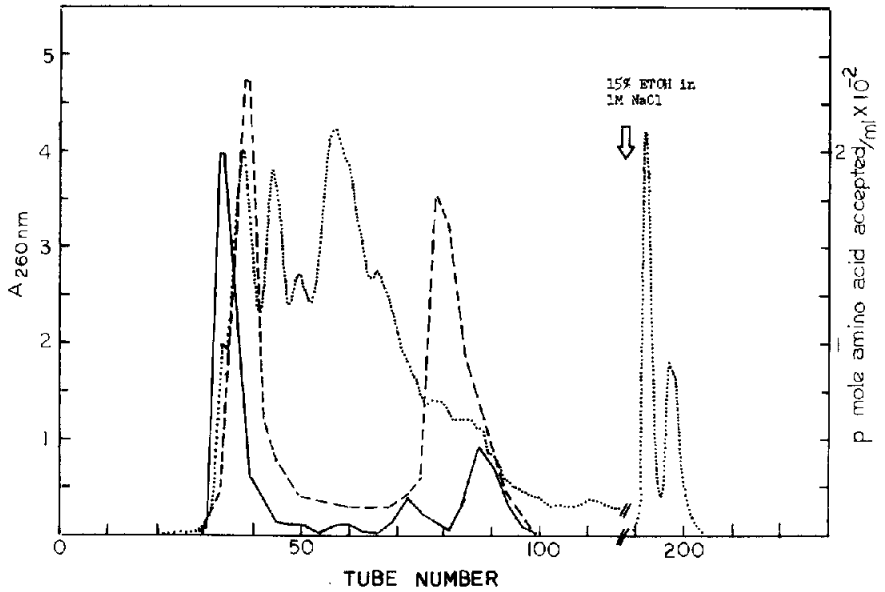

Fig. 1. Chromatography of yeast tRNA on BD-cellulose (for details, see text). Each fraction was measured for absorbance at 260 nm, ... Amino acid acceptor activity was scanned by incubating 25 μl of every third fraction with 0.1 ml of a mixture containing 0.1 M tris (pH 7.4), 5 mM β-mercaptoethanol, 15 mM $MgCl_2$, 0.1 mM EDTA, 2.0 mM ATP, 5 μl of crude aminoacyl tRNA synthetase solution and 25 nCi of ^{14}C-leucine ——, ^{14}C-methionine - - - -, or other ^{14}C amino acids (not shown in this figure).

3.2. *Purification of $tRNA_a^{Leu}$*

Having observed the results described above, we expanded the chromatographic scale with a shallower gradient of NaCl so that $tRNA_a^{Leu}$ could be separated from $tRNA^{fMet}$ with minimum overlap. 5 g crude yeast tRNA was applied to a BD-cellulose column (4.2 X 140 cm). The column was eluted with a linear gradient of NaCl from 0.4 to 0.6 M in 0.05 M NaOAc, pH 5.0 at 4°. The total volume of the eluent was 6 l. Fractions were collected at a flow rate of 12 ml per 20 min and were measured for absorbance at 260 nm and for leucine acceptor activity. The early part of the chromatographic pattern is shown in fig. 2. A first sharp peak contained leucine acceptor activity. Since Gillam et al. [7] reported that $tRNA^{fMet}$ was the first peak to be eluted when crude yeast tRNA was fractionated on BD-cellulose in 10 mM $mgCl_2$ at room temperature, fractions under the first peak were also measured for acceptor activity for other amino acids. Certain amounts of $tRNA^{Val}$ and $tRNA^{Ile}$ were detected. No measurable amount of $tRNA^{Met}$ was found. The acceptor activity of leucine increased 2-fold after the solution was incubated at 60° in 10 mM $MgCl_2$ and cooled slowly to room temperature. Fractions containing $tRNA_a^{Leu}$ were combined and deactivated by a modification of the procedure of Lindahl et al. [2]. The combined solution was dialyzed against 1 mM EDTA, concentrated to a small volume, and redialyzed against 0.01 M tris at pH 6.9 containing 0.5 mM EDTA. This thoroughly dialyzed solution was heated at 60° for 5 min and rapidly cooled to 2° in an ice bath. The solution was then adjusted to 0.02 M $MgCl_2$ and incubated at 25° for 30 min. $tRNA_a^{Leu}$ trapped in denatured form was purified on a Sephadex G-100 column (3 X 120 cm) pre-equilibrated with 0.15 M NaCl, 0.01 M tris pH 6.9, 0.5 mM EDTA and 5 mM $MgCl_2$ in the cold room. The column then was eluted with the same buffer. Fractions of 7.5 ml were collected at the rate of 15 min per tube and measured for absorbance at 260 nm and for leucine acceptor activity. Fig. 3 shows the elution profile. It is evident that the leucine acceptor activity coincides with the 260 nm absorbance in the first peak, indicating a constant specific leucine acceptor activity. Acceptor activities for many other amino acids under this peak as well as for the following peak were also measured and compared

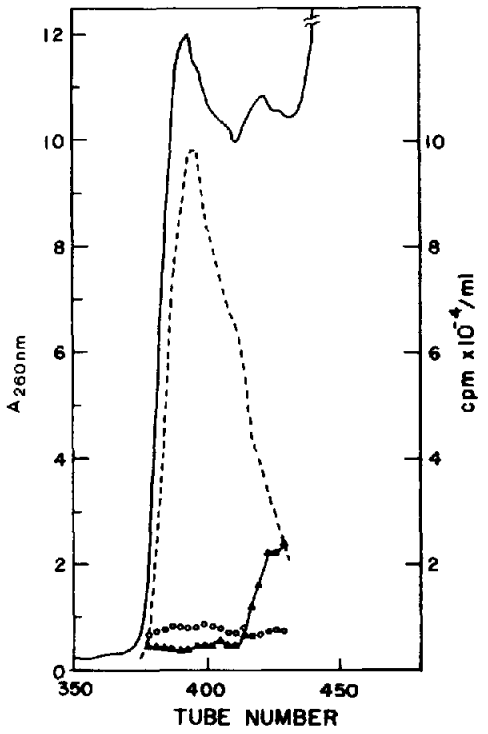

Fig. 2. Isolation of yeast $tRNA_a^{Leu}$ by chromatography of crude yeast tRNA in a BD-cellulose column. Details are in the text. The acceptor activity was scanned by incubating 50 µl from every fourth tube with 50 µl of a mixture containing 0.1 M tris (pH 7.4), 5 mM β-mercaptoethanol, 20 mM $MgCl_2$, 0.1 mM EDTA, 2.5 mM ATP, 5 µl of crude aminoacyl tRNA synthetase solution and 0.05 µCi of ^{14}C-leucine------, ^{14}C-valine ○○○○, or ^{14}C-methionine △-△-△. 50 µl of the incubated mixture was applied to a filter paper disc, washed, and counted [5].

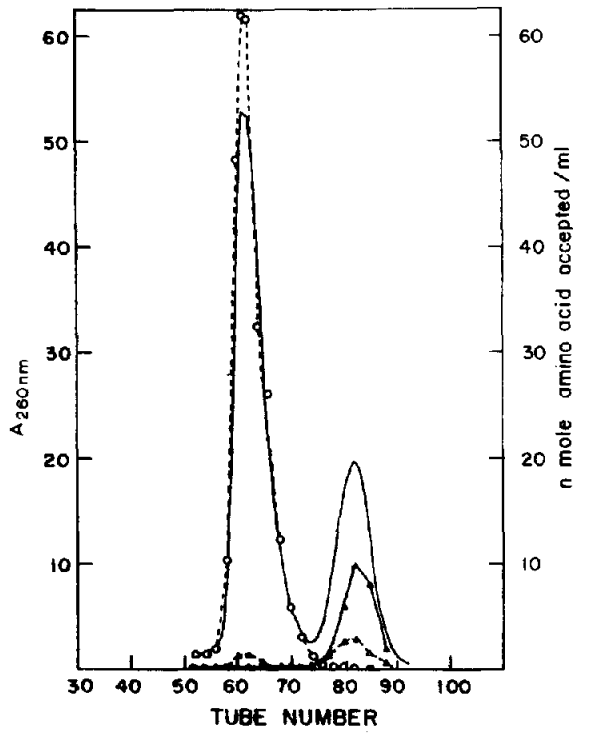

Fig. 3. Purification of $tRNA_a^{Leu}$ by gel filtration on Sephadex G-100. (for details, see text). The acceptor activity was quantitatively assayed by incubating 0.05 absorbance unit of tRNA from every other tube (pre-activated) in 0.2 ml of solution containing 0.1 M tris (pH 7.5), 5 mM ATP, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 µl of crude aminoacyl synthetase solution and 0.3 µCi of ^{14}C-leucine ○----○, ^{14}C-valine ▲----▲, or ^{14}C-glycine △----△. 100 µl of incubated solution was applied to a filter paper disc, washed and counted [5].

with those of crude yeast tRNA. Results in table 1 show that the $tRNA_a^{Leu}$ is at least 70% pure. As pointed out by Lindahl et al. [2], this figure is more properly indicative of > 90% purity. Virtually no other amino acid acceptor activity was observed except 1.5% for glycine and 4.7% for isoleucine. The recovery of $tRNA_a^{Leu}$ from Sephadex G-100 was quantitative. From 5.0 g of crude yeast tRNA, 100 mg of purified $tRNA_a^{Leu}$ was recovered.

3.3. *Nucleotide sequence of the fragments obtained by degradation of $tRNA_a^{Leu}$ with pancreatic RNase.*

$tRNA_a^{Leu}$ purified as described above (557 absorbance units in 3 ml of 0.02 M tris pH 7.4) was incubated with 3 mg of pancreatic RNase at 37° for 12 hr. The degraded mixture was made 7 M with urea and applied to a DEAE-cellulose column (1 × 70 cm) pre-equilibrated with 7 M urea, 0.02 M tris at pH 7.4. The column was eluted with a linear gradient from zero to 0.4 M NaCl in 7 M urea buffered with 0.02 M tris at pH 7.4 (total volume, 2·l). Fractions at 2.5 ml were collected at the rate of 10 min per fraction. Absorbance at 260 nm was measured. Fig. 4 shows the chromatographic pattern. Further elution of the column with 1 M NaCl in 7 M urea released no nucleotidic material. Fractions under each peak were combined, and total absorbance at 260 nm for each peak was measured. Urea and NaCl were removed by pas-

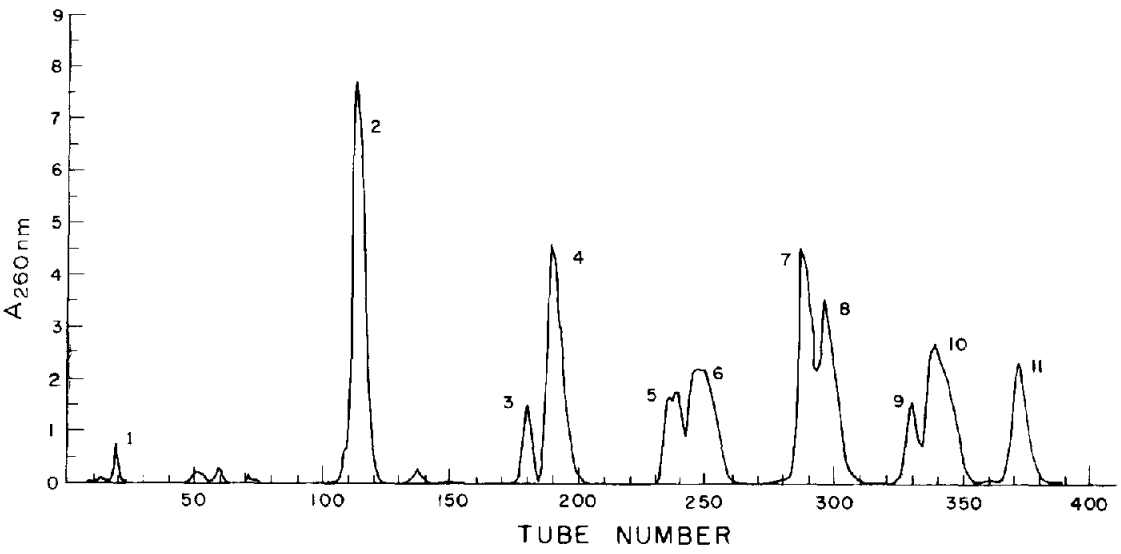

Fig. 4. Chromatographic pattern of pancreatic RNase digest of $tRNA_a^{Leu}$ on DEAE cellulose. For details, see text. Identities of peaks are listed in table 2.

sing the solution through Biogel P-2. Components in each peak were further separated and their nucleotide sequence was analyzed according to the procedure described in the structural studies of yeast $tRNA^{Phe}$ [8, 9]. Mononucleotides in peak 2 were separated by paper chromatography in solvent (A). The separated nucleotides were then eluted and characterized quantitatively by their spectrophotometric properties. The later peaks contained oligonucleotides. The components under each peak were first incubated with bacterial alkaline phosphatase. These 3′-dephosphorylated oligonucleotides were then separated by chromatography in solvent (A) or on DEAE cellulose at pH 3.2 [10]. The purified oligonucleotides were subjected to snake venom phosphodiesterase digestion to identify the 5′-terminal nucleoside, or with T_1 ribonuclease followed by snake venom phosphodiesterase digestion for sequence determination. Table 2 summarizes the results of these analyses. These data show that $tRNA_a^{Leu}$ has a chain length of 85 nucleotides (including the adenosine at the 3′-terminus), indicating that $tRNA_a^{Leu}$ has the S region miniloop [11] between the anticodon loop and TΨCG loop. Similar to most yeast tRNAs, the 3′-terminal pA in $tRNA_a^{Leu}$ is missing [8]. It is interesting that the 5′-terminus of $tRNA_a^{Leu}$ is pGpGpU---, as in yeast $tRNA^{Val}$ [12] and yeast $tRNA^{Ile}$ [13] because these three amino acids are close analogues. The other tRNA which has a pGpGpU--- terminal sequence is $tRNA^{Trp}$ from *E. coli* [14, 15]. It is worthy of note that $tRNA_a^{Leu}$ has no modified adenosine. Whether or not this tRNA contains a modified nucleotide next to the anticodon remains to be determined. Sequence analysis on fragments obtained from T_1 RNase degradation of $tRNA_a^{Leu}$ and its total sequence derivation are in progress in our laboratory.

Table 1
Amino acid acceptor[a] activities of purified $tRNA_a^{Leu}$.

Amino acid	Moles amino acid accepted/ mole tRNA[b]		
	Tube 62[c]	Tube 82[c]	Crude yeast tRNA
Alanine	< 0.001	< 0.001	0.037
Asparatic acid	< 0.001	< 0.001	0.034
Glycine	0.015	0.025	0.033
Glutamic acid	< 0.001	0.001	0.016
Histidine	< 0.001	< 0.001	0.028
Isoleucine	0.047	0.038	0.027
Leucine	0.695	0.006	0.032
Lysine	< 0.001	0.001	0.020
Methionine	0.008	0.008	0.014
Threonine	0.002	0.006	0.044
Valine	0.001	0.23	0.034

[a] Acceptor activities were assayed under the conditions described in the legend to fig. 3.
[b] Average molar absorbance of tRNA is assumed to be 600×10^3 at 260 nm.
[c] Peak tubes from fig. 3.

Table 2
Nucleotide sequences of fragments obtained by degradation of $tRNA_a^{Leu}$ with pancreatic RNase.

Peak	Identify	Molar[a] ratio	Peak	Identify	Molar[a] ratio
1	Cytidine	0.93	6	ApGpCp	1.65
2	Ψp	1.03		ApApCp	1.06
	Cp	7.90		$GpN^2meGpCp$	0.82
	Up	7.10	7	GpApApUp	1.05
	Ac5meCp	0.82		ApAp1meGpCp	1.05
	DiHUp	1.03	8	GpApGpCp	1.23
3	N^2dimeGpCp	1.03		ApGpGpUp	1.21
4	GpUp	2.37	9	pGpGpUp	1.00
	Gp5meCp	1.06	10	ApApGpGpCp	1.00
	ApUp	1.21		ApApGpApUp	1.00
	ApCp	0.98	11	ApApGpApGpTp	0.97
5	GpGpDiHUp	1.09			
	GpApΨp	0.91			

[a] pGpGpUp was used as the basic unit to calculate the molar ratio of each component.
See fig. 4 for chromatographic separation of these fragments.

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