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Primary Sequence of $tRNA_1^{Val}$ from *Escherichia coli* B. II. Isolation of Large Fragments by Limited Digestion with RNases, and Overlapping of Fragments to Deduce the Total Primary Sequence*

Fumiko Kimura, Fumio Harada, and Susumu Nishimura†

ABSTRACT: The total primary sequence of *Escherichia coli* $tRNA_1^{Val}$ was unambiguously deduced from the overlapping of the sequences of large fragments obtained by limited digestion of tRNA with either *Bacillus subtilis* RNase or pancreatic RNase. The nucleotide sequences of large fragments were determined using a combination of two-dimensional thin-layer chromatography and DEAE-Sephadex A-25 column chromatography monitored with a sensitive automatic ultraviolet recorder. Using these procedures, 1–2 optical density units of fragments were sufficient for characterization. Thus many fragments obtained in relatively low yields could be characterized. In addition, a large number of fragments could be assayed in a much shorter time than with the procedure used previously for nonradioactive tRNA.

The preceding paper described the sequences of oligonucleotides obtained from *Escherichia coli* $tRNA_1^{Val}$ by digestion with RNase T_1 and pancreatic RNase. To connect their sequences and so obtain the total primary sequence of tRNA, large fragments with overlapping sequences were required (Holley, 1968). To obtain these we used limited digestion of *E. coli* $tRNA_1^{Val}$ with *Bacillus subtilis* RNase, since it was already predicted that *B. subtilis* RNase splits tRNA into two fragments at the position of the anticodon (Oda *et al.*, 1969). Limited digestion with *B. subtilis* RNase was performed in the presence of 0.02 M magnesium chloride at 37°. A high temperature was chosen because at this temperature less enzyme was required and specific cleavage was obtained as well as at 0°. Then limited digestion with pancreatic RNase

* From the Biology Division, National Cancer Center Research Institute, Chuo-ku, Tokyo, Japan. *Received March 22, 1971.* The work was partly supported by research grants from the Japanese Ministry of Education.

† To whom to address correspondence.

was carried out in a similar fashion to obtain large fragments by cleavage at different positions.

Characterization of large fragments is tedious and time consuming using conventional methods with nonradioactive tRNA. Moreover, relatively large quantities of samples are required to obtain reliable results. This paper describes the application of two-dimensional thin-layer chromatography and DEAE-Sephadex A-25 column chromatography monitored with a sensitive automatic ultraviolet recorder in characterization of the sequences of large fragments. For this method less than 2 optical density units of material were required. Therefore, many fragments obtained in low yield could be characterized. In addition, using this technique, samples could be assayed much more rapidly than with the procedure used for nonradioactive tRNA.

A preliminary account of part of this work has been published (Harada *et al.*, 1969b).

Materials and Methods

Materials. E. coli $tRNA_1^{Val}$, *B. subtilis* RNase, pancreatic RNase, RNase T_1, RNase T_2, DEAE-Sephadex A-25, and thin-layer plates were as described in the preceding paper. DEAE-cellulose (Whatman DE-23; capacity, 1 mequiv/g) was obtained from W. & R. Balston, Ltd.

Partial hydrolysis of $tRNA_1^{Val}$ by B. subtilis RNase or Pancreatic RNase. E. coli $tRNA_1^{Val}$ was treated with *B. subtilis* RNase as described previously (Oda *et al.*, 1969). For a typical treatment 800 optical density units of $tRNA_1^{Val}$ in 40 ml of 0.02 M $MgCl_2$ in 0.1 M Tris-HCl (pH 7.5) was treated with 100 units of *B. subtilis* RNase (Nishimura, 1966) at 37° for 13 min. After the incubation, the reaction mixture was cooled in an ice–water bath and extracted with 20 ml of 88% phenol saturated with 0.02 M Tris-HCl (pH 7.5)–0.02 M $MgCl_2$. The aqueous layer was extracted four times with 20 ml of ether. The RNase-treated tRNA was precipitated from the aqueous solution containing 0.4 M NaCl by adding three volumes of ethanol and stored at −15° for 18 hr. Then it was precipitated by centrifugation at 9000 rpm for 10 min and dissolved in 7 ml of distilled water. The yield was 500 optical density units.

For treatment of $tRNA_1^{Val}$ with pancreatic RNase, 940 optical density units of $tRNA_1^{Val}$ was incubated with 37 μg of bovine pancreatic RNase in 24 ml of 0.02 M $MgCl_2$ in 0.1 M Tris-HCl (pH 7.5) at 37° for 10 min. Then the treated $tRNA_1^{Val}$ was isolated by the procedure described above. The yield was 680 optical density units.

Separation of Large Fragments. The treated-tRNA solution was mixed with one-tenth volume of 0.1 M EDTA (pH 7.0), heated for 4 min at 85°, and then quickly cooled. Then solid urea was added to the solution to a concentration of 7 M. The sample was loaded onto a column of DEAE-cellulose (Whatman DE23, column size, 0.5 × 150 cm), and fractionated as described previously (Oda *et al.*, 1969). The tRNA fragments separated by chromatography were directly precipitated by addition of three volumes of ethanol. The mixture was allowed to stand overnight at −15°. The fragments were collected by centrifugation, dissolved in 2 ml of 7 M urea, and subjected to DEAE-Sephadex A-25 column chromatography at pH 2.7 as described previously (Oda *et al.*, 1969). The fractions containing the purified fragments were mixed with three volumes of ethanol, stored at −15° overnight, and precipitated by centrifugation. Then they were dissolved in a small volume of distilled water and extensively dialyzed against water at 4°.

Two-Dimensional Thin-Layer Chromatography for Separation of Oligonucleotides. The tRNA fragments (0.5–2 optical density units) were dissolved in 0.01–0.2 ml of 0.05 M triethylammonium bicarbonate (pH 7.8) and incubated with 1 μg of RNase T_1 and 4 μg of pancreatic RNase for 18 hr at 37°. The hydrolysate was applied to a thin-layer plate coated with Avicel SF cellulose (10 × 10 cm). Chromatography was carried out in the first dimension with isobutyric acid–0.5 N NH_4OH (5:3, v/v, solvent E) for 6 hr at 23° and in the second dimension with *tert*-butyl alcohol–ammonium formate buffer, pH 3.8 (1:1, v/v), as described by Rushizky and Knight (1960) for 6 hr at 23°. Better resolution is obtained when several thin-layer plates are put in a tank at the same time.

To determine the base composition of an oligonucleotide in a spot, the spot was scratched off and suspended in 0.5 ml of water with vigorous shaking for 2 min. After standing for 2 hr, the supernatant was collected by centrifugation, evaporated almost to dryness, and hydrolyzed extensively with RNase T_2. The hydrolysate was subjected to two-dimensional thin-layer chromatography using the solvent system described in the preceding paper (Harada *et al.*, 1971). The nucleotide in each spot was scratched off and suspended in 0.5 ml of water. The ultraviolet absorbance of the supernatant was measured in a Shimazu MPS-50L spectrophotometer. It was not necessary to obtain a completely clear supernatant when the spectrophotometer is used as specified.

To identify the oligonucleotide in each spot on the chromatogram, 20 optical density units of intact $tRNA_1^{Val}$ was digested with RNase T_1 or pancreatic RNase, and the digests were chromatographed on Toyo-Roshi No 51A paper (30 × 30 cm) in the same way as for thin-layer chromatography except that the time of development was 48 hr in each dimension. The chromatographic pattern was identical with that of the thin-layer chromatogram of the digest of 2 optical density units of $tRNA_1^{Val}$. Each spot was cut out, eluted with water, and hydrolyzed with RNase T_2. The base composition of the digest was determined by two-dimensional thin-layer chromatography.

Oligonucleotide Analysis by DEAE-Sephadex A-25 Column Chromatography. The procedure used to characterize *E. coli* $tRNA^{fMet}$ fragments (Seno *et al.*, 1969) was adopted. Usually 5 optical density units of intact $tRNA_1^{Val}$ or 0.5–2 optical density units of tRNA fragments were hydrolyzed by either RNase T_1 or pancreatic RNase. The hydrolysate was applied to a DEAE-Sephadex A-25 column (0.5 × 150 cm). Linear gradient elution was performed with 400 ml of 0.14 M NaCl in 0.02 M Tris-HCl (pH 7.5)–7 M urea in the mixing chamber, and 400 ml of 0.7 M NaCl in 0.02 M Tris-HCl (pH 7.5)–7 M urea in the reservoir. The ultraviolet absorbance of the effluent from the column was recorded continuously using an automatic ultraviolet recorder (Jeol, Model JLC 3BC). The flow rate was 18 ml/hr.

Results

E. coli $tRNA_1^{Va}$. was partially hydrolyzed by either *B. subtilis* RNase or pancreatic RNase in the presence of magnesium ion at 37° to obtain large fragments. Limited digestion of $tRNA_1^{Val}$ could be achieved by adding magnesium ion as reported earlier (Nishimura and Novelli, 1965; Penswick and Holley, 1965). These fragments were separated by DEAE-cellulose column chromatography, and then fractionated by DEAE-Sephadex column chromatography under acidic conditions. The typical examples in Figures 1–4 show that many large homogeneous fragments were obtained. The nucleotide sequences of these fragments were deduced by analysis of RNase digests of the fragments by two-dimensional thin-

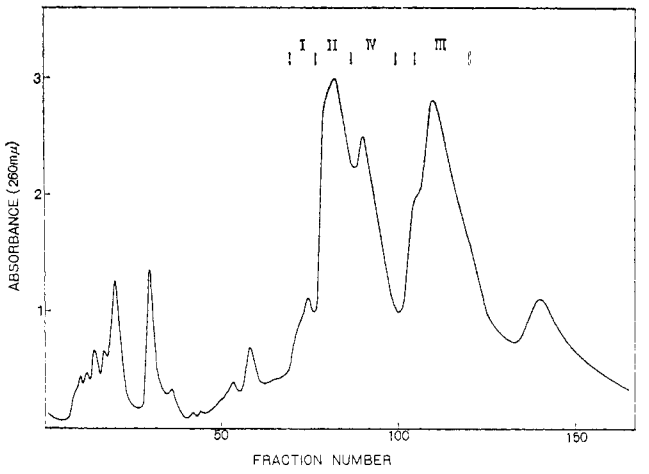

FIGURE 1: Fractionation of a partial *B. subtilis* RNase digest of $tRNA_1^{Val}$ by DEAE-cellulose column chromatography. The partial digest prepared as described in the Experimental section was applied to a column (0.5 × 150 cm) of DEAE-cellulose (DE-23). Elution was performed with a linear salt gradient obtained by placing 800 ml of 0.25 M NaCl–0.02 M Tris-HCl (pH 8.0)–7 M urea in the mixing chamber and 800 ml of 0.65 M NaCl–0.02 M Tris-HCl (pH 8.0)–7 M urea in the reservior. Fractions of 4 ml of effluent were collected at a flow rate of 11 ml/hr.

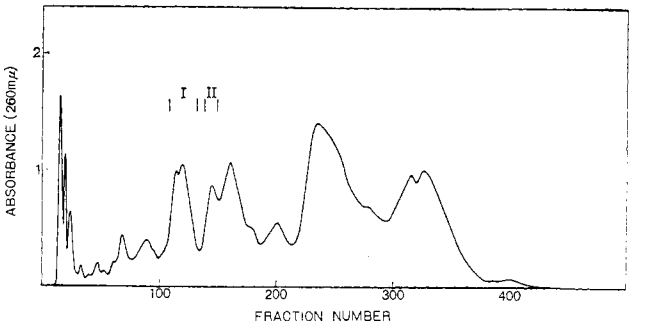

FIGURE 3: Fractionation of a partial digest of $tRNA_1^{Val}$ with pancreatic RNase by DEAE-cellulose column chromatography. The partial digest prepared as described in the Experimental Section was applied to a column (0.5 × 150 ml) of DEAE-cellulose. The column was washed with 100 ml of 0.25 M NaCl–0.02 M Tris-HCl (pH 8.0)–7 M urea. Then elution was performed with a linear salt gradient obtained by placing 1 l of 0.25 M NaCl–0.02 M Tris-HCl (pH 8.0)–7 M urea in the mixing chamber and 1 l. of 0.5 M NaCl–0.02 M Tris-HCl (pH 8.0)–7 M urea in the reservior. Fractions of 3 ml of effluent were collected at a flow rate of 11 ml/hr.

layer chromatography and DEAE-Sephadex A-25 chromatography, as described in the Experimental Section. Figure 5 shows a schematic chromatogram of the separation of RNase T_1 and pancreatic RNase digests of intact $tRNA_1^{Val}$ by two-dimensional thin-layer chromatography. Oligonucleotides which were smaller than hexanucleotides were quite well separated from each other by this method. Figures 6 and 7 show typical examples of the microanalysis of the compositions of fragments by DEAE-Sephadex A-25 column chromatography. The elution profiles were obtained by continuous measurement of ultraviolet absorption of the effluent from the column with an automatic recorder, as described in the Experimental Section. As shown in Figures 6 and 7, when a hydrolysate of intact $tRNA_1^{Val}$ was subjected to this assay, the chromatographic profiles obtained were identical with those reported in the preceding paper (see Figures 1 and 8 in Harada *et al.*, 1971) obtained on large-scale separation of oligonucleotides. Thus, the presence of m^7G-U-C-Gp, C-D-Gp, T-ψ-C-Gp, C-U-C-A-Gp, U-C-A-U-C-A-C-C-C-A-C-C-A, and C-A-C-C-U-C-C-C-U-V-A-C-m^6A-A-Gp in the RNase T_1 digest, and pG-G-G-Up, G-G-G-A-G-A-G-Cp, and m^6A-A-G-G-A-G-G-G-G-m^7G-Up in the pancreatic RNase digest could readily be deduced by this procedure.

Figure 8 shows the sequences of the large fragments used to identify the total primary sequence of $tRNA_1^{Val}$ unambiguously. The sequences of these fragments were deduced from the following results.

Fragment F (*A-U-C-C-C-G-U-C-A-U-C-A-C-C-C-A-C-C-A*). Fragment F was obtained by partial hydrolysis with *B. subtilis* RNase as shown in Figure 2c. Digestion of fragment F (0.6 optical density unit) with pancreatic RNase showed

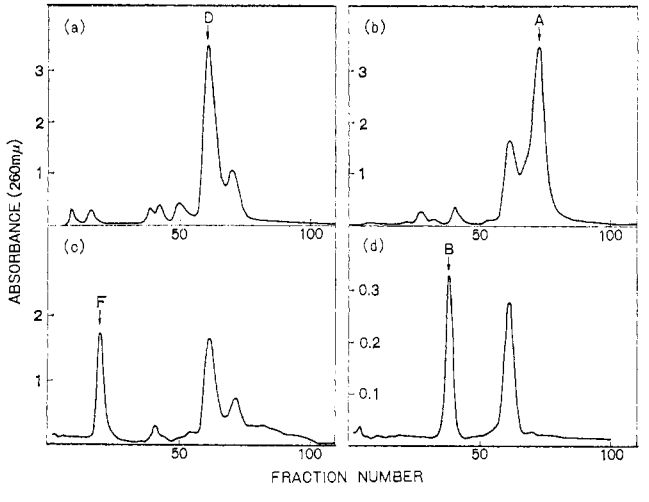

FIGURE 2: Further purification of large fragments from a *B. subtilis* RNase digest by DEAE-Sephadex A-25 column chromatography at pH 2.7. Partially purified fractions, II, III, IV, and I described in Figure 1 was applied separately to columns (0.5 × 100 cm) of DEAE-Sephadex A-25. Elution was performed with a linear salt gradient obtained by placing 200 ml of 0.06 M HCl–7 M urea in the mixing chamber and 200 ml of 0.6 M NaCl–0.06 M HCl–7 M urea in the reservior. Fractions of 3 ml of effluent were collected at a flow rate of 15 ml/hr. (a) Fraction II, (b) fraction III, (c) fraction IV, and (d) fraction I.

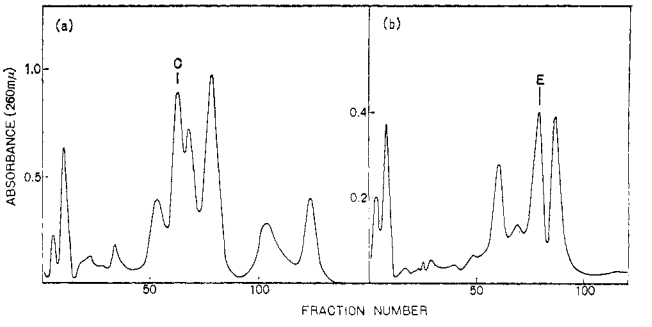

FIGURE 4: Further purification of large fragments from the pancreatic RNase digest by DEAE-Sephadex A-25 column chromatography at pH 2.7. Partially purified fractions I and II described in Figure 3 were applied separately to columns (0.3 × 50 cm) of DEAE-Sephadex A-25. Elution was performed with a linear salt gradient obtained by placing 100 ml of 0.06 M HCl–7 M urea in the mixing chamber and 100 ml of 0.4 M NaCl–0.06 M HCl–7 M urea in the reservior. Fractions of 1 ml of effluent were collected at a flow rate of 20 ml/hr. (a) Fraction I and (b) fraction II.

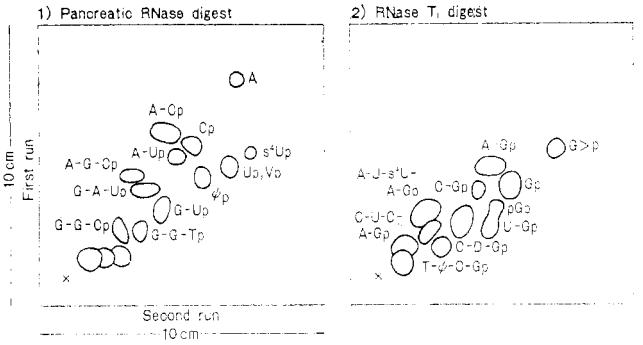

FIGURE 5: Two-dimensional thin-layer chromatography of digests of *E. coli* $tRNA_1^{Val}$ with RNase T_1 and pancreatic RNase.

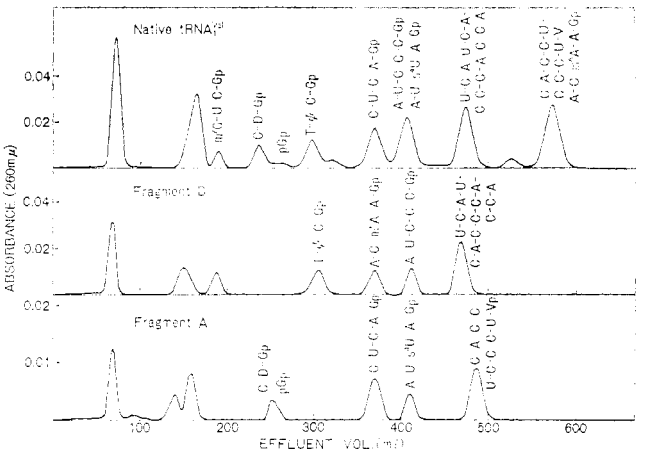

FIGURE 6: Microanalysis of the oligonucleotide composition of complete RNase T_1 digest of intact $tRNA_1^{Val}$, fragment D, and fragment A by DEAE-Sephadex A-25 column chromatography.

that it contained A-Cp, A-Up, G-Up, Cp, and adenosine, indicating that it was derived from the 3′-hydroxy end. A specimen (2 optical density units) was hydrolyzed by RNase T_1, and the products were separated by descending paper chromatography with solvent A as described in the preceding paper. Two spots were obtained. The slower moving product was digested with RNase T_2 to yield adenosine, Ap, Cp, and Up in the ratio of 1.1:3.0:7.2:2.3, indicating that its sequence is U-C-A-U-C-A-C-C-C-A-C-C-A at the 3′-hydroxy end. The faster moving product was digested with RNase T_2 yielding Ap, Up, Cp, and Gp in the ratio of 1.1:1.1:3.0:0.63. Thus this material must be A-U-C-C-C-Gp since data on the oligonucleotide sequences of a complete RNase T_1 digest of $tRNA_1^{Val}$ showed that only this sequence has this base composition. Thus it was concluded that A-U-C-C-C-G- must be placed at the 5′-hydroxy end of the sequence, U-C-A-U-C-A-C-C-C-A-C-C-A. Figure 9 shows actual ultraviolet absorption spectra of nucleotides eluted from two-dimensional thin-layer chromatograms which were used for determination of nucleotide composition of the two oligonucleotides.

Fragment E (*G-G-T-ψ-C-G-A-U-C-C-C-G-U-Cp*). This fragment was obtained by limited digestion of $tRNA_1^{Val}$ with pancreatic RNase as shown in Figure 4b. The oligonucleotides shown in Table I were found in digests of the fragment with RNase T_1 or pancreatic RNase. By overlapping the sequences of these oligonucleotides, it was concluded that the sequence of fragment E must be either (1) G-G-T-ψ-C-G-A-U-C-C-C-G-U-Cp or (2) G-A-U-C-C-C-G-G-T-ψ-C-G-U-Cp. There is only one A-U-C-C-C-G- sequence in the $tRNA_1^{Val}$ molecule, located at the 5′-hydroxy end of the U-C- sequence in fragment F, so the sequence of fragment E was deduced to be 1. Thus the G-G-T-ψ-C-G- sequence of fragment E was placed at the 5′-hydroxy end of fragment F.

Fragment D (*A-C-m⁶A-A-G-G-A-G-G-G-G-m⁷G-U-C-G-G-C-G-G-T-ψ-C-G-A-U-C-C-C-G-U-C-A-U-C-A-C-C-C-A-C-C-A*). Fragment D was obtained by limited digestion with *B. subtilis* RNase as shown in Figure 2a. Olignucleotide analysis by thin-layer chromatography and DEAE-Sephadex A-25 column chromatography showed that fragment D is a half-molecule from the 3′-hydroxy end. The evidence that this fragment was obtained by cleavage of the phosphodiester bond of uridin-5-oxyacetic acid is (1) on digestion of the fragment with pancreatic RNase no 3′-nucleotide of uridin-5-oxyacetic acid was obtained, (2) 3 moles of A-Cp was found in a pancreatic RNase digest of the fragment, and (3) no C-A-C-C-U-C-C-C-U-V-A-C-m⁶A-A-Gp was present in a RNase T_1 digest of the fragment, but a new smaller oligonucleotide was obtained by column chromatography, as shown in Figure 6. The sequence of this fragment from G-G-T- to -C-C-A has already been deduced as shown in fragments F and E. In addition, sequence the G-C-A-C-C-U-C-C-C-U-V-A-C-m⁶A-A-G-G-A-G-G-G-G-m⁷G-U-C-Gp has already been obtained by overlapping the sequences of oligonucleotides from RNase T_1 and pancreatic RNase digest of $tRNA_1^{Val}$ (see Table VI of the preceding paper). Therefore the remaining trinucleotide, G-G-Cp, from a pancreatic RNase digest of the fragment must be placed between the 3′-hydroxy end of -m⁷G-U-C and the 5′-hydroxy end of the G-G-T- sequence.

Fragment C (*A-G-C-D-G-G-G-A-G-A-G-C-A-C-C-U-Cp*). This fragment was obtained from the pancreatic RNase digest as shown in Figure 4c. In addition to usual methods of analysis, 6 optical density units of the fragment was digested with RNase T_1 and separated by two-dimensional paper chromatography. The nucleotide composition of the hexanucleotide obtained was found to be Ap, 4Cp, and Up. Its nucleotide sequence must be C-A-C-C-U-Cp, since the oligonucleotide sequence of the complete RNase T_1 digest of intact $tRNA_1^{Val}$ showed that this is the only plausible sequence for an oligonucleotide with this nucleotide composition. Furthermore C-A-C-C-U-Cp must be at the 3′-hydroxy end

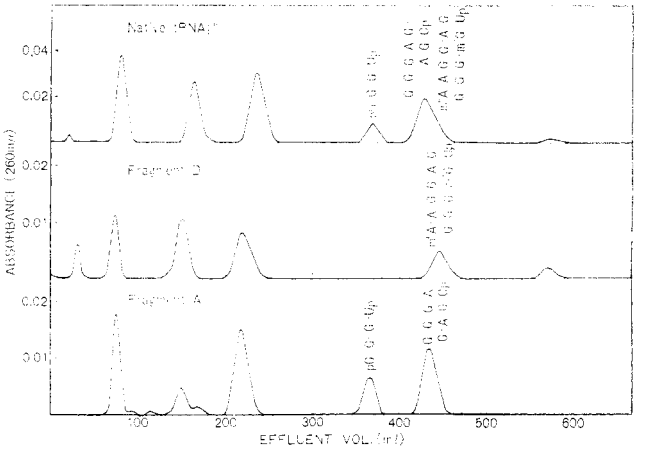

FIGURE 7: Microanalysis of the oligonucleotide compositon of complete pancreatic RNase digests of intact $tRNA_1^{Val}$, fragment D, and fragment A by DEAE-Sephadex A-25 column chromatography.

Fragment A pG-G-G-U-G-A-U-s^4U-A-G-C-U-C-A-G-C-D-G-G-G-A-G-A-G-C-A-C-C-U-C-C-C-U-V_p

Fragment B pG-G-G-U-G-A-U-s^4U-A-G-C-U-C-A-G_p

Fragment C A-G-C-D-G-G-G-A-G-A-G-C-A-C-C-U-C_p

Fragment D A-C-m^6A-A-G-G-A-G-G-G-G-m^7G-U-C-G-G-C-G-G-T-ψ-C-G-A-U-C-C-C-G-U-C-A-U-C-A-C-C-C-A-C-C-A

Fragment E G-G-T-ψ-C-G-A-U-C-C-C-G-U-C-A-U-C-A-C-C-C-A-C-C-A

Fragment F A-U-C-C-C-G-U-C-A-U-C-A-C-C-C-A-C-C-A

FIGURE 8: Large oligonucleotides obtained by limited digestion with *B. subtilis* RNase and pancreatic RNase used to elucidate the total primary sequence of *E. coli* t RNA_1^{Val}.

of fragment C. Thus the products of digestion of the fragments with RNase T_1 were identified as Gp, A-Gp, C-D-Gp, and C-A-C-C-U-Cp. Analysis of a pancreatic RNase digest of fragment C showed the presence of Cp, Up, A-Cp, A-G-Cp, and G-G-G-A-G-A-G-Cp. The Y-A-G sequence was not found in an RNase T_1 digest, so the sequence of A-G-C- must be at the 5′-hydroxy end. The -D-G sequence must be next to the A-G-C sequence, since C-D-Gp is the only oligonucleotide in the RNase T_1 digest with pyrimidine at the 5′-hydroxy end, except C-A-C-C-U-Cp which must be at the 3′-hydroxy end. The remaining -G-G-A-G-A-G- sequence was placed between the A-G-C-D-G- and -C-A-C-C-U-Cp sequences.

Fragment B (*pG-G-G-U-G-A-U-s^4U-A-G-C-U-C-A-Gp*). This fragment was obtained from the *B. subtilis* RNase digest, as shown in Figure 2d. Digestion of fragment B, with RNase T_1 yielded Gp, U-Gp, pGp, C-U-C-A-Gp, and A-U-s^4U-A-Gp, and digestion of the fragment with pancreatic RNase gave Cp, Up, s^4Up, A-Gp, A-G-Cp, G-A-Up, and pG-G-G-Up. The pG-G-G-U sequence must be at the 5′-hydroxy end of this fragment. U-Gp is the only oligonucleotide in the RNase T_1 digest with U at the 5′-hydroxy end, so G must be at the 3′-hydroxy end of the pG-G-G-U- sequence. The overlapping sequence, Y-G-A-U-s^4U-A-G-C- has already been obtained by comparison of complete digests of $tRNA_1^{Val}$ with RNase T_1 and pancreatic RNase so the -A-U-s^4U-A-G-C- sequence must be placed at the 3′-hydroxy end of the pG-G-G-U- sequence. Thus the remaining sequence, -U-C-A-Gp was placed at the 3′-hydroxy end of fragment B.

Fragment A (*pG-G-G-U-G-A-U-s^4U-A-G-C-U-C-A-G-C-D-G-G-G-A-G-A-G-C-A-C-C-U-C-C-C-U-Vp*). This fragment was a half-molecule from the 5′-hydroxy end obtained by limited digestion of $rRNA_1^{Val}$ with *B. subtilis* RNase, as shown in Figure 2b. The presence of pGp in the RNase T_1 digest, and of uridin-5-oxyacetic acid in a pancreatic RNase digest were confirmed to two-dimensional thin-layer chromatography. To identify the terminal nucleotide at the 3′-hydroxy end, 20 optical density units of the fragment were exhaustively hydrolyzed with RNase T_1, and the hydrolysate was separated by two-dimensional paper chromatography. The oligonucleotide which remained near the origin was completely hydrolyzed by RNase T_2 to determine its nucleotide composition. The products were Vp, Up, Ap, and Cp in a ratio of 1.0:1.96:0.87:6.85. Only one molecule of Ap was found in this oligonucleotide, so fragment A must have uridin-5-oxyacetic acid at the 3′-hydroxy end.

From analyses of the oligonucleotides in RNase T_1 and pancreatic RNase digests and from the overlapping of these sequences, the three following possible sequences of fragment A were considered: (1) pG-G-G-U-G-A-U-s^4U-A-G-C-U-C-A-G-C-D-G-G-G-A-G-A-G-C-A-C-C-U-C-C-C-U-Vp, (2) pG-G-G-U-G-G-G-A-G-A-G-C-D-G-A-U-s^4U-A-G-C-U-C-C-A-G-C-A-C-C-U-C-C-C-U-Vp, and (3) pG-G-G-U-G-A-U-s^4U-A-G-C-D-G-G-G-A-G-A-G-C-U-C-A-G-C-A-C-C-U-C-C-C-C-U-Vp. Sequences 2 and 3 can be excluded by overlapping of the sequences of either fragment B or fragment C. Finally connection of fragment A and fragment D to obtain the total primary sequence of $tRNA_1^{Val}$ was achieved by overlapping these sequences with that of peak 11 from a complete digest of RNase T_1 with $tRNA_1^{Val}$.

Discussion

The total primary sequence of *E. coli* $tRNA_1^{Val}$ was unambiguously determined from the overlapping of sequences of large fragments obtained by limited digestions of tRNA with *B. subtilis* RNase and pancreatic RNase. The sequence can

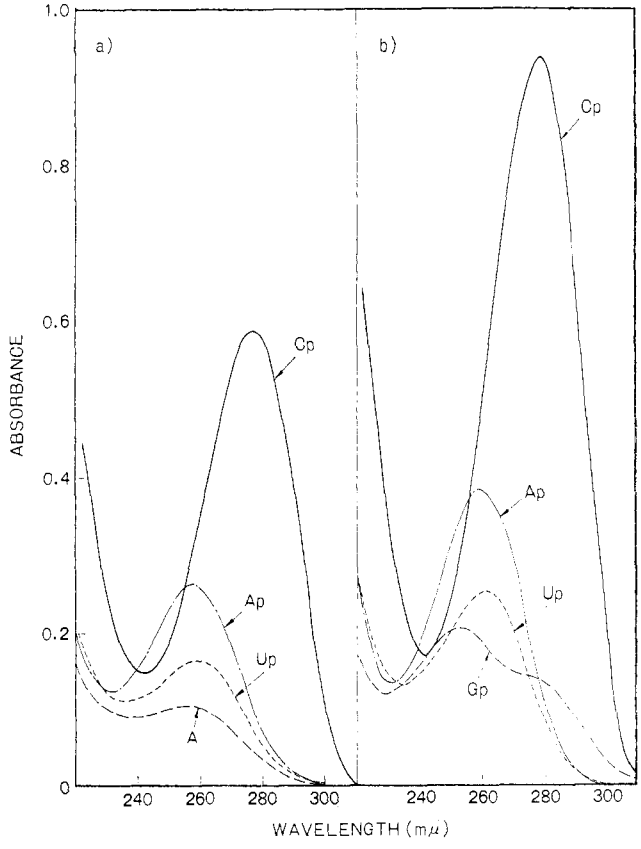

FIGURE 9: Ultraviolet absorption spectra of nucleotides eluted from two-dimensional thin-layer chromatograms of RNase T_2 digests of oligonucleotides. (a) RNase T_2 digest of U-C-A-U-C-A-C-C-C-A-C-C-A and (b) RNase T_2 digest of A-U-C-C-C-Gp.

TABLE I: Identity of Oligonucleotides from RNase T_1 and Pancreatic RNase Digests of Fragment e.

Digestion	Identity of Oligonucleotide	Method of Characterization	Amt of Sample Used for Assay (Optical Density Unit)
Pancreatic RNase	Cp, Up, G-Up, G-G-Tp, G-A-Up	Thin-layer chromatography	0.5
RNase T_1	Gp, U-Cp, T-ψ-C-Gp, A-U-C-C-C-Gp	DEAE-Sephadex A-25 column chromatography	1.0
RNase T_1	Gp, U-Cp, T-ψ-C-Gp, A-U-C-C-C-Gp	Thin-layer chromatography	0.8

be arranged in a cloverleaf structure like that common to all other known tRNAs, as shown in Figure 10. The sequence is in complete agreement with that reported by Yaniv and Barrell (1969) which was obtained by a quite different method. We also fully characterized the minor nucleosides located in the sequence. As described in separate papers from our laboratory, a minor nucleoside located in the 3′-hydroxy end of the anticodon was fully characterized as N^6-methyladenosine (Saneyoshi *et al.*, 1969), and a new minor nucleoside located in the first position of the anticodon was subsequently identified as uridin-5-oxyacetic acid (Murao *et al.*, 1970). This is the first detection of N^6-methyladenosine in a position at the 3′-hydroxy end of the anticodon, and it has not yet been found in other tRNA. Uridin-5-oxyacetic acid seems to have a specific role for codon recognition of *E. coli* $tRNA_1^{Val}$. *E. coli* $tRNA_1^{Val}$ was recognized by G-U-U with 20% of the efficiency of G-U-A and G-U-G in an experiment on tRNA–ribosome binding (Kellog *et al.*, 1966). Uridin-5-oxyacetic acid has wobbling characteristics to recognize U in addition to A and G in the codon sequence. It is interesting that *E. coli* $tRNA_1^{Ser}$ also contains uridin-5-oxyacetic acid in the first position of the anticodon, and this $tRNA_1^{Ser}$ recognized U-C-U with 20% of the efficiency of U-C-A and U-C-G (Ishikura *et al.*, 1971). It has also been suggested that recognition of the UCU codon by $tRNA_1^{Ser}$ is actually involved in f_2 coat protein synthesis *in vitro* (Roufa *et al.*, 1970).

Single cleavage of $tRNA_1^{Val}$ by *B. subtilis* RNase has been achieved by splitting the phosphodiester bond of uridin-5-oxyacetic acid. It was previously reported that excess *B. subtilis* RNase attacks the phosphodiester bond of uridine (Whitfeld and Witzel, 1963). In fact, the enzyme has been used for specific cleavage of the phosphodiester bond of uridine in the oligonucleotides obtained by RNase T_1 digestion, as described in the preceding paper. It was observed that the phosphodiester bond of uridin-5-oxyacetic acid is much more susceptible than that of normal uridine, as described in the preceding paper.

The valine-acceptor activity was fully restored by combining two fragments obtained by limited hydrolysis of *E. coli* $tRNA_1^{Val}$ with *B. subtilis* RNase (Oda *et al.*, 1969). The fragments designated II and III in that paper are the same as fragments D and A, respectively, reported here. The restoration of valine-acceptor activity was temperature and time dependent, and no decrease of ultraviolet absorbance was observed during the course of renaturation. This is a striking characteristic of the reconstitution of *E. coli* $tRNA_1^{Val}$ fragments as compared to the reconstitutions of fragments from yeast $tRNA^{Val}$, yeast $tRNA^{Tyr}$, wheat germ $tRNA^{Phe}$, and *E. coli* $tRNA^{fMet}$ (Bayev *et al.*, 1967a; Hashimoto *et al.*, 1969; Thiebe and Zachau, 1969; Seno *et al.*, 1969). As shown in Figure 11, the two fragments may form a secondary structure separately. Thus the total amounts of base pairing in these molecules are not much less than that of the reconstituted molecule. Therefore, the formation of the reconstituted molecule might have a very high activation energy and a high incubation temperature might be required to achieve a measurable rate of rearrangement.

It is interesting to compare the nucleotide sequences of *E. coli* $tRNA_1^{Val}$ and yeast $tRNA^{Val}$ (Bayev *et al.*, 1967b; Takemura *et al.*, 1968) with regard to the recognition site of tRNA for aa-tRNA synthetase, since Val-tRNA synthetase from *E. coli* and yeast recognize $tRNA^{Val}$ from both species (Lagerkvist and Waldenström, 1964). The only nucleotide sequence which is common to both *E. coli* $tRNA_1^{Val}$ and yeast $tRNA^{Val}$

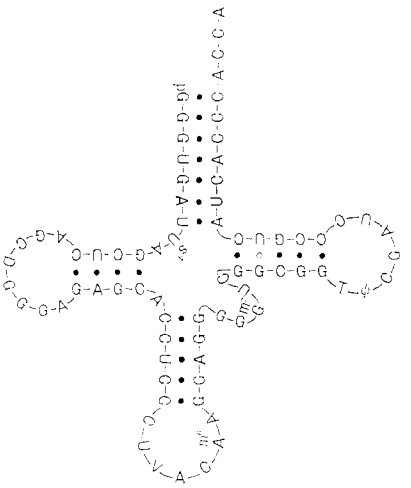

FIGURE 10: Cloverleaf structure of *E. coli* $tRNA_1^{Val}$.

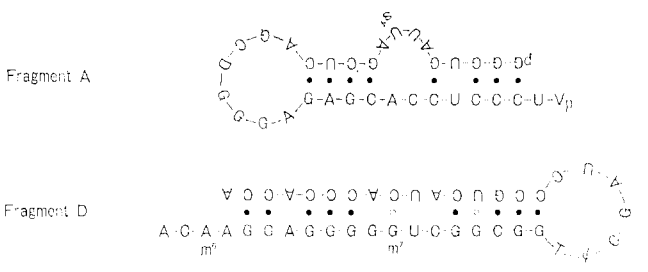

FIGURE 11: Possible secondary structures of fragment A and fragment D.

is a nonanucleotide in the TψC loop, as we (Harada *et al.*, 1969a,b) and Yaniv and Barrell (1969) have already reported. The TψC loop probably does not serve as a recognition site for aa-tRNA synthetase, since $tRNA^{Ser}$'s (Zachau *et al.*, 1966; Staehelin *et al.*, 1968) and $tRNA^{Tyr}$ (Goodman *et al.*, 1968; RajBhandary *et al.*, 1969) have almost the same sequence in this loop. Thus comparison of the nucleotide sequences of the two $tRNA^{Val}$'s suggests that the three-dimensional structure, rather than the nucleotide sequence of a particular region of tRNA, is involved in recognition of tRNA by the synthetase.

Overlapping of oligonucleotide sequences to obtain the total primary sequence was facilitated by the combined use of thin-layer chromatography and column chromatography monitored with a sensitive automatic ultraviolet recorder in characterization of large fragments. It should be emphasized that these techniques are feasible for sequence determination of other tRNAs. Oligonucleotides smaller than hexanucleotides are usually quite well separated by the thin-layer chromatography, since limited number of oligonucleotides are produced from purified tRNA by the RNase digestion. In fact, this procedure was found to be useful with any purified tRNA such as *E. coli* $tRNA_2^{Glu}$, $tRNA^{His}$, $tRNA_1^{Ser}$, $tRNA^{Leu}$, $tRNA^{Asp}$, and $tRNA_2^{Tyr}$. Each oligonucleotide in the chromatogram is first characterized by separate experiment using paper chromatogram of the RNase digest of intact tRNA. Then, type of oligonucleotide derived from the RNase digest of large fragment can be deduced from the location of spot in the thin-layer chromatogram, without doing the further characterization. In this case, 0.5–1 optical density unit of the fragment is sufficient for characterization. When quantitative analysis of oligonucleotide is required for characterization of the fragment, the spot in a chromatogram is eluted with water for spectrophotometric analysis. For this purpose, material corresponding to more than 0.05 optical density unit/nucleotide residue is required. Characterization of longer oligonucleotides can be carried out by column chromatographic analysis monitored with a sensitive automatic ultraviolet recorder which detects 0.05 optical density unit in a full-scale range. For this method, 0.5–2 optical density units of tRNA fragment are required for obtaining reliable chromatographic profile. It is suggested that DEAE-Sephadex A-25 column chromatography at pH 2.9 can be used for the detection of oligonucleotides which cannot be separated from each other at neutral pH. It is noteworthy to mention that these techniques are now successfully being used in our laboratory to obtain total primary sequences of *E. coli* $tRNA_2^{Glu}$ and $tRNA^{His}$.

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