Nucleotide sequence of threonine tRNA from Bacillus subtilis

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ABSTRACT

A threonine tRNA was purified from Bacillus subtilis W168 by a combined use of column chromatographic systems. The nucleotide sequence was determined to be pG-C-C-G-G-U-G-U-A-G-C-U-C-A-A-U-D-G-G-D(U)-A-G-A-G-C-A-
U-C-U-U-G-C-C-G-C-A-C-A-A, where about 40% of D2o remained unmodified as U2o. It consists of 76 nucleotides including a new minor nucleoside, 5-methoxyuridine (mo5U), which occupies the wobble position of anticodon.

INTRODUCTION

We have recently proposed anticodon sequences of tRNAAla, tRNAThr and tRNAVal from Bacillus subtilis [1]. In these three tRNAs, 5-methoxyuridine (mo5U)* commonly locates at the wobble position of anticodons. Here, we describe purification and total nucleotide sequence of tRNAThr, one of these tRNAs.

MATERIALS AND METHODS

Purification of tRNAThr. Growth of B. subtilis W168 and preparation of crude tRNA were reported previously [2]. Threonine tRNA was purified by a successive use of following column chromatographic systems: DEAE-Sephadex A-50 (pH 7.5) [3], Sepharose 4B (pH 4.5) with a reverse gradient of ammonium sulfate [4], and, for some preparations, BD-cellulose (pH 6.0) [5].

* Abbreviations. m1A: 1-methyladenosine, t5A: N-((9-β-D-ribofuranosylpurin-6-yl)carbamoyl)threonine, m5A: N-((9-β-D-ribofuranosylpurin-6-yl)-β-methylcarbamoyl)threonine, m3C: 3-methylcytidine, m7C: 5-methylcytidine, m5G: 5-methylguanosine, m2G: N2-methylguanosine, m7G: 7-methylguanosine, I:inosine, ho5U: 5-hydroxymethyluridine, mo5U: 5-methoxy-
uridine. 1 A260 unit is defined as an amount of material which gives an absorption of 1.0 at 260 nm when dissolved in 1 ml of water and measured with a 1 cm of light path.

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Sequencing Techniques. Methods of fractionation of oligonucleotides produced by complete digestion with RNase T1 or pancreatic RNase as well as general procedures used for sequence determination were essentially the same as described by Harada et al. [6]. 5'-Terminal nucleoside of each oligonucleotide was determined by digestion with nuclease P1 from Pentatium citrinum [7]. For sequence analysis of oligonucleotides longer than pentanucleotide, digestion with nuclease SW [8,9] was useful. It was done as follows. A mixture of 1.0 A260 unit of an oligonucleotide and 5 to 15 units of the enzyme in 20 μl of 50 mM sodium carbonate buffer (pH 10.3)-5 mM magnesium acetate-0.1 M NaCl was incubated at 37° for 3 hr. Resulting fragments were separated by two-dimensional paper chromatography on Toyo filter paper No.51A. Solvent systems were, for first dimension, isobutyric acid-0.5 M ammonia (5:3 by vol.) and, for second, tert-butyl alcohol-0.078 M ammonium formate buffer (pH 3.5) (1:1 by vol.). Each oligonucleotide was analyzed by digestion with RNase T2.

Limited Digestion with RNases. Limited digestion with RNase T1 was performed by incubating 360 A260 units of purified tRNA with 30 μg of RNase T1 in 10 ml of 0.1 M Tris-HCl buffer (pH 7.5) for 15 min. at 37°.

In case of limited digestion with pancreatic RNase, following three conditions were used for each 200 A260 units of tRNA: (1) enzyme/tRNA: 1/500 (by wt.), 50 mM MgCl2, at 37° for 10 min., (2) enzyme/tRNA: 1/1000 (by wt.), no MgCl2, at 37° for 5 min., (3) enzyme/tRNA: 1/500 (by wt.), no MgCl2, at 0° for 20 min. All were incubated in 0.1 M Tris-HCl buffer (pH 7.5). After reaction was stopped by phenol extraction, all three limited digests were combined and subjected to column chromatography.

Large oligonucleotides produced were separated by DEAE-cellulose (DE-23) (pH 7.5) column chromatography followed by DEAE-Sephadex A-25 (pH 2.7) column chromatography.

Chemical Cleavages. Threonine tRNA was chemically cleaved at the site of m7G or D according to Simsek et al. [10] and Beltchev and Grunberg-Manago [11], respectively. In each case, 150 A260 units of tRNAThr were treated. Fragments were separated as described above.

Fingerprinting. Purified fragments were analyzed, after complete digestion with RNase T1 or pancreatic RNase, by fingerprinting technique [12] developed for analysis of [32P]-labeled RNA by Sanger et al. [13]. Usually 2 to 6 A260 units of a fragment was digested and applied to a cellulose acetate strip. For analysis of large fragments, it was necessary to have fingerprint patterns of complete digests of tRNAThr with RNase T1.
RESULTS

Purification of tRNA^Thr.

Crude tRNAs from *B. subtilis* were fractionated on a DEAE-Sephadex A-50

Fig. 1. Purification of tRNA^Thr by column chromatography.

(a) DEAE-Sephadex A-50 (pH 7.5) column chromatography of crude tRNA from *B. subtilis*.

Column size: 2.7 x 127 cm. Amounts charged: 21,000 A260 units.
Linear gradient of NaCl and MgCl₂. Mixing chamber (3:1): 0.375 M NaCl-8 mM MgCl₂-20 mM Tris-HCl (pH 7.5). Reservoir (3:1): 0.525 M NaCl-16 mM MgCl₂-20 mM Tris-HCl (pH 7.5). Flow rate: 50 ml/hr. Each fraction: 18 ml. Fractions indicated by arrows were collected.

(b) Sepharose 4B column chromatography of tRNA^Thr-rich fraction.

Column size: 2.6 x 62 cm. Amounts charged: 8,950 A260 units of tRNA^Thr-rich fraction from (a). Decreasing linear gradient of ammonium sulfate. Mixing chamber (936 ml): 10 mM sodium acetate (pH 4.5)-10 mM MgCl₂-6 mM 2-mercaptoethanol-1 mM EDTA-1.3 M (NH₄)₂SO₄. Reservoir (1:1): the same except no (NH₄)₂SO₄. Flow rate: 70 ml/hr. Each fraction: 8.3 ml.
(pH 7.5) column (Fig. 1a). Fractions of a major tRNA\textsuperscript{Thr} indicated by arrows were pooled and applied to a Sepharose 4B column (Fig. 1b). Threonine tRNA was eluted as a single peak in the beginning of a reverse gradient of ammonium sulfate (pH 4.5) and separated from other tRNAs. Usually, purity of tRNA\textsuperscript{Thr} reached nearly 90\% at this step. Occasionally, a small amount of tRNA\textsuperscript{Gly} was a contamination. In that case, the preparation was further purified by BD-cellulose (pH 6.0) column chromatography.

This tRNA\textsuperscript{Thr} from \textit{B. subtilis} can accept threonine with almost equal efficiency by crude aminoacyl-tRNA synthetase preparations from \textit{B. subtilis} W168 and \textit{E. coli} Q13.

Analysis of Oligonucleotides Obtained by Complete Digestion with RNases.

Column chromatographic behavior of the products by complete digestion of tRNA\textsuperscript{Thr} with RNase T\textsubscript{1} was previously reported [1]. By two-dimensional paper chromatography, peaks 2-1 and 2-2 gave single spots and peak 3 was separated into two components. Peaks 4, 6 and 7 were separated into three, two and three, respectively, by DEAE-Sephadex A-25 column chromatography under acid condition. Sequence determination of peak 5 as A-C-U-mo\textsuperscript{5}U-Gp was already reported [1]. Peak 7-3 was sequenced as follows. Digestions with RNase T\textsubscript{2} and with nuclease P\textsubscript{1} indicated that this fragment is U-(3C-,3U-)Gp. By digestion with nuclease SW, U-C-C-U\textsubscript{OH}, pU-C-U\textsubscript{OH}, pC-U-C\textsubscript{OH}, pC-(U-,C-)U\textsubscript{OH} and pU-U-Gp were obtained. Therefore, the sequence was determined to be U-C-C-U-C-U-Gp. A fingerprint pattern of complete digest of tRNA\textsuperscript{Thr} with RNase T\textsubscript{1} is shown in Fig. 2a. Sequencing data of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Fingerprints of complete digests of tRNA\textsuperscript{Thr} with RNases. (a) RNase T\textsubscript{1} digest. (b) Pancreatic RNase digest.}
\end{figure}
all the RNase Tₐ fragments are summarized in Table I.

The oligonucleotides obtained by complete digestion with pancreatic RNase were separated into eleven fractions by DEAE-Sephadex A-25 (pH 7.5) column chromatography operated at 65°. Peaks 5, 6 and 7 were separated into three, six and two components, respectively, by column chromatography under acid condition. Other fractions were composed of single components. Fig. 2b shows a fingerprint pattern of complete digest of tRNA-Thr with pancreatic RNase. Sequencing data of these pancreatic RNase fragments are summarized in Table II.

Experimental and theoretical molar ratios of the oligonucleotides are listed in Table III. Molar ratio of U-A-Gp to D-A-Gp was found to be 4 to 1, and that of G-G-Up to G-G-Dp, 7 to 3.

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TABLE I

Summary of Nucleotide Sequence Determination of RNase Tₐ Endproducts

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541
## TABLE II

### Summary of Nucleotide Sequence Determination of Pancreatic RNase Endproducts

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**Legend for TABLE I and TABLE II**

- U₇G₇p, pU₇G₇p, and m₇G₇p were determined as degradation products of m₇Gp, m₇Gp, and m₇G, respectively.
- S Dp, pD, and pG were detected only by phosphate coloration on thin-layer chromatograms and not determined quantitatively.
- T P₄ and pU were not separated from m₇G₄U and pmo₇U, respectively, by two-dimensional thin-layer chromatography used.
- H Molar extinction coefficient of m₇G₄U was from [27].
- §§ τ₆A was not separated from τ₆A by two-dimensional thin-layer chromatography used and their amounts were calculated from spectral data.
- * This oligonucleotide was hydrolyzed exhaustively with 380 units of RNase T₁ per A₂₆₀ unit in 0.05 M Tris-HCl buffer (pH 7.5) at 37° for 15 hr.

**Abbreviations.**
- T₁: RNase T₁ digestion.
- P₁: Nuclease P₁ digestion.
- T₂: RNase T₂ digestion.
- T₄: Nuclease T₄ digestion.
- P: Pancreatic RNase digestion.
- SW: Nuclease SW digestion.

### Limited Digestion with RNases, Chemical Cleavages and Deduction of Primary Structure of tRNA-Thr

By limited digestion with RNase T₁, tRNA-Thr was severed at a site in the anticodon and 5'- and 3'-halves were obtained. A part of 5'-half molecules were devoid of 5'-terminal pGp. By limited digestion with pancreatic RNase, relatively small oligonucleotides were obtained. Chemical scission at m₇G gave a 5'-two-thirds molecule and a 3'-one-third molecule. By chemical cleavage at D, a 3'-three-fourths molecule was obtained. We could not, however, attain definitive results in 5'-terminal analysis of this fragment. It may be partly due to incomplete modification at D₂₀. Of these products obtained by limited digestion, the fragments used for derivation of total nucleotide sequence are shown in Fig. 3, with deduced primary structure of tRNA-Thr.
TABLE III Molar Ratios of Endproducts of Complete Digestion with RNases

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Fig. 3. Nucleotide sequences of tRNA^Thr and large fragments.
(a) Nucleotide sequence of tRNA^Thr from B. subtilis.
(b) Sequences established by combining the data obtained by complete digestion with RNase T1 and pancreatic RNase.
(c) Oligonucleotides obtained by limited digestion with RNase T1.
(d) Oligonucleotides obtained by limited digestion with pancreatic RNase.
(e) Oligonucleotides obtained by chemical cleavage at m7G or D.

A part of the nucleotide sequence of 5'-terminal amino acid acceptor stem of this tRNA cannot be unambiguously ordered from the available oligonucleotides. An alternative sequence of this part is pG-C-C-G-U-G-G-U-. This sequence, however, produces base-pair mismatches at two positions in the acceptor stem. We believe that this tRNA^Thr has the sequence of pG-C-C-G-U-G-U- in this part. The total sequence illustrated by a cloverleaf model (Fig. 4) is grouped into type D4V5 (class I) [14]. Partial dis-
placements of U with D were observed in oligonucleotides U-A-Gp and G-G-Up. As we did not detect this type of displacement in the other oligonucleotides, it is concluded that this substitution occurs solely at the 20th position from 5'-end. Calculation from analytical data shows that about 60% of analyzed tRNA\(^{\text{Thr}}\) has D and the rest has U at this site.

* Bacillus subtilis* tRNA\(^{\text{Thr}}\) thus sequenced is composed of 76 nucleotides including 6 kinds of modified nucleosides, D, Ψ, T, m\(^7\)G, t\(^6\)A and a new constituent, mo\(^5\)U. A modified nucleoside, t\(^6\)A, occupies the position adjacent to 3'-end of anticodon. The nucleoside, mo\(^5\)U, locates at the first position of anticodon of tRNA\(^{\text{Thr}}\) as tRNA\(^{\text{Ala}}\) and tRNA\(^{\text{Val}}\) from the same origin [1]. Determination of total nucleotide sequence of tRNA\(^{\text{Thr}}\) is the first instance as a tRNA containing mo\(^5\)U. We reported in the previous paper [1] that it was unsuccessful to separate mo\(^5\)Up from Up, and mo\(^5\)U from U, by usual solvent systems. By further investigation we found that mo\(^5\)U and U can be separated by two-dimensional thin-layer chromatography reported by Rogg et al. [15]. Fig. 5 shows a chromatogram of a roughly equimolar mixture of mo\(^5\)U and other four usual nucleosides on TLC aluminium roll cellulose sheet.
Fig. 5. Separation of 5-methoxyuridine from uridine by two-dimensional thin-layer chromatography on aluminium roll cellulose plate. Solvent systems, 1st dimension: 1-butanol/isobutyric acid/conc. ammonia water/water (75/37.5/2.5/25 by vol.), 2nd dimension: saturated ammonium sulfate/0.1 M sodium acetate (pH 6.0)/2-propanol (79/19/2 by vol.).

values of \( m^{5}U \) were 0.22 in the first dimension and 0.50 in the second (solvent systems A and B, respectively, in reference [15]).

**DISCUSSION**

Threonine tRNA is one of the tRNA species which exhibit a change in the ratio of existing peaks between the profiles of tRNAs from vegetative cells and spores of *B. subtilis* by RPC-5 column chromatography [16]. Chromatographic behaviors on RPC-5 [16] and DEAE-Sephadex A-50 (Fig. 1a) columns show the presence of at least three isoacceptor threonine tRNAs. The tRNA\(^{Thr}\) sequenced here is a species present as major isoacceptors in both vegetative cells and spores.

The nucleotide sequences of threonine tRNAs from *E. coli* [17] and brewer's yeast [18,19] have been reported. Comparison of the sequence of *B. subtilis* tRNA\(^{Thr}\) with those of others shows that 28 nucleotides are different between *B. subtilis* and *E. coli* (Fig. 6a) and 29 between *B. subtilis* and yeast (both tRNA\(^{Thr}_{1a}\) and tRNA\(^{Thr}_{1b}\)) (Fig. 6b). If post-transcriptional modifications of nucleosides are disregarded in comparison, 37 residues are common to all of these three threonine tRNAs (Fig. 6c). The common sequence from \( U_{14} \) to \( A_{21} \) in D loop is found also in several tRNAs specific to other amino acids [20]. The common sequences seen in anticodon loop, \( G_{35} \) to \( A_{38} \) and in terminal region may be related to recognition by threonyl-tRNA synthetase.

As predicted previously [21-23], \( t^{6}A \) occupies the position adjacent to
Fig. 6. Comparison of structures among threonine tRNAs.
(a) Structure of tRNAThr from E. coli.
(b) Structure of tRNAThr and tRNAThr from brewer's yeast. Nucleosides common to those of tRNAThr from B. subtilis are enclosed with brackets.
(c) Composite structure of threonine tRNAs from B. subtilis, E. coli and brewer's yeast. Differences in the state of modification were disregarded for this comparison.

3'-end of anticodon of tRNA\textsuperscript{Thr} from B. subtilis. E. coli tRNA\textsuperscript{Thr} contains \textit{mt}^6A as well as \textit{t}^6A at this position [17,24]. In B. subtilis tRNA\textsuperscript{Thr}, we did not detect \textit{mt}^6A. Void has recently detected an unknown methylated nucleoside in B. subtilis tRNA hydrolyzates [25,26]. We were informed that
mo\textsuperscript{5}U eluted at the same position as this nucleoside by Aminex A-6 column chromatography [Vold, B. S.: personal communication]. Singhal and Vold also reported that hydrolysates from \textit{B. subtilis} tRNA contained 5-hydroxy-uridine (ho\textsuperscript{5}U) as judged from its eluting position on Aminex A-6 [26]. We did not detect ho\textsuperscript{5}U in tRNA\textsuperscript{Thr} from \textit{B. subtilis}.

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