Primary Sequence of $tRNA_1^{Val}$ from *Escherichia coli* B. I. Oligonucleotide Sequences of Digests of *Escherichia coli* $tRNA_1^{Val}$ with RNase T_1 and Pancreatic RNase*

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ABSTRACT: $tRNA_1^{Val}$ from *Escherichia coli* B was digested with RNase T_1 or pancreatic RNase and the oligonucleotides produced were separated and their nucleotide sequences were determined. Several methods were used for determination of the sequences of particular oligonucleotides. (1) *Bacillus subtilis* RNase and RNase U_2 were frequently used in sequence determinations. (2) To determine the sequences of the tridecanucleotide, U-C-A-U-C-A-C-C-C-A-C-C-A and the pentadecanucleotide, C-A-C-C-U-C-C-C-U-V-A-C-m^6A-A-Gp, derived from the RNase T_1 digest, stepwise degradation by periodate oxidation was adopted. (3) Limited digestion with snake venom phosphodiesterase was used to determine the sequences of T-ψ-C-G and the pentadecanucleotide. In these cases, the degradation was completely blocked at the positions of the minor nucleoside pseudouridine and uridin-5-oxyacetic acid, respectively. (4) To determine the sequence of the undecanucleotide, m^6A-A-G-G-A-G-G-G-G-m^7G-Up from the pancreatic RNase digest, this oligonucleotide was partially digested with silkworm nuclease, since it was not attacked by snake venom phosphodiesterase. The results obtained from the RNase T_1 digest and the pancreatic RNase digest are in excellent agreement. *E. coli* $tRNA_1^{Val}$ consists of 76 nucleotides, including 7 minor nucleosides, such as ribothymidine, pseudouridine, dihydrouridine, 7-methylguanosine, 4-thiouridine, N^6-methyladenosine, and uridin-5-oxyacetic acid.

Several years ago, we found that *Escherichia coli* tRNA could be altered by treatment with *Bacillus subtilis* RNase or RNase T_1 so as to retain its ability to accept amino acids, but not to transfer them to protein, suggesting that the anticodon region of tRNA was selectively cleaved by the RNase (Nishimura and Novelli, 1965). In fact, we have recently shown that purified *E. coli* $tRNA_1^{Val}$ is split into two fragments at the presumed anticodon by treatment with *B. subtilis* RNase (Oda *et al.*, 1969). When the two fragments were combined and annealed in the presence of magnesium ion, the valine-acceptor activity was fully restored. To examine the mechanism of this renaturation process in more detail, it became necessary to obtain the total primary sequence of *E. coli* $tRNA_1^{Val}$. In addition, since the primary structure of yeast $tRNA^{Val}$ has been determined (Bayev *et al.*, 1967; Takemura *et al.*, 1968), and Val-tRNA synthetase from *E. coli* and yeast recognize tRNAs from both species (Lagerkvist and Waldenström, 1964), it seemed interesting to compare the sequence of *E. coli* $tRNA^{Val}$ to that of yeast $tRNA^{Val}$ to obtain information on the recognition site for aa-tRNA synthetase.

This paper reports the nucleotide sequence of oligonucleotides derived from *E. coli* $tRNA_1^{Val}$ either by RNase T_1 digestion or by pancreatic RNase digestion. Special methods were adopted to determine the oligonucleotide sequence. These consisted of (1) use of RNase U_2 to split the molecule at the position of adenylic acid, (2) use of *B. subtilis* RNase to split the chain at the position of uridylic acid, (3) application of periodate oxidation, (4) use of snake venom phosphodiesterase to obtain oligonucleotide terminating with a minor nucleotide, and (5) use of silkworm nuclease for partial hydrolysis of the oligonucleotide.

Preliminary results on the sequence of oligonucleotides obtained by RNase T_1 digestion have been published (Harada *et al.*, 1969a,b). The sequence of oligonucleotides reported in this communication is in complete agreement with that reported by Yaniv and Barrell (1969). The accompanying paper (Kimura *et al.*, 1971) describes the overlapping of the sequences of large fragments obtained by limited digestion with either *B. subtilis* RNase or pancreatic RNase, and the total primary sequence of *E. coli* $tRNA_1^{Val}$ thus elucidated.

Materials and Methods

Materials. E. coli $tRNA_1^{Val}$ was prepared as described previously (Nishimura *et al.*, 1967; Oda *et al.*, 1969; Nishimura, 1971). This $tRNA_1^{Val}$ accepted 1.6 mμmoles of [^{14}C]-valine/1 optical density unit[1] of tRNA. Chromatography of the RNase T_1 digest or pancreatic RNase digest of $tRNA_1^{Val}$ indicated that the preparation was more than 95% pure. This $tRNA_1^{Val}$ was recognized mainly by G-U-A and G-U-G, and less well by G-U-U when tested in tRNA–ribosome binding experiments (Kellog *et al.*, 1966; Oda *et al.*, 1969). RNase T_1 and T_2 were gifts from Dr. H. Okazaki of the Central Research Laboratories, Sankyo Co., Ltd. RNase U_2 was a gift from Dr. F. Egami of the University of Tokyo. Silkworm nuclease was kindly provided by Dr. J. Mukai of Kyushu University. *E. coli* alkaline phosphomonoesterase was a gift from Dr. K. Tanaka of Shionogi Research Laboratory, Shionogi Co., Ltd. *B. subtilis* cyclic phosphodiesterase was a gift from Dr. Y. Sugino of the University of Kyoto. *B. subtilis* RNase was

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[1] Abbreviations used are: m^6A, N^6-methyladenosine; m^7G, 7-methylguanosine; D, dihydrouridine; s^4U, 4-thiouridine; V, uridin-5-oxyacetic acid; N, 2-methylthio-N^6-isopentenyladenosine; N*, *N*-[9(β-D-ribofuranosyl)purin-6-ylcarbamoyl]threonine; Q, unknown nucleoside located in the first position of the anticodon of *E. coli* $tRNA_2^{Tyr}$; Y, pyrimidine nucleoside; optical density unit, an amount of material which has an absorbance of 1.0 at 260 mμ when dissolved in 1 ml of water and measured with a 1-cm light path.

prepared as described previously (Nishimura, 1960; Nishimura and Ozawa, 1962). Pancreatic RNase (five-times recrystallized) was purchased from Sigma Chemical Co., and snake venom phosphodiesterase was obtained from Worthington Biochemicals. DEAE-Sephadex A-25 (capacity, 35 ± 0.5 mequiv/g; particle size, 40–120 μ) was a product of Pharmacia Fine Chemicals. DEAE-cellulose was obtained from Brown Co. Dowex 1-X2 and Dowex 50W-X8 were purchased from Dow Chemical Co. Bio-Gel P-2 was a product from Bio-Rad Laboratories. Thin-layer glass plates coated with Avicel SF cellulose were purchased from Funakoshi Pharmaceutical Co. Synthetic 7-methylguanosine was a gift from Dr. R. H. Hall of McMaster University.

General Conditions for Enzymatic Degradations. All incubations were conducted at 37°. For RNase T_1 digestion, the mixture contained 10 optical density units of oligonucleotide and 5 μg of RNase T_1 in 0.5 ml of 0.05 M Tris-HCl buffer (pH 7.8). The solution was incubated for 12–18 hr. For pancreatic RNase digestion, 10 optical density units of oligonucleotide and 10 μg of enzyme were dissolved in 0.5 ml of 0.05 M Tris-HCl buffer (pH 7.8) and incubated for 12–18 hr. For RNase T_2 digestion, 10 optical density units of oligonucleotide and 1 unit of enzyme (Uchida and Egami, 1966) were dissolved in 0.2 ml of 0.05 M potassium acetate buffer (pH 4.7) and incubated for 3 hr. For *E. coli* alkaline phosphatase digestion, 10 optical density units of oligonucleotide and 300 units of the enzyme (Torriani, 1966) were dissolved in 0.5 ml of ammonium bicarbonate buffer (pH 7.8) and incubated for 3 hr. For RNase U_2 digestion, the mixture contained 40 optical density units of oligonucleotide and 0.1 unit of enzyme (Arima *et al.*, 1968) in 0.5 ml of 0.05 M potassium acetate buffer (pH 4.7). The solution was incubated for 20 hr. It is necessary to use proper amounts of RNase U_2 so that terminal nucleotide of products are remained as cyclic phosphate. Incubation with excess amounts of RNase U_2 resulted in nonspecific cleavage at the positions of cytidylic acid and uridylic acid residues (Uchida *et al.*, 1970). In some cases as described in Results section, the RNase U_2 digests were treated with 0.1 M HCl for 18 hr at 4° in order to open cyclic phosphate. For *B. subtilis* RNase digestion, 10 optical density units of oligonucleotide and 50 μg of enzyme (Nishimura, 1966) were dissolved in 0.5 ml of 0.05 M triethylammonium bicarbonate buffer (pH 7.9) and incubated for 7 hr. Amounts of the enzyme added in the incubation mixture must be controlled to avoid cleavage at the position of cytidylic acid residue, since excess amounts of the enzyme can also attack phosphodiester bond of cytidine in addition to uridine (Whitfeld and Witzel, 1963). For digestion with snake venom phosphodiesterase, 5 optical density units of oligonucleotide and 5 μg of the enzyme were dissolved in 0.5 ml of ammonium bicarbonate buffer (pH 7.8) and incubated for 5 hr. For digestion with *B. subtilis* cyclic phosphodiesterase, 4 optical density units of nucleoside 2′,3′-cyclic phosphate and 20 units of enzyme (Shimada and Sugino, 1969) were dissolved in 0.2 ml of Tris-HCl buffer (pH 7.5) and incubated for 1 hr.

Alkaline Hydrolysis. Oligonucleotide (10 optical density units) was dissolved in 0.3 ml of 0.3 M NaOH and incubated for 18 hr at 37°. After the digestion the material was neutralized with Dowex 50 (H^+ form) resin. The resin was removed by filtration, and the filtrate and washings were evaporated for chromatography.

Desalting of Pooled Fractions. To remove urea and salts from the tri- and higher oligonucleotides, pooled fractions were diluted 10-fold with water and applied to a column of DEAE-cellulose (0.8 × 5 cm) (Rushizky and Sober, 1962). The column was washed with 15 ml of 0.01 M triethylammonium bicarbonate buffer (pH 7.8) and then the oligonucleotides were eluted with 2 M triethylammonium bicarbonate. Triethylammonium bicarbonate was removed in a rotary evaporator. The mono- and dinucleotides were desalted using a column of Bio-Gel P-2 (Uziel and Cohn, 1965) or a small column of DEAE-Sephadex A-25 (0.8 × 5 cm). With DEAE-Sephadex A-25, 0.1 M triethylammonium bicarbonate was used as washing buffer, and oligonucleotides were eluted with 2 M triethylammonium bicarbonate.

Paper Chromatography and Thin-Layer Chromatography. Paper chromatography was carried out on Toyo-Roshi No. 51A paper with the following solvent systems: (A) isobutyric acid–concentrated NH_4OH–H_2O (66:1:33, v/v), solvent B, 1-propanol–concentrated NH_4OH–H_2O (55:10:35, v/v); solvent C, 1-butanol–H_2O–concentrated NH_4OH (86:14:5, v/v); solvent D, ethanol–1 M ammonium acetate, pH 7.5 (70:30, v/v); solvent E, isobutyric acid–0.5 N NH_4OH (5:3, v/v); solvent F, isopropyl alcohol–concentrated HCl–H_2O, (70:15:15, v/v). The descending technique was used except with solvents E and F which were used for two-dimensional paper chromatography. Spots were usually detected on chromatograms under an ultraviolet lamp at 2537 Å. For detection of 4-thiouridylic acid, an ultraviolet lamp at 3650 Å was used. Nucleotidic material was eluted from paper chromatograms with water.

Two-dimensional paper chromatography was performed as follows. A sample of alkaline hydrolysate, RNase T_2 hydrolysate or snake venom phosphodiesterase hydrolysate (−15 optical density units) were spotted on Toyo-Roshi No. 51A paper (30 × 30 cm) and chromatographed with solvent E for 16 hr. Then, the paper was air-dried and chromatographed for 30 hr with solvent F. The pattern of $tRNA_1^{Val}$ after digestion with RNase T_2 was described previously (Saneyoshi *et al.*, 1969). Each spot was cut into small pieces (about 0.4 × 0.4 cm), transfered to a test tube, and eluted with 0.01 N HCl (1–2 ml). Two-dimensional thin-layer chromatography on an Avicel SF plate (10 × 10 cm) was also carried out with solvents E and F. The pattern was the same as that of the two-dimensional paper chromatogram. In this case, −3 optical density units of hydrolysate were used and the chromatogram was developed for about 3 hr in each dimension. Each spot was removed from the plate with a spatula and transferred to a small test tube (0.5 × 6 cm). Then 0.5 ml of 0.01 N HCl was added and the tubes were shaken for 2 min, stood for 2 hr, and centrifuged for 2 min at 2000 rpm to remove cellulose powder. In all cases, a similar sized area without ultraviolet-absorbing material was also eluted and used as a blank.

Results

Products of Digestion with RNase T_1. A sample of $tRNA_1^{Va}$ (1000 optical density units) was dissolved in 5 ml of 0.05 M Tris-HCl buffer (pH 7.8) containing 500 μg of RNase T_1. The solution was incubated for 16 hr at 37°. Then solid urea was added to the solution to a concentration of 7 M. The mixture was adsorbed on a column of DEAE-Sephadex A-25, and the column was eluted with a linear gradient of sodium chloride (Rushizky *et al.*, 1964). A typical elution pattern is shown in Figure 1. Further elution of the column with 1 M NaCl did not release any nucleotidic material. The fractions in each peak were pooled, freed of urea and salt, and characterized as described below. The identities and quantities of materials in the peaks are summarized in Table I.

PEAK 1 (G>p). This was identified as guanosine 2′,3′-

TABLE I: Analysis of Products Obtained by Degradation of $tRNA_1^{Val}$ with RNase T_1.

Peak	Composition	Molar Ratio[a]
1	G>p	1.4
2	Gp	8.8
3-1	C-Gp	1.2
3-2	A-Gp	2.7
3-3	U-Gp	0.9
4	m^7G-U-C-Gp	0.8
5	C-D-Gp	1.1
6	pGp	1.0
7	T-ψ-C-Gp	1.0
8	C-U-C-A-Gp	1.0
9-1	A-U-C-C-C-Gp	0.9
9-2	A-U-s^4U-A-Gp	0.5
10	U-C-A-U-C-A-C-C-C-A-C-C-A	0.8
11	C-A-C-C-U-C-C-C-U-V-A-C-m^6A-A-Gp	0.8

[a] Extinction coefficients of mononucleotides were taken from the literature (Holley *et al.*, 1965; Madison *et al.*, 1967; Katz and Dudock, 1969). In addition, the following millimolar extinction coefficients at pH 7 and 260 mμ were adopted: s^4U, 2.5 and V, 3.8.

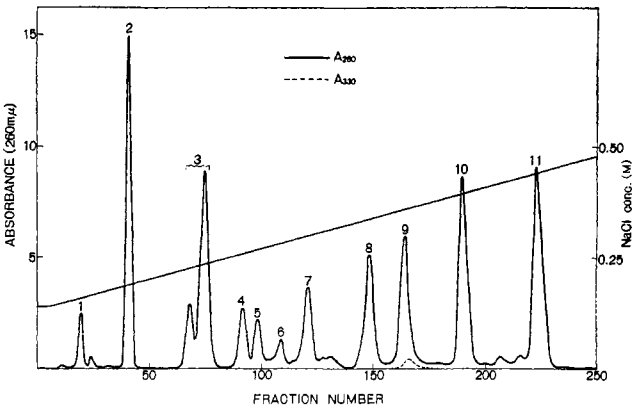

FIGURE 1: Chromatography of an RNase T_1 digest of $tRNA_1^{Va}$ (1000 optical density units) on a column (0.6 × 150 cm) of DEAE-Sephadex A-25. Elution was carried out with a linear gradient obtained with 1000 ml each of 0.14 and 0.7 M sodium chloride in the presence of 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The flow rate was 10 ml/hr. Fractions of 5.2 ml of effluent were collected.

cyclic phosphate from its spectrum and mobility in solvent A. The nucleoside obtained by treatment with cyclic phosphodiesterase was also identified as guanosine from its spectrum and mobility in solvent C.

PEAK 2 (Gp). This was identified as Gp from its spectrum and mobility on two-dimensional paper chromatography. In addition, guanosine was found to be the sole product after alkaline phosphomonoesterase treatment, judging from its mobility on paper chromatography with solvent C.

PEAK 3 (C-Gp, A-Gp, U-Gp). This peak was a mixture of C-Gp, A-Gp, and U-Gp. These dinucleotides were completely separated by Dowex 1 column chromatography (Ishikura *et al.*, 1966; Neelon *et al.*, 1967) and identified by digestion with RNase T_2 and subsequent two-dimensional paper chromatography.

PEAK 4 (m^7G-U-C-Gp). On two-dimensional paper chromatography of an RNase T_2 digest of this peak, Cp, Up, Gp, and m^7Gp were identified in a molar ratio of 1.1:1.2:1.0:0.6. A small amount of m^7Gp* (degradation product of m^7Gp) (Jones and Robins, 1963; Brookes and Lawley, 1961; Haines *et al.*, 1962) was also found between the spots of Gp and Cp. The ultraviolet absorption spectra of m^7Gp and m^7Gp* were identical with those of the corresponding synthetic nucleoside. Digestion of the oligonucleotide with pancreatic RNase and subsequent separation of the products with solvent A showed that Cp, Gp, and m^7G-Up were present in equimolar amounts. The oligonucleotide was treated with *E. coli* alkaline phosphomonoesterase. The dephosphorylated tetranucleotide was freed from enzyme by chromatography with solvent B. It was found that m^7G in the oligonucleotide was converted to m^7G* during this chromatography. The product was digested with snake venom phosphodiesterase and separated by two-dimensional paper chromatography. The 5′ terminal was determined to be m^7G*.

PEAK 5 (C-D-Gp). An RNase T_2 digest was separated by two-dimensional paper chromatography. Cp and Gp were obtained in the ratio of 1.1 to 1.0. In addition, Dp was detected on the chromatogram by spraying with ammonium molybdate reagent (Hanes and Isherwood, 1949). The dihydrouridine content was determined from the decrease in absorbance at 235 mμ after addition of NaOH to 0.02 M to the oligonucleotide solution (RajBhandary *et al.*, 1968b). The ratio of the amounts of these components was Dp:Cp:Gp = 0.9:1.1:1.0. The dephosphorylated oligonucleotide was digested with snake venom phosphodiesterase and the digest was developed by two-dimensional paper chromatography. Cytidine was obtained as the sole nucleoside.

PEAK 6 (pGp). This was identified as pGp. Its spectrum, position of elution from the column, and R_F on two-dimensional paper chromatography supported this conclusion. In addition, on dephosphorylation with *E. coli* alkaline phosphomonoesterase only guanosine was obtained.

PEAK 7 (T-ψ-C-Gp). On two-dimensional paper chromatography of an RNase T_2 digest, Gp, Cp, ψp, and Tp were obtained in the ratio of 1.0:1.2:0.9:1.1. The dephosphorylated oligonucleotide (8 optical density units) was dissolved in 0.09 ml of 0.02 M NH_4HCO_3 and incubated with 180 μg of snake venom phosphodiesterase at 37° for 10 min. The reaction mixture was then heated for 1.5 min at 100°, and chromatographed with solvent A. Two spots were obtained. The material with the higher mobility was identified as pC. The more slowly moving component was treated with alkaline phosphomonesterase and the digest was chromatographed with solvent A. Guanosine and T-ψ were obtained in the ratio of 1.0:0.9.

PEAK 8 (C-U-C-A-Gp). The products of RNase T_2 digestion were Gp, Cp, Ap, and Up in the ratio of 1.0:2.2:1.1:1.1. After dephosphorylation of peak 8 complete digestion with snake venom phosphodiesterase showed that cytidine was the 5′-terminal nucleoside. The complete sequence was determined by partial digestion with snake venom phosphodiesterase as described by Holley *et al.* (1964). The dephosphorylated pentanucleotide (19 optical density units) was dissolved in 0.3 ml of 0.05 M NH_4HCO_3 and incubated with 160 μg of snake venom phosphodiesterase at 37° for 7 min. The mixture was then heated for 1.5 min at 100°, and fractionated by DEAE-Sephadex A-25 column chromatography. Three main peaks were obtained. The first peak was further treated with alkaline phosphomonoesterase, and the digest was chromato-

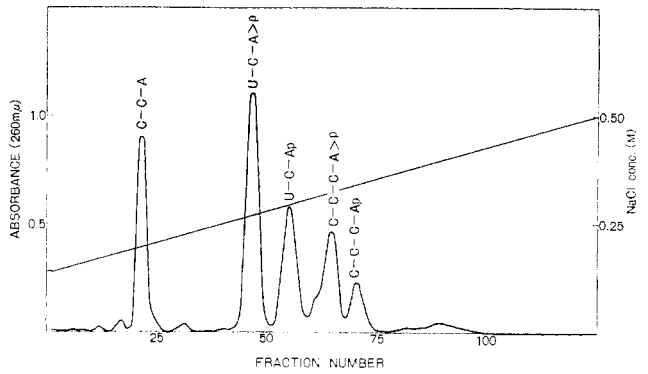

FIGURE 2: Chromatography of an RNase U_2 digest of peak 10 (30 optical density units) on a column (0.5 × 100 cm) of DEAE-Sephadex A-25. Elution was carried out with a linear gradient obtained from 250 ml each of 0.14 and 0.7 M sodium chloride in the presence of 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The flow rate was 10 ml/hr. Fractions of 2.7 ml of effluent were collected.

graphed with solvent A. Three spots were obtained. They were identified as adenosine, guanosine, and C-U-C, respectively. The second and third peaks were identified as C-U-C-A and C-U-C-A-G, respectively.

PEAK 9 (A-U-C-C-C-Gp, A-U-s^{4}U-A-Gp). The compounds in this peak was separated into peak 9-1 and peak 9-2 by column chromatography on DEAE-Sephadex A-25 in the presence of 7 M urea (pH 2.7) (Rushizky *et al.*, 1964). The RNase T_2 digest of peak 9-1 contained Ap, Cp, Up, and Gp in the ratio of 1.0:3.0:1.0:0.9. Digestion of dephosphorylated peak 9-1 with snake venom phosphodiesterase yielded adenosine as the sole nucleoside. The products of digestion of peak 9-1 with pancreatic RNase were Cp, Gp, and A-Up in the ratio of 3.0:1.0:1.0.

The poor yield of peak 9-2 was due to the presence of a 4-thiouridine residue in this oligonucleotide. The products of RNase T_2 hydrolysis were Gp, Ap, Up, and s^{4}Up in the ratio of 1.0:1.9:1.3:0.4. The low yield of s^{4}Up and high yield of Up is explained as due to partial conversion of 4-thiouridine to uridine during the isolation procedure. The products of digestion with pancreatic RNase were separated by chromatography on DEAE-Sephadex A-25. Two main peaks were obtained. The first peak was further separated into s^{4}Up and A-Up by chromatography with solvent D. The second peak was identified as A-Gp. The complete sequence of this pentanucleotide was determined by analysis of the products obtained by RNase U_2 digestion. Separation of the products by DEAE-Sephadex A-25 column chromatography gave three main peaks. They were identified as A>p, Gp, and U-s^{4}U-A>p, respectively.

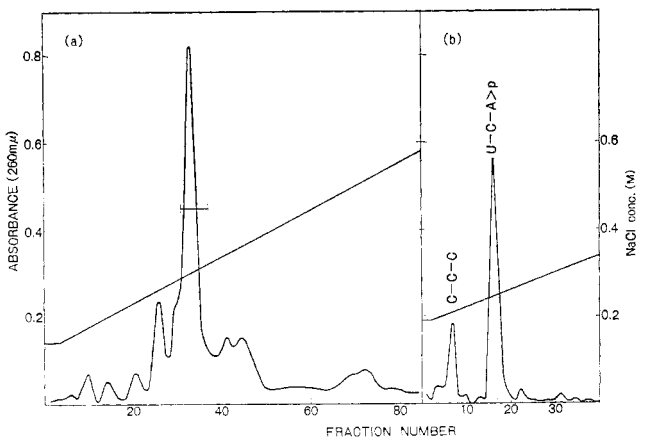

FIGURE 3: DEAE-Sephadex A-25 column chromatography (0.3 × 50 cm). Elution was carried out with an NaCl gradient from 0.1 to 0.7 M (100 ml × 2) in 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The flow rate was 10 ml/hr. Fractions of 2 ml of effluent were collected. (a) Periodate oxidation products of peak 10. (b) Fractions 31–37 of (a) were pooled and digested with RNase U_2.

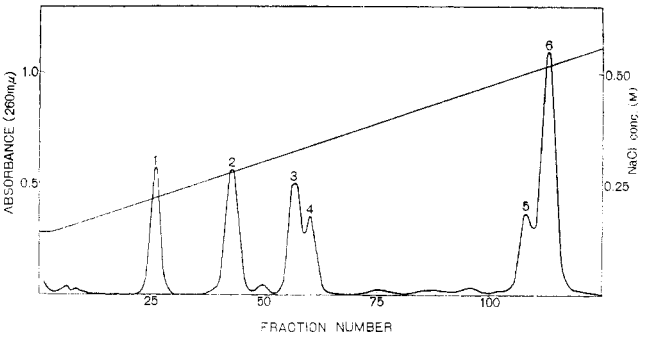

FIGURE 4: Chromatography of an RNase U_2 digest of peak 11 (40 optical density units) on a column (0.5 × 100 cm) of DEAE-Sephadex A-25. The conditions for chromatography were as in Figure 2 except that 3-ml fractions were collected.

PEAK 10 (U-C-A-U-C-A-C-C-C-A-C-C-A). The RNase T_2 digest gave Ap, Cp, Up, and adenosine in the ratio of 3.0:7.2:1.9:0.9. Uridine was identified as the 5′-terminal nucleoside by digestion of the oligonucleotide with snake venom phosphodiesterase. The digest of the oligonucleotide with pancreatic RNase contained adenosine, Cp, Up, A-Cp, and A-Up in the ratio of 1.0:4.9:1.0:1.6:0.8. The RNase U_2 digest was treated with 0.1 M HCl for 18 hr at 4°, and separated by DEAE-Sephadex A-25 column chromatography. C-C-A, U-C-Ap, and C-C-C-Ap were obtained in the ratio of 1.0:2.1:1.0 (Figure 2). Thus, the partial sequence of the oligonucleotide was U-C-A(U-C-A,C-C-C-A)C-C-A. The complete sequence was determined using periodate oxidation by the method of Khym and Uziel (1968) with the modifications that NaCl and CTABr were omitted. The mixture was desalted on a DEAE-cellulose column, and the bases released were not analyzed. Periodate oxidation followed by alkaline phosphomonoesterase treatment was carried out four times. The shortened oligonucleotide was reisolated by DEAE-Sephadex A-25 column chromatography in the presence of 7 M urea, at pH 7.5 (Figure 3a). Fractions in the main peak was pooled, desalted, and digested with RNase U_2. The products were separated by DEAE-Sephadex A-25 column chromatography. Two peaks were obtained as shown in Figure 3b. The first was found to be C-C-C, and the second peak U-C-A>p. The ratio of the amounts of C-C-C and U-C-A>p was 1.0:1.8.

PEAK 11 (C-A-C-C-U-C-C-C-U-V-A-C-m^{6}A-A-Gp). The RNase T_2 digest contained Gp, Cp, Ap, Up, m^{6}Ap, and Vp in the ratio of 1.0:7.3:2.7:2.0:1.0:1.0. Complete digestion with snake venom phosphodiesterase showed that cytidine was the 5′-terminal nucleoside. A-Cp (2 moles) and m^{6}A-A-Gp (1 mole) were obtained on digestion with pancreatic RNase. On chromatography of the RNase U_2 digest after the acid treatment on DEAE-Sephadex A-25 six peaks were obtained (Figure 4). The first peak contained Gp and a little Ap, while the second peak only contained C-Ap. The third and fourth peaks were both C-m^{6}A-Ap judging from analysis of RNase T_2 digests. The separation of this oligonucleotide into two

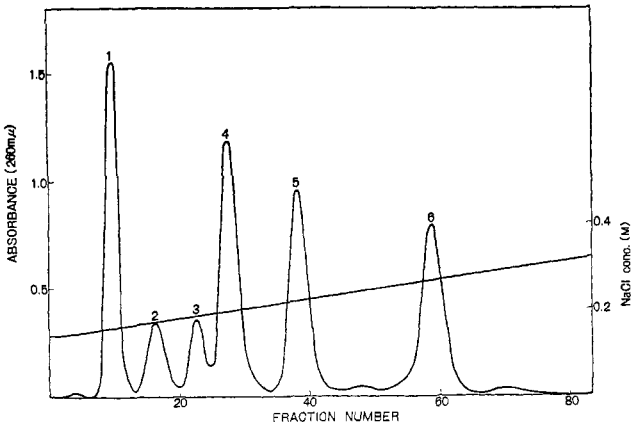

FIGURE 5: Chromatography of a *B. subtilis* RNase digest of peak 6 of Figure 4 (16 optical density units) on a column (0.3 × 100 cm) of DEAE-Sephadex A-25. Elution was carried out with a linear gradient obtained with 150 ml each of 0.14 and 0.7 M NaCl in 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The flow rate was 8 ml/hr. Fractions of 1.2 ml were collected.

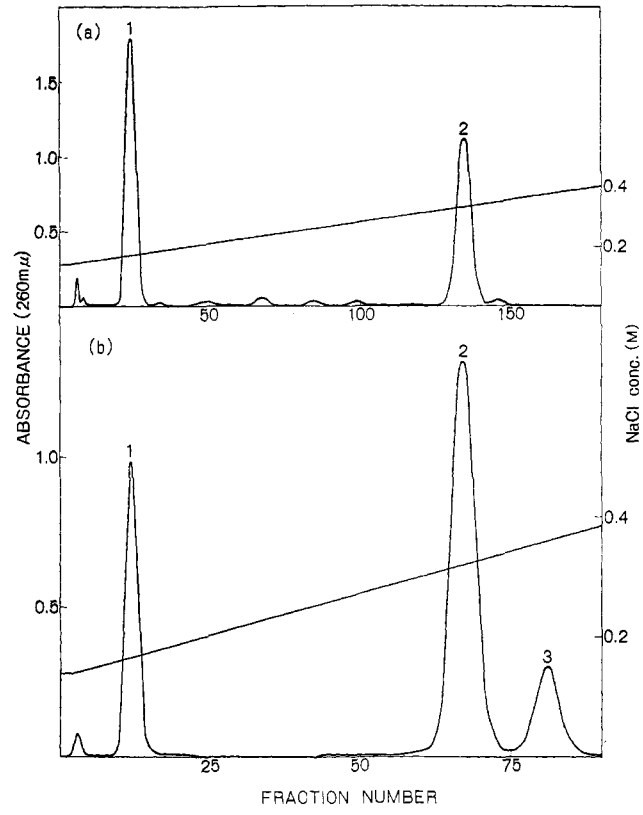

FIGURE 6: (a) Chromatography of a controlled digest of pentadecanucleotide (38.5 optical density units) with snake venom phosphodiesterase on a column (0.5 × 100 cm) of DEAE-Sephadex A-25. Elution was carried out with a linear gradient obtained with 250 ml each of 0.14 and 0.4 M NaCl in 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The flow rate was 10 ml/hr and the fraction size 2.8 ml. (b) Chromatography of an RNase U_2 digest of peak 2 of (a) (18 optical density units) on a column (0.3 × 50 cm of DEAE-Sephadex A-25. The conditions of chromatography were as for Figure 3 except that 1.7-ml fractions were collected.

peaks is due to the fact that the oligonucleotide in the third peak has terminal cyclic phosphate. The fifth and sixth peaks were dephosphorylated and the dephosphorylated oligonucleotides were hydrolyzed with either RNase T_2 or snake venom phosphodiesterase to determine the 3′ and 5′ end of the nucleotides. Results indicated that the fifth peak was $C(C_4,U_2)Vp$, while the sixth peak was $C(C_4,U_2,V)Ap$. The relative proportions of Ap in the first peak and of oligonucleotide in the fifth peak also suggests that the fifth peak was a degradation product of the sixth peak (Table II). The partial sequence of the sixth peak was determined by digestion with *B. subtilis* RNase. Chromatography of the products on DEAE-Sephadex A-25 gave six peaks, as shown in Figure 5. Each peak was desalted, treated with 0.1 N HCl for 18 hr at 4° to open the cyclic phosphate, and then treated with the alkaline phosphomonoesterase to remove 3′-terminal phosphate. The nucleotide sequences of each peak were elucidated by analysis of digests with RNase T_2 or snake venom phosphodiesterase and are summarized in Table III. It seems probable that C-C-U>p in the fourth peak and C-C-C-U>p in the fifth peak were derived from $C(C_4,U)Up$ in the last peak. These results indicate that the partial sequence of the pentadecanucleotide must be [C-A,(C-C-U,C-C-C-U)V-A]C-m^6A-A-Gp. Its complete sequence was finally determined by controlled digestion of the pentadecanucleotide with snake venom phosphodiesterase. Minor nucleosides are generally resistant to snake venom phosphodiesterase (RajBhandary *et al.*, 1968a; Neelon *et al.*, 1967) so if conditions are carefully controlled, the digestion should stop at the position of the minor nucleoside. The dephosphorylated pentadecanucleotide (38.5 optical density units) was dissolved in 0.7 ml of 0.05 M ammonium bicarbonate buffer (pH 7.8) and incubated with 330 μg of snake venom phosphodiesterase at 37° for 10 min. The reaction mixture was then heated at 100° for 1.5 min. The hydrolysate was fractionated by DEAE-Sephadex A-25 column chromatography.

TABLE II: Analysis of Products Obtained by Degradation of the Pentadecanucleotide with RNase U_2.

Peak	Composition	Molar Ratio
1	Gp	1.0
	Ap	0.3
2	C-Ap	0.9
3	C-m^6A-A>p	0.9
4	C-m^6A-Ap	0.9
5	$C(C_4,U_2)Vp$	0.3
6	$C(C_4,U_2,V)Ap$	0.7

TABLE III: Analysis of Products Obtained by Degradation of $C(C_4,U_2,V)Ap$ with *B. subtilis* RNase.

Peak	Composition	Molar Ratio
1	Ap	1.0
2	C-C>p[a]	0.2
	C-U>p[a]	0.2
3	V>p	1.1
4	C-C-U>p	0.8
5	C-C-C-U>p	0.5
6	$C(C_4,U)U>p$	0.3

[a] These dinucleotides must be degradation products of peak 5.

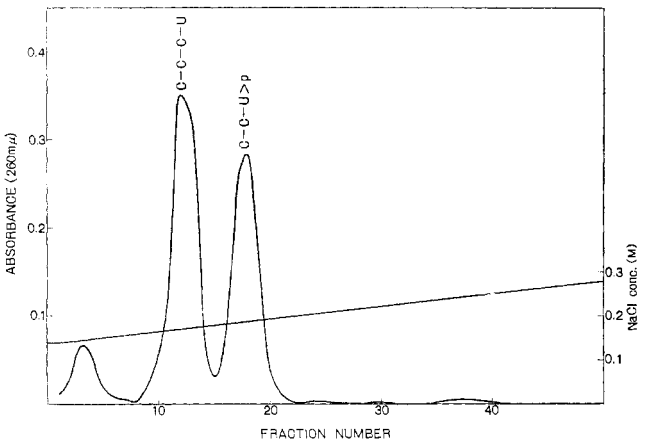

FIGURE 7: Chromatography of a *B. subtilis* RNase digest of (C_5,U)U (4 optical density units) on a column (0.3 × 50 cm) of DEAE-Sephadex A-25. Elution was carried out with a linear gradient obtained with 100 ml each of 0.14 and 0.4 M NaCl in 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The flow rate was 10 ml/hr and the fraction size 2.0 ml.

As shown in Figure 6a, only two peaks were obtained. The first peak was found to be a mixture of nucleoside 5′-monophosphates. The second peak was digested with RNase U_2 and chromatographed on a DEAE-Sephadex A-25 column. Three peaks were obtained (Figure 6b). They were C-A>p, C(C_4,U_2)V, and the original oligonucleotide, respectively. From these results, the partial sequence of the pentadecanucleotide must be C-A(C-C-U,C-C-C-U)V-A-C-m^{6}A-A-Gp. To determine the order of the oligonucleotides (shown in parentheses), C(C_4,U_2)V was treated with periodate, followed by alkaline phosphomonoesterase. The oligonucleotide was isolated and digested with *B. subtilis* RNase. Chromatography of the digest on DEAE-Sephadex A-25 gave two main peaks (Figure 7), which were characterized as C-C-C-U and C-C-U>p, respectively. It was often observed that oligonucleotide terminated with cyclic phosphate was eluted later as expected from a column of DEAE-Sephadex A-25. It apparently does not lose entirely one negative charge as compared to corresponding oligonucleotide terminated with 3′,(2′)-phosphate. Thus C-C-U>p was eluted later than C-C-C-U, resulting in the separation of the two oligonucleotides. The same phenomenon has been observed in the case of separation of C-C-C-A>p from U-C-Ap (Figure 2), and C-m^{6}A-A>p from C-Ap (Figure 4).

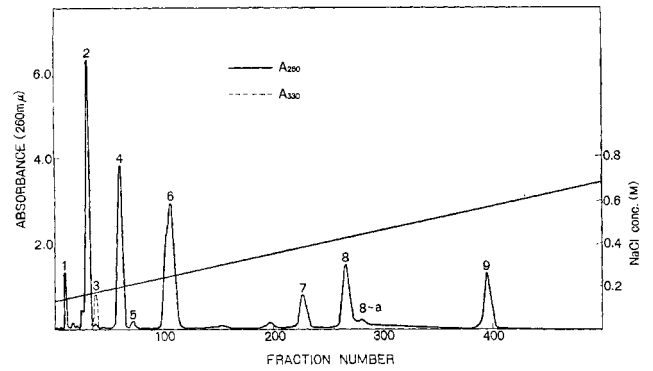

FIGURE 8: Chromatography of a pancreatic RNase digest of $tRNA_1^{Val}$ (300 optical density units). The conditions of chromatography were as for Figure 1 except that 3.5-ml fractions were collected.

TABLE IV: Analysis of Products Obtained by Degradation of $tRNA_1^{Val}$ with Pancreatic RNase.

Peak	Composition	Molar Ratio
1	A	1.0
2-1	Cp	14.5
2-2	Dp	0.9
2-3	ψp	0.9
2-4	Up	3.0
3	s^{4}Up	1.0
4-1	A-Cp	4.0
4-2	A-Up	1.0
4-3	G-Up	1.2
5	Vp	1.1
6-1	A-G-Cp	2.0
6-2	G-G-Cp	1.0
6-3	G-A-Up	2.0
6-4	G-G-Tp	1.0
7	pG-G-G-Up	1.0
8	G-G-G-A-G-A-G-Cp	0.9
9	m^{6}A-A-G-G-A-G-G-G-G-m^{7}G-Up	0.6

[a] A value of 17.0 was tentatively used as the millimolar extinction coefficient of s^{4}Up at 330 mμ and pH 7.

Products of Digestion with Pancreatic RNase. A sample of tRNA (300 optical density units) was dissolved in 1.8 ml of 0.05 M Tris-HCl buffer (pH 7.8) and incubated with 600 μg of pancreatic RNase at 37° for 16 hr. After incubation, solid urea was added to the reaction mixture to a concentration of 7 M. The hydrolysate was adsorbed on a column of DEAE-Sephadex A-25 and eluted with a linear gradient of sodium chloride (Figure 8). The compositions of the peaks are given in Table IV. Analyses of the fragments are described below.

PEAK 1 (A). This was identified as adenosine from its spectrum.

PEAK 2 (Cp, Dp, ψp, Up). The fractions in this peak containing 7 M urea and salt were pooled, diluted tenfold with water, and adsorbed on a column of Dowex 1 (X2 formate form, 200–400 mesh). Nucleotidic materials were eluted with a parabolic gradient attained by placing 100 ml of solution in each chamber (Offengand *et al.*, 1961). The nucleotides thus separated were identified from their spectra and elution positions (Figure 9). The absorbance at 235 mμ due to Dp was measured as follows. A sample of 0.95 ml of eluate was mixed with 0.05 ml of 10 M NaOH and its absorbance at 235 mμ was recorded immediately. Then the mixture was incubated for 30 min at 20°, and its absorbance at 235 mμ was remeasured. The contribution of Dp to the absorbance at 235 mμ was calculated from the difference between these values.

PEAK 3 (s^{4}Up). This was identified as s^{4}Up from its spectrum.

PEAK 4 (A-Cp, A-Up, G-Up). This peak contained A-Cp, A-Up, and G-Up. These nucleotides were completely separated on Dowex 1 (X2, chloride form, 200–400 mesh) as with peak 3 of the RNase T_1 digest. Their base compositions were determined by two-dimensional paper chromatography of RNase T_2 digests.

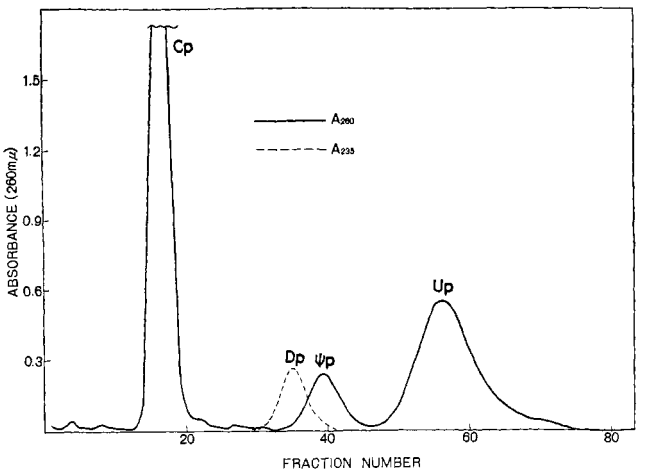

FIGURE 9: Chromatography of the mononucleotide fraction of Figure 8 (57 optical density units) on a column (0.5 × 30 cm) of Dowex 1-X2 formate form (200–400 mesh). Elution was carried out with a parabolic gradient established with 100 ml of 0.035 M ammonium formate (pH 3.7) in each of the two mixing chambers and 100 ml of 0.6 M ammonium formate (pH 3.7) in the reservoir chamber. The flow rate was 8 ml/hr and the fraction size 2.0 ml.

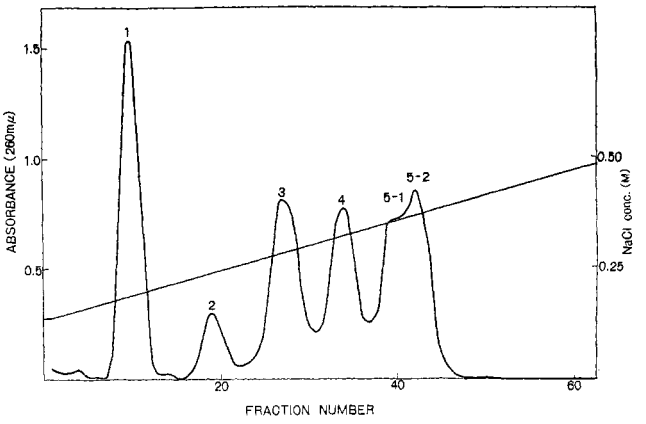

FIGURE 10: Chromatography of a limited digest of dephosphorylated octanucleotide (30 optical density units) with snake venom phosphodiesterase on a column (0.3 × 50 cm) of DEAE-Sephadex A-25. The conditions for chromatography were as for Figure 3.

PEAK 5 (Vp). This was identified as Vp from its spectrum, elution position on column chromatography, and R_F value on two-dimensional paper chromatography. Vp has an extra negative charge. Therefore, it was eluted next to the dinucleotides.

PEAK 6 (A-G-Cp, G-G-Cp, G-A-Up, G-G-Tp). This peak was found to be a mixture of A-G-Cp, G-G-Cp, G-A-Up, and G-G-Tp. It was treated with alkaline phosphatase and the trinucleoside diphosphates were completely separated by Dowex 1 (X2, chloride form, 200–400 mesh) column chromatography. Each peak was desalted, digested with snake venom phosphodiesterase or RNase T_2, and analyzed by two-dimensional paper chromatography.

PEAK 7 (pG-G-G-Up). The products of RNase T_2 digestion were pGp, Gp, and Up in the ratio of 1.0:1.9:0.8.

PEAK 8 (G-G-G-A-G-A-G-Cp). The products of alkaline hydrolysis were Cp, Ap, and Gp in the ratio of 0.9:2.5:5.2. RNase T_1 digestion of the oligonucleotide yielded Gp, A-Gp, and Cp in the ratio of 3.0:1.8:1.0. The complete sequence was obtained by partial digestion with snake venom phosphodiesterase. The dephosphorylated octanucleotide (30 optical density units) was dissolved in 0.7 ml of 0.05 M NH_4HCO_3 and incubated with 400 μg of the enzyme for 5 min at 37°. Then the reaction mixture was heated at 100° for 1.5 min and fractionated by DEAE-Sephadex A-25 column chromatography. Five peaks were obtained as shown in Figure 10. On paper chromatography of the first peak with solvent A, G-G-G was separated from mononucleotides. The next three peaks were identified as G-G-G-A, G-G-G-A-G, and G-G-G-A-G-A, respectively, by two-dimensional paper chromatography of RNase T_2 digests. The last peak contained two components. The first half, peak 5-1, was identified as G-G-G-A-G-A-G and the latter half, peak 5-2, as G-G-G-A-G-A-G-C.

PEAK 9 (m^6A-A-G-G-A-G-G-G-G-m^7G-Up). This oligonucleotide seemed to be an aggregate, since it was eluted much later than the octanucleotide and was extremely resistant to RNase T_1, RNase T_2, and snake venom phosphodiesterase. Peak 8-a, eluted just after peak 8, had a base composition identical with that of peak 9, suggesting that peak 8-a is the same oligonucleotide as peak 9, but is not aggregated. Another observation supporting this idea is that column chromatography of a pancreatic RNase digest of $tRNA_1^{Val}$ on DEAE-Sephadex A-25 at four times the normal flow rate resulted in a higher yield of peak 8-a with concomitant decrease in the yield of peak 9. The products of alkaline hydrolysis were Up, m^7G*p, Gp, Ap, and m^6Ap in the ratio of 1.0:1.2:5.9:2.0:1.0. From the oligonucleotide sequences of the RNase T_1 digest shown in Table I, it was concluded that the 5′ and 3′ terminals must be m^6A-A-Gp and G-m^7G-Up, respectively. To obtain the complete sequence of the undecanucleotide, it was partially digested with silkworm nuclease (Mukai, 1965; Mukai and Soeta, 1967) since it was not attacked by snake venom phosphodiesterase. The undecanucleotide (60 optical density units) was dissolved in 2.5 ml of 0.05 M sodium carbonate–sodium bicarbonate buffer (pH 10.5) containing 0.1 M NaCl and 0.0005 M magnesium acetate and incubated with 5 μg of the enzyme for 1 hr at 37°. The hydrolysate was chromatographed on a DEAE-Sephadex A-25 column (Figure 11). The first and second peaks were found to be single components, and determined as m^6A-A and m^6A-A-G, respectively. The next three peaks were treated with alkaline phos-

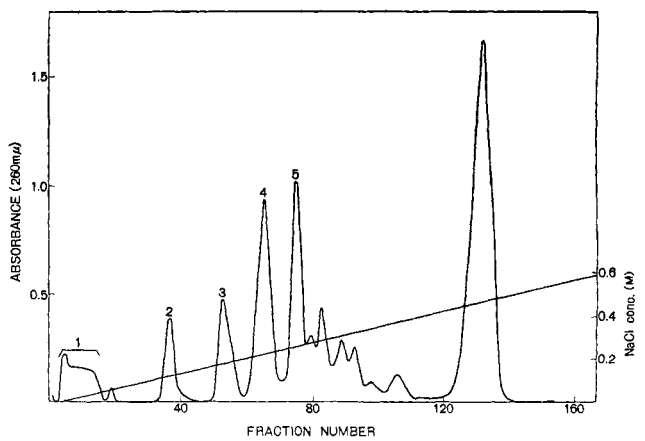

FIGURE 11: Chromatography of a silkworm nuclease digest of undecanucleotide (60 optical density units) on a column (0.3 × 50 cm) of DEAE-Sephadex A-25. Elution was carried out with an NaCl gradient from 0 to 0.7 M (two 200-ml portions) in 0.02 M Tris-HCl (pH 7.5) and 7 M urea. The flow rate was 10 ml/hr and the fraction size was 2 ml.

TABLE V: Nucleotide Composition of m^6A Containing Oligonucleotide Obtained by Degradation of Undecanucleotide with Silkworm Nuclease.[a]

Peak	Composition	Analysis[b]
1	m^6A-A	m^6Ap:A = 1.0:1.0
2	m^6A-A-G	m^6Ap:Ap:G = 1.0:1.0:1.0
3	m^6A-A-G-G	m^6Ap:Ap:Gp:G = 1.0:1.1:1.2:1.0
4	m^6A-A-G-G-A	m^6Ap:Ap:Gp:A = 1.0:1.1:1.9:0.9
5	m^6A-A-G-G-A-G	m^6Ap:Ap:Gp:G = 1.0:2.6:2.1:0.9

[a] Each peak was desalted, dephosphorylated, and chromatographed with solvent B. Each spot was fractionated further by paper chromatography with solvent A. [b] Each spot was digested with RNase T_2, and subjected to two-dimensional thin-layer chromatography.

phomonoesterase. The products of each were purified by paper chromatography with solvent B and solvent A, successively. The spots were digested with RNase T_2, and their base composition and 3′-terminal nucleoside were determined by two-dimensional thin-layer chromatography. The third peak was identified as m^6A-A-G-G, and the fourth and fifth peaks as m^6A-A-G-G-A and m^6A-A-G-G-A-G, respectively. Details of the exact base compositions of each oligonucleotide are shown in Table V.

Discussion

The sequences of products obtained by complete digestion with RNase T_1 or pancreatic RNase were determined. The sequences of long fragments in the RNase T_1 digest were effectively determined by treatment with purine-specific RNase U_2 and then digestion with *B. subtilis* RNase. Perfectly controlled digestion with snake venom phosphodiesterase was applied in identification of T-ψ-C-G and C-A-C-C-U-C-C-C-U-V-A-C-m^6A-A-G. Digestion stopped completely at the position of pseudouridine and V, resulting in the formation of T-ψ and C-A-C-C-U-C-C-C-U-V, respectively. Similar results were obtained with A-C-U-Q-U-A-N-A-ψ-C-U-G of *E. coli* $tRNA^{Tyr}$ (F. Harada, F. Kimura, and S. Nishimura, 1968, unpublished data) and C-U-N*-A-G of *E. coli* $tRNA^{Ser}$ (H. Ishikura, Y. Yamada, and S. Nishimura, unpublished data). In these cases, A-C-U-Q-U-A-N-A-ψ and C-U-N*, respectively, were obtained. Thus this method is very useful for obtaining shorter oligonucleotides terminated by a minor nucleoside at the 3′-hydroxy end for sequence determination. Periodate oxidation is also useful for determination of the sequences of oligonucleotides (Uziel and Gassen, 1969). We used this method with the tridecanucleotide. On partial degradation of the undecanucleotide with silkworm endonuclease, we obtained di- to hexanucleotides containing 5′-terminal nucleoside. Thus this enzyme is very useful in determination of the sequences of oligonucleotides which are not attacked by snake venom phosphodiesterase. The use of this enzyme in determining the total sequence of a decanucleotide containing 5-methylaminomethyl-2-thiouridine has also been reported recently from our laboratory (Ohashi *et al.*, 1970).

TABLE VI: Overlapping Sequences Obtained from RNase T_1 and Pancreatic RNase Fragments.

Y-G-G-T-ψ-C-Gp
Y-G-A-U-s^4U-A-G-Cp
Y-G-A-U-C-C-C-Gp
G-C-U-C-A-G-Cp
G-C-A-C-C-U-C-C-C-U-V-A-C-m^6A-A-G-G-A-G-G-G-G-m^7G-U-C-Gp

The results of the two analyses are in excellent agreement and overlapping sequences obtained by the two methods are shown in Table VI. *E. coli* $tRNA_1^{Val}$ contains 1 mole each of 4-thiouridine, dihydrouridine, 6-methyladenosine, 7-methylguanosine, pseudouridine, ribothymidine, and the new minor component V. This is the first report of the detection of 6-methyladenosine in a purified tRNA. The structural determination of this compound was described previously (Saneyoshi *et al.*, 1969). The structure of the new minor component V was determined as uridin-5-oxyacetic acid, as reported previously (Murao *et al.*, 1970).

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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*

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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

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