**Nucleotide Sequence Determination of Bacteriophage T4 Leucine Transfer Ribonucleic Acid***

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**SUMMARY**

The nucleotide sequence of T4 tRNA{sub}Leu, one of several transfer RNAs specifically coded for by bacteriophage T4, has been determined using {sup}32P-labeled material from T4-infected cultures of *Escherichia coli*. The purified RNA species which has been sequenced has been shown to have leucine acceptor activity, and to hybridize well to T4 DNA. The sequence is: PGCGAGAAUGGUCAADDGmGUAGGCACAGCACUNAAA * A q GCUGCGGAAUGAUUCC - UUGUGGG'IYKGAGUCCCACUUCUCGCACCA-OH. The 87 nucleotide length is the same as that of the two E. coli leucine tRNAs, the sequences of which have been reported. The molecule can be arranged in the classic cloverleaf pattern. The sequence further shows that the anticodon of the T4 tRNA{sub}Leu is -N-A-A-, in which N is a modified form of U. Thus, the molecule might be expected to recognize the leucine codons UUA or UUG, or both together, but trinucleotide binding studies by Scherberg and Weiss (1972) Proc. Nat. Acad. Sci. U. S. A. 69, 114) indicate that it recognizes only UUA.

Infection of *Escherichia coli* with bacteriophage T4 results in two kinds of change in the tRNA complement of the infected cell. At least one of the normal host species, a tRNA{sub}Leu which recognizes the codon CUG, is degraded by endonucleolytic cleavage after T4 infection (3-5). In addition to this inactivation of a host tRNA, T4 itself produces some 7 or 8 new tRNA{sub}s, including a tRNA{sub}Leu which are transcribed directly from its own genome (6-12).}

Polyacrylamide gel electrophoresis of {sup}32P-labeled tRNA{sub} extracted from T4-infected cells results in the characteristic band pattern seen in Fig. 4 (2, 13). Bands 3, 4, and 0 of this pattern have each been found to be a single pure species of tRNA and therefore can be easily isolated and purified. Band 3 is T4 tRNA{sub}Leu, and has been sequenced by McClain and Barrell. Band 6, the T4 tRNA{sub}Gln, has been sequenced by Stahl et al. We report here the details of the sequence determination of band 4, the T4 tRNA{sub}Leu.

**MATERIALS AND METHODS**

**Enzymes**

Spleen phosphodiesterase, pancreatic ribonuclease, snake venom phosphodiesterase, and bacterial alkaline phosphatase were obtained from Worthington Biochemical Corporation. U1 ribonuclease (14, 15), an enzyme with the same properties as T1 ribonuclease, was donated by C. Dekker. T1 and T2 ribonucleases were obtained from Calbiochem. U2 ribonuclease was a gift from the Sankyo Chemical Co. The silkworm nuclease (16, 17) was donated by J. Mukai. The modified e-carboxymethyllysine-41-pancreatic RNase (18-20) was donated by R. Himinkrion and J. Goldstein, and came to us via W. Salser. U4 ribonuclease was donated by A. Blank (21). E. coli aminocoyl tRNA synthetases were prepared from E. coli MRE 600 according to the procedures of Muench and Berg (22).

**Bacterial and Bacteriophage Strains**

*E. coli* strains CA274 (su-) and CA275 (suf{sub}II) were obtained from S. Brenner. *E. coli* strain W3110 was obtained from M. Green. Wild type bacteriophage T4D was obtained from S. Brenner. Bacteriophage T4 am tA3 was isolated on E. coli strain 2130. The amber mutation in gene t restricts lysis of (su-) hosts, more so at 30° than at 37°. Bacteriophage T4 psul{sup} was obtained from W. McClain. This mutant of T4 produces tRNA{sub}Leu but not the T4 tRNA{sub}Gln (see Fig. 6).

**Low Phosphate Growth Media**

The low phosphate medium (Medium A) used in the preparation of {sup}32P-labeled bacterial tRNA was as described in Landy et al. (24).

A special low phosphate medium (Medium B) was used for the preparation of {sup}32P-labeled T4 tRNA. The stock solution was 0.1 M Tris, pH 7.5, containing 1.5 g of KCl, 9.0 g of NaCl, and 1.0 g of NH{sub}4Cl per liter. For use, the desired volume was brought to 0.4% glucose, 0.001 M MgSO{sub}4, 0.1 mM sodium phosphate.

1 W. McClain and B. Barrell, manuscript in preparation.
2 S. Stahl, G. Paddock and J. Abelson, manuscript in preparation.
3 W. McClain and B. Barrell, unpublished results.
phate, then 0.01 volume of phosphate-free (see below) peptone was added.

Phosphate-free peptone was prepared by dissolving 20 g of Difco Bacto-peptone in approximately 100 ml of distilled water, and bringing to pH 10 with concentrated NH₄OH. After addition of 1.0 ml of 1 M MgCl₂, the precipitate containing magnesium-ammonium-phosphate was removed by centrifugation and the supernatant was brought to neutral pH with concentrated HCl and autoclaved. Final volume varied from 120 to 170 ml.

Radioisotopes

Carrier-free H₃²P₀₄ was obtained from New England Nuclear Corporation. [¹⁴C]Leucine uniformly labeled at 312 mCi per mmole was obtained from Amersham/Searle Corporation. [¹³C]Leucine uniformly labeled at 312 mCi per mmole was obtained from Amersham/Searle Corporation. [W]Leucine uniformly labeled at 312 mCi per mmole was obtained from Amersham/Searle Corporation.

Materials for Column Chromatography

DEAE-cellulose Whatman DE 32 was prepared as directed by the manufacturer. BD-cellulose was prepared as in Gillam et al. (25). Material for RPC2 (26) was a gift of G. D. Novelli and B. Vold.

Buffers

Standard DEAE-buffer for use with DEAE-cellulose columns was 0.01 M Tris pH 7.4, 0.002 M mercaptoethanol. NaN₃ was added as specified.

Standard BD-buffer for use with BD-cellulose columns was 0.01 M sodium acetate pH 5.0, 0.01 M MgCl₂, 0.002 M mercaptoethanol. NaN₃ and ethanol were added as specified.

Standard RPC buffer for use with RPC2 columns was 0.01 M sodium acetate, pH 5.0, 0.01 M MgCl₂, 0.001 M EDTA, 0.002 M mercaptoethanol. NaN₃ was added as specified.

Gel Materials

Acrlylamide was obtained from Matheson. It was purified by twice repeated recrystallization from ethyl acetate, washed with hexane, and vacuum dried. N,N'-Methylene bisacrylamide and N,N',N',N'-tetramethylethylenediamine were obtained from Eastman Kodak.

Sequencing Materials

Standard materials used in nucleotide sequencing have been described (27-29). In addition, cellulose acetate strips (2.5 x 17 inches) was used for making autoradiographs of ³²P-labeled materials fractionated on paper, thin layer DEAE-cellulose plates, and polyacrylamide gels.

Preparation of Nucleic Acids

Bacteriophage T₄ DNA—T₄D DNA was phenol-extracted as needed, from 5 ml of phage at 2 X 10⁹ per ml according to procedures described by Thomas and Abelson (30).

³²P-Labeled T₄ tRNA—A 400-ml culture of CA274 or W3110 was grown at 37° in Medium B to an A₆₅₀ of 0.3. Fifty micrograms per ml of L-tryptophan were added, and the culture was infected with T₄ am tX₃ at a nominal multiplicity of 10 phage per bacterium. At 2 min after infection, 100 nCi of H₃²P₀₄ were added. At 8 min after infection, 50 µg per ml of chloramphenicol were added. Growth was continued, now at 30°, for 90 to 120 min.

The cells were harvested by centrifugation and the bacterial pellets were resuspended in a total volume of 5.0 ml of 0.01 M Tris, pH 7.4, 0.1 M NaCl, 0.002 M mercaptoethanol. Normally, 80-90% of the radioactivity added to the infected culture was recovered.

The resuspended cells were extracted twice with an equal volume of phenol, and the aqueous layer transferred to a siliconized screw-capped tube. It was brought to 0.2 M NaCl and the nucleic acids precipitated with twice the volume of absolute ethanol. The ethanol precipitate was chilled in a freezer for at least 30 min.

The ethanol precipitate was pelleted in a clinical centrifuge, and resuspended in 1.0 ml of standard DEAE-buffer without salt. The material was loaded onto a small DEAE-cellulose column in a Pasteur pipette and washed with 20 ml of 0.3 M NaCl DEAE-buffer to remove very low molecular weight material. The tRNA was then eluted with 1.0 M NaCl DEAE buffer in small (20 to 25 drop) fractions. The bulk of the ³²P-labeled tRNA eluted in one or two small fractions, and the two or three most radioactive fractions were pooled.

For final processing of ³²P-labeled RNA for sequencing, see below.

³²P-Labeled E. coli tRNA—³²P-labeled bacterial tRNA was prepared by growing E. coli CA274 in a Medium A in the presence of 0.1 mCi per ml of ³²P and allowing growth to continue for several generations. The cells were harvested by centrifugation and resuspended in 3.0 ml of 0.01 M Tris buffer, pH 7.4. The tRNA was phenol-extracted, purified on DEAE-cellulose, stripped, and fractionated on BD-cellulose as described above for T₄ ³²P-labeled tRNA.

Carrier E. coli CA244 tRNA—Freeze-dried E. coli CA244 tRNA was obtained from B. F. C. Clark. It was further purified in 200-µg lots by phenol extraction and batchwise DEAE-cellulose chromatography followed by standard ethanol precipitation.

Reversed Phase Column Chromatography

To the "BD hydrophobic fraction" of ³²P labeled T₄ tRNA, 2.4 mg of carrier tRNA were added. The sample was ethanol
precipitated, pelleted, and resuspended in 3.0 ml of 0.4 M NaCl RPC buffer. It was then fractionated by elution from an RPC2 column (26) using a 1000-ml salt gradient, from 0.4 to 1.0 M NaCl RPC buffer. The column (1 × 100 cm) was maintained at 30°. Fractions of approximately 8 ml were collected, at a flow rate of 10 min per fraction. Pooled fractions of radioactive material, diluted to less than 0.3 M NaCl, were reconstituted by siphoning onto a small DEAE-cellulose column and eluting in small volumes of 1.0 M NaCl, 0.01 M sodium acetate pH 5, 0.002 M mercaptoethanol.

**Polyacrylamide Gel Electrophoresis**

Ten per cent polyacrylamide slab gels (20 × 40 cm) were prepared, and samples of [32P]tRNA prepared and loaded according to the procedures of De Wachter and Fiers (31). Electrophoresis was at 400 volts for 12 to 16 hours. Electrophoresis buffer was 0.1 M Tris-acetate pH 8.5. An autoradiograph of the resulting gel was used to make a template to cut out radioactive bands. Material from gel bands was eluted by homogenizing with 0.3 M NaCl, using a total volume of 3.0 ml. Gel material was removed by centrifugation, and the supernatant filtered through a 0.45-μm Millipore filter.

**Aminoacylation of tRNA**

Aminoacylating activity of the E. coli tRNA synthetases, and conditions for optimum acylation were established using E. coli CA244 tRNA. Reaction mixes contained 0.1 M sodium cacodylate, pH 5.0, the derivatized material was resuspended in 0.3 M NaCl, 0.01 M of 14C]leucine at 312 mCi per mmole. Incorporation of [14C]leucine was assayed by collecting the trichloroacetic acid precipitate of the reaction mixture on nitrocellulose filters, and counting in a scintillation counter.

**Phenoxacyctyl Derivatization of Leucyl-tRNA**

The phenoxacyctyl ester of N-hydroxysuccinimide was prepared as described by Gillam et al. (32).

Acylated tRNA was recovered from the above reaction mixes by phenol extraction and ethanol precipitation. The final pellet was reconstituted in 0.5 ml of distilled H2O and reacted with the phenoxacyctyl ester as described in Gillam et al. (32). After two ethanol precipitations in the presence of 0.2 M sodium acetate, pH 5.0, the derivatized material was resuspended in 0.3 M NaCl BD-buffer and chromatographed on BD-cellulose. Elution was with BD-buffers containing 0.3 M NaCl; 1.0 M NaCl; 1.0 M NaCl, 10% ethanol; 1.5 M NaCl, 15% ethanol.

**Hybridization of RNA with DNA**

RNA-DNA hybridization was carried out on nitrocellulose filters as described by Gillespie and Spiegelman (33).

Acylated and derivatized [32P]tRNA recovered from BD-cellulose chromatography in 1.5 M NaCl. Ethanol BD-buffer (15%) was ethanol precipitated, stripped of amino acids, and finally resuspended in distilled H2O for use in hybridization experiments.

**Final Processing of [32P]tRNA for Sequencing**

T4 [32P]tRNALeu purified by the various procedures described above—RPC2 chromatography, polyacrylamide gel electrophoresis, BD-cellulose chromatography of acylated and derivatized tRNA—was prepared for sequencing by several cycles of ethanol precipitation from 0.2 M sodium acetate, pH 5, to reduce residual NaCl. Where necessary, carrier tRNA was added to at least 20 μg per ml to ensure precipitation. The final pellet was gently air-dried and finally resuspended in 50 to 100 μl of distilled H2O.

**Sequencing**

Procedures used in sequencing the T4 tRNALeu were as described in Sanger et al. (27), Brownlee and Sanger (28), and Barrell (29), with some modifications and additions.

In some experiments, first dimensional electrophoresis of total and partial enzymatic digests of T4 tRNA was run on 5%-cm strips of cellulose acetate obtained from Schleicher and Schuell, instead of on 8.5-cm strips cut from rolls obtained from Oxo, Ltd.

**Enzymic Digestions**—T1 ribonuclease which has the same specificity as T1 ribonuclease was used instead of the latter for fingerprints and partial digestions.

Digestion of oligonucleotides with silkworm nuclease were carried out in 10 μl of the enzyme at 125 μg per ml in 0.5 M Na2CO3-NaHCO3, pH 10.5, 0.1 M NaCl, 0.005 M magnesium acetate. Incubation was at 37° for 60 min. Combined silkworm nuclease-bacterial alkaline phosphatase digestions were performed by including 5 to 10 μg of alkaline phosphatase in the digestion mix. Digestion products in either case were separated by electrophoresis at pH 3.5 on DEAE-paper.

Partial enzymatic digestions of intact tRNA were carried out using U1, pancreatic, and modified e-carboxymethyllysine-41-pancreatic ribonucleases. The modified pancreatic enzyme cleaves most frequently at -GpA or -UmP sequences (20).

Partial U1 ribonuclease digestions were carried out in 0.01 M Tris, pH 7.4, 0.02 M MgCl₂, 0.1 mg per ml of bovine serum albumin, at 4° for 20 min. Enzyme to substrate ratios of 1:1000, 1:100, and 1:200 were used.

Partial pancreatic ribonuclease digestions were carried out in 0.01 M Tris, pH 7.4, 0.02 M MgCl₂, 0.1 mg per ml of bovine serum albumin at 4°, usually for 30 min. Enzyme to substrate ratio of 1:500 was used.

Partial digestions with the e-carboxymethyllysine-41-pancreatic ribonuclease (20) were carried out in 0.01 M sodium acetate, pH 4, 0.001 EDTA. Enzyme to substrate ratios of 1:100, 1:50, and 1:20 were used. Incubations were for 30 min at room temperature.

All partial digestions were in 2- or 3-μl volumes. Spleen phosphodiesterase digestions were carried out in 10 μl of enzyme, 1 mg per ml, in 0.1 M ammonium acetate, pH 5.7, 0.002 M EDTA, 0.05% Tween 80 at 37° for 16 hours.

**Identification of Modified Nucleotides**—Modified nucleotides frequently found in tRNAs were identified essentially as in Sanger et al. (27). T, Ψ, D, and GmG were identifiable by their mobilities at pH 3.5 on both Whatman 32 and DEAE-papers, and T, Ψ, and GmG were further identified by paper chromatography in isopropanol-NH₃ (27). Nucleotide A, 2-methylthio-N₄(γ,γ-dimethylallyl) adenosine, was identified by its electro-
phoretic mobility at pH 3.5 on Whatman No. 3MM paper, and at pH 3.5 on DEAE-paper (34, 35). All nucleotides were further characterized by running on cellulose thin layer plates developed with isobutyric acid-NH₃ or 2-propanol-HCI (46). Some nucleotides were also run in a 1-butanol-formic acid system (36). In addition, GmG was digested with spleen phosphodiesterase and the products obtained were identified as G and Gm on a cellulose thin layer developed with 2-propanol-HCl.

RESULTS

Purification of T4 tRNA\(^{Leu}\)

To purify the T4 tRNA\(^{Leu}\) we at first employed the procedures developed by Gillam et al. (32). \(^{32}\)P-labeled T4 tRNA was chromatographed on BD-cellulose, and the 1.0 M NaCl, 10% ethanol "hydrophobic fraction" was acylated with leucine and phenoxyacetyl derivatized as described under “Materials and Methods.” A deacetylated sample was also prepared. The two samples were then rechromatographed on BD-cellulose with elution buffers as shown. The shaded peak in the lower leucine-charged sample, eluting in 1.5 M NaCl, 15% ethanol BD-buffer is T4 tRNA\(^{Leu}\). The peak still eluting at 1.0 M NaCl, 10% ethanol is T4 tRNA\(^{Ser}\) plus some unacylated T4 tRNA\(^{Leu}\). The peak eluting at 1.0 M NaCl is assumed to be nonhydrophobic material incompletely eluted in the original BD-cellulose fractionation, or hydrophobic material altered by experimental manipulation.

To characterize the \(^{32}\)P-labeled T4 tRNA\(^{Leu}\) eluted from the BD-cellulose column with 1.5 M NaCl, 15% ethanol, we performed RNA-DNA hybridization experiments and fingerprint analysis. Fig. 2 shows that 70% of the T4 \(^{32}\)P-labeled tRNA\(^{Leu}\) can hybridize to T4 DNA whereas E. coli \(^{32}\)P-labeled tRNA\(^{Leu}\) purified by the same procedure did not hybridize. In experiments not shown here, we found that bulk E. coli tRNA does not compete in the hybridization of T4 \(^{32}\)P-labeled tRNA\(^{Leu}\) with T4 DNA.

A U1 fingerprint of the T4 tRNA\(^{Leu}\), prepared as described above, was similar to the one presented in Fig. 8A, but these preparations often contained contaminating oligonucleotides which were later identified as originating from T4 tRNA\(^{Ser}\). Yields in this acylation and derivatization procedure were low.

To obtain pure preparations of T4 tRNA\(^{Leu}\), we resorted to chromatography on the reversed phase system RPC2 (36). T4 \(^{32}\)P-labeled tRNA was chromatographed on BD-cellulose and the "hydrophobic" fraction was further fractionated by RPC2 chromatography as shown in Fig. 3. It can be seen that three peaks, Peaks I, II, and III, are obtained, all of which elute from the column after most of the E. coli tRNA. Acylation with leucine and derivatization of Peak I resulted in a much larger percentage (about 60%) of the material, now eluting from BD-cellulose in 1.5 M NaCl, 15% ethanol. Peak I is thus a less contaminated form of T4 tRNA\(^{Leu}\) than the original "hydrophobic" fraction used in Fig. 1. Some T4 tRNA\(^{Leu}\) is also found in Peak II. The remainder of Peak II is T4 tRNA\(^{Ser}\), as is Peak III (see below).
be seen for T4 pSul- in Fig. 6.

... and Peak III migrates as Band 3. Band 3 RNA has been sequenced by Mainland and Barrell and is T4 tRNA$_{\text{ser}}$.

... are convenient to use in the preparation of T4 tRNA$_{\text{leu}}$ because the "hydrophobic fraction" of their tRNA contains only T4 nucleotides, and gives only band 4 on polyacrylamide gels as can be seen for T4 pSul$^-$ in Fig. 6.

Sequence Analysis of T$_4$ tRNA$_{\text{leu}}$

Pancreatic Ribonuclease Digestion Products—T$_4$ [p$^32$P]tRNA$_{\text{leu}}$ was digested to completion with pancreatic ribonuclease, and the products were separated using the standard two-dimensional system developed by Sanger et al. (27). Fig. 7A shows the resulting fingerprint, while Fig. 7B identifies the oligonucleotides corresponding to each spot.

Analysis of the pancreatic digestion products and their sequence is shown in Table I. All but five nucleotides, p10, p12, p16, p17 and p18, were sequenced by analysis of 3'-dephosphorylated versions of the four nucleotides. Material eluted from an ordinary pancreatic fingerprint was incubated with bacterial alkaline phosphatase to remove the 3'-phosphates. The dephosphorylated oligonucleotides were purified by electrophoresis on DEAE-paper at pH 3.5. Separate aliquots of each were subjected to alkaline digestion which produces 3'-nucleotides, and to digestion with snake venom phosphodiesterase which produces 5'-nucleotides. The results in Table I establish the sequences of the four nucleotides as shown.

Nucleotide p12 contains the modified base A*, 2-methylthio-N$^4$-$\gamma$,-$\gamma$-dimethylallyl) adenosine (39-41). As indicated in the legend to Fig. 7, nucleotide p12, AAA*A*W, occurs in several forms with distinctly different mobilities. This effect has been observed before by Gefter and Russell (34), and is due to heterogeneity in the modification of A*.

This result is reminiscent of those obtained by Waters and Novelli (9) who demonstrated two new peaks of tRNA$_{\text{leu}}$ in RPC2 chromatography following T$_4$ infection. These peaks eluted at higher salt concentrations than any of the E. coli tRNA$_{\text{leu}}$ species.

The sequence analysis to be presented has not given evidence for two distinct sequences, therefore we assume that the two peaks of tRNA$_{\text{leu}}$ are caused by heterogeneity in the modification of the tRNA. Such heterogeneity has been observed in E. coli tRNA$_{\text{tyr}}$ produced in $\phi$SodSuIII-infected cells (34).

Some of the sequence analysis was performed with material obtained from RPC2 Peak I, but during the course of the work it was found that a much simpler purification could be obtained by electrophoresis of T$_4$ [p$^32$P]tRNA on 10% polyacrylamide gels.

... when unfractionated T$_4$ [p$^32$P]tRNA is subjected to electrophoresis on a 10% polyacrylamide gel. The autoradiograph of the gel reveals seven sharp bands. Six of these bands (0, 1, 2, 3, 4, 6) are pure species of RNA. Band 5, which has the same mobility as bulk E. coli tRNA, is a mixture of four species of tRNA. All of these RNA species hybridize specifically to T$_4$ DNA (37).

Fig. 5 shows an acrylamide gel electrophoresis of T$_4$ [p$^32$P]tRNA and of various fractions obtained from BD-cellulose chromatography. The 1.0 m NaCl fraction is shown in Sample b. The hydrophobic fraction, Sample c, consists only of Bands 3 and 4. The T$_4$ tRNA$_{\text{leu}}$ obtained in 1.5 m NaCl, 15% ethanol in Fig. 1, is shown in Sample d. It is mostly Band 4 with some contaminating Band 3.

RPC2 Peak I (Fig. 3) migrates as Band 4 in polyacrylamide gel electrophoresis. Peak II gives a mixture of Bands 3 and 4, and Peak III migrates as Band 3. Band 3 RNA has been sequenced by McClain and Barrell and in T$_4$ tRNA$_{\text{ser}}$.

 Mutants of T$_4$ can be obtained in which the T$_4$ tRNA$_{\text{ser}}$ is not made, such as T$_4$ pSul$^-$ and T$_4$ pSul$^-\chi$ (38). Such mutants are convenient to use in the preparation of T$_4$ tRNA$_{\text{leu}}$ because the "hydrophobic fraction" of their tRNA contains only T$_4$ tRNA$_{\text{leu}}$, and gives only band 4 on polyacrylamide gels as can be seen for T$_4$ pSul$^-$ in Fig. 6.

FIG. 3. Reversed phase column chromatography of T$_4$ [p$^32$P]tRNA. Carrier tRNA (2.4 mg) was added to the 1.0 m NaCl, 10% ethanol "hydrophobic fraction" obtained from BD-cellulose chromatography of T$_4$ [p$^32$P]tRNA. The sample was chromatographed by gradient elution from an RPC2 column as described under "Materials and Methods." Fractions of about 8 ml were collected at a flow rate of 10 min per fraction. Percentage of transmission at 260 nm was continuously monitored. Radioactivity of even-numbered fractions from 12 through 88 was assayed by counting 10-$\mu$l aliquots in a Nuclear Chicago scintillation counter. ——, percentage of transmission; ——, counts per min X $10^4$ of p$^32$P in 10-$\mu$l aliquots of each fraction (e.g. Fraction 54 in Peak I contained about $2\times10^4$ cpm per ml); ——, NaCl gradient.

FIG. 4. Polycrylamide gel electrophoresis of T$_4$ [p$^32$P]tRNA. Unfractionated T$_4$ [p$^32$P]tRNA purified only through the DEAE-cellulose step was electrophoresed on a 10% polyacrylamide gel as described under "Materials and Methods." Distance migrated is shown in centimeters on the left. Bands of radioactivity are numbered 0 to 6. BPB indicates the position of migration of the bromphenol blue dye marker.
the low yield of A*p in the alkaline digestion products of p12 shown in Table I, and for the low yield of p12 itself seen in Table II. The sequence shown in Table I for p12, AAA*A¥, depends on later results (Table V-D) obtained from the silkworm nuclease digestion of the U1 oligonucleotide u15C which contains p12.

Nucleotide (p5) is subsumed under p4 in Table I and together they are shown as having the variable sequence AAAA. Both spots usually appear in fingerprints of oligonucleotides produced by pancreatic RNAase digestion as seen in Fig. 7, A and B, but their combined molar yield is only 1.1 (see Table II). There is only one AAA“U” sequence in the entire tRNA, as shown by pancreatic digestion of U1 nucleotides, and the U1 nucleotide u14 (see Table III) in which it occurs cannot possibly contain 6 Ap residues. We conclude that our preparations of T4 tRNA leu contain some unmodified U in this position.

Experimentally determined molar yields of the pancreatic nucleotides of Fig. 7 and Table I are shown in Table II. As discussed above, the most discrepant result is that shown for p12 for which the experimental yield is very low.

U1 Ribonuclease Digestion Products—A standard two-dimensional fingerprint of the U1 ribonuclease digestion products of T4 tRNA leu is shown in Fig. 8, A and B. Nucleotide u5, the 3'-end of the molecule, CACCA—OH, does not show on the fingerprint, but can be seen in Fig. 8E. Fig. 8, C and D, shows a U1 fingerprint in which the second dimension has been developed by homochromatography on a thin layer DEAE-cellulose plate.

The quantitative data from alkaline and pancreatic digestion alone were sufficient to establish the results shown in the fourth
Fig. 7. Standard two-dimensional fingerprint of pancreatic ribonuclease digest of T4 [azaP]tRNA\textsuperscript{44}. Material used was from single band in "hydrophobic fraction" of Fig. 6. A, autoradiograph of fingerprint on DEAE-paper. B, diagram identifying pancreatic nucleotide products. Broken line circle below pl0 is a derivative of pl8, AAA\textsuperscript{4}A\textsuperscript{4}. Broken line circle near p80 is also C, probably C cyclic. Products pl, "U\textsuperscript{5}" include D and \Psi, as well as U.

### Table I

**Analysis of pancreatic ribonuclease digestion products***

Pancreatic RNase digestion products from fingerprints as shown in Fig. 7 were analyzed by standard alkaline and enzymatic digests. The heading "Nucleotide" of the first column refers to any pancreatic digestion product, either mononucleotide or oligonucleotide.

| Nucleotide | Alkaline Digestion Products | U1 Ribonuclease Digestion Products | Products of Alkaline & Snake Venom Digestion of 3'-OH Nucleotides | Deduced Sequence |
|------------|-----------------------------|-----------------------------------|---------------------------------------------------------------|-----------------|
| p1         | Up\textsuperscript{5}        | A-Cp                              | AIK: 3 Ap + 2 Gp                                              | Up\textsuperscript{5} |
| p2         | 0.9 Gp + 1.0 Cp              | 1 Gp + 1 Cp                       | SV: 2 pA + 2 pG + 1 pC                                       | A-Cp            |
| p3         | 0.8 Ap + 0.8 Gp + 1.0 Cp     | 1.3 A - Gp + 1.0 Cp               | A - A - A - Cp                                              | G-Cp            |
| p4         | 3.1 Ap + 1.0 Cp              | A - A - A - Gp                    | A - A - A - A - \Psi p                                       | A - C - G - Cp  |
| p5         | 3.1 Ap + 1.0 Up              | 1 pGp + 1 Cp                     | AIK: 3 Ap + 2 Gp                                              | G-\Up            |
| p6         | 1.3 Gp + 1.0 Up              | 1.7 A - Up + 1.0 Gp               | SV: 2 pA + 2 pG + 1 pC                                       | A - A - A - G - C - p |
| p7         | 0.5 Gp + 1.0 Gp              | 1.5 A - Gp + 1.1 Gp + 1.0 Up      | AIK: 1 Ap + 2 Gp                                              | G - G - \Up      |
| p8         | 1.9 Gp + 1.0 Up              | 1.0 A - A - Up + 1.7 Gp           | AIK: 2 Ap + 2 Gp                                              | G - G - G - \Up  |
| p9         | 2.8 Ap + 1.7 Gp + 1.0 Cp     | 1.0 A - A - Up + 0.9 A - Gp + 1.1 Gp | AIK: 3 Ap + 2 Gp                                              | G - G - G - A - A - Up |
| p10        | 3.2 Ap + 0.6 A*p + 1.0 \Psi p |                               |                                                               |                 |
| p11        | 0.5 Gm - Gp + 1.0 Gp         |                               |                                                               |                 |
| p12        | 0.9 Ap + 1.0 Gp + 1.0 Up     |                               |                                                               |                 |
| p13        | 1.9 Gp + 1.0 Up              |                               |                                                               |                 |
| p14        | 1.0 Gp + 1.0 Up              |                               |                                                               |                 |
| p15        | 1.0 Gp + 1.0 Gp              |                               |                                                               |                 |
| p16        | 1.9 Gp + 1.0 Gp + 1.0 Up     |                               |                                                               |                 |
| p17        | 1.9 Gp + 1.0 Gp + 1.0 Up     |                               |                                                               |                 |
| p18        | 1.0 Gp + 1.0 Gp + 1.0 Up     |                               |                                                               |                 |
| p19        | 1 Ap + 1.0 Cp                |                               |                                                               |                 |
| p20        | 1 Ap + 1.0 Cp                |                               |                                                               |                 |

* Dashes between bases indicate phosphodiester bonds. Relative yields were determined by measuring the radioactivity of the paper containing the nucleotide products in a Nuclear Chicago scintillation counter; or, where shown as integers, by visual inspection of intensities of spots on autoradiographs.

* Products were identified by their electrophoretic mobilities at pH 3.5 on Whatman no. 52 paper. Identifications of modified bases Tp, \Psi p, Dp, and Gmp Gp were verified by isopropanol-ammonia paper chromatography.

* All products other than mononucleotides were analyzed by alkaline digestion. Nucleotides p19, p4, and p12 were unaffected by U1 ribonuclease digestion as expected, and had electrophoretic mobilities expected for products containing 1, 3, and 4 Ap residues, respectively.

* 3'-Dephosphorylated nucleotides p10, p16, p17, and p18 were prepared as described in text. Alkaline and snake venom digestion products were identified by electrophoretic mobilities at pH 3.5 on Whatman No. 52 paper. AIK = alkaline digestion product; SV = snake venom digestion product.

* Nucleotide p1, more than one spot in most fingerprints, includes \Psi p and Dp.

* Nucleotides p4 and (p5) are shown here as one variably modified product. See also Table II and text.

* Base A*p, 2-methylthio-N\textsuperscript{4}(\gamma,\gamma-dimethylallyl) adenosine, was identified by its electrophoretic mobility at pH 3.5 on Whatman No. 3MM paper (R\textsubscript{amp} = 1.35) and by its electrophoretic mobility on DEAE-paper at pH 3.5 (R\textsubscript{amp} = 0.78). These values agree with those reported for an authentic sample of A*p by Gefter (35). For the sequence determination of p12 see text and Table V-D.
Table II
Pancreatic ribonuclease digestion products
Relative yields of products obtained in fingerprints such as those in Fig. 7.

| Nucleotide | Sequence | Exponentially Determined* (3 expts.) | Molar Yield | Expected from Final Structure |
|------------|----------|--------------------------------------|-------------|-----------------------------|
| p1         | Up       | 11.9                                 | 12          |
| p20        | Cp       | 8.2                                  | 10          |
| p19        | A-Cp     | 4.1                                  | 4           |
| p2         | G-Cp     | 3.6                                  | 3           |
| p3         | A-G-Cp   | 1.0                                  | 1           |
| p6[a]      | A-Cp     | 1.1                                  | 1           |
| p6         | G-Up     | 1.3                                  |             |
| p10        | p-Cp     | 0.9                                  | 1           |
| p9         | G-Cp     | 1.4                                  |             |
| p12        | A-5-A-U-U-Cp | 0.9                              | 1           |
| p13        | Gm-G-Dp  | 1.0                                  |             |
| p15        | G-T-G-Dp | 1.1                                  |             |
| p16        | G-C-G-Dp | 1.3                                  |             |
| p17        | G-G-A-Dp | 1.2                                  |             |
| p18        | G-A-G-A-A-Dp | 0.7                              | 1           |

*Yields were determined by measuring the radioactivity of the paper containing the nucleotide in a Beckman low-background gas-flow counter.

[a] Nucleotide p6 here includes both p6 and (p6) shown in Table I, as explained in the text.

The low yield of product p12 is caused by variable modification of Ap, 3'-phosphodiesterase sensitive and Ala, and is explained in the text; this results in variable mobility of the product so that not all of it is found in the actual position of p12 in the fingerprints.

Both alkaline and pancreatic digestion of this nucleotide should generate both free Up and free Up. In the experiments shown in Table III, however, the two were not resolved and the free U's of u14 are therefore shown as "U." As mentioned earlier, the D modification is present in variable yield.

The final deduced sequence of u13 is CACUUC. The alkaline and pancreatic digestion of this nucleotide should generate both free Up and free Up. In the experiments shown in Table III, however, the two were not resolved and the free U's of u14 are therefore shown as "U." As mentioned earlier, the D modification is present in variable yield.

The final deduced sequence of u17 is G-G-A-Dp. The alkaline and pancreatic digestion of this nucleotide should generate both free Up and free Up. In the experiments shown in Table III, however, the two were not resolved and the free U's of u14 are therefore shown as "U." As mentioned earlier, the D modification is present in variable yield.

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Fig. 8. Two-dimensional fractionations of U1 ribonuclease digests of T4 [32P]tRNA\(^{\text{uo}}\), and a T6 [32P]tRNA. A, standard fingerprint on DEAE-paper, of T4 material from an RPC2, Peak I as in Fig. 3; B, diagram identifying nucleotides in A; C, fingerprint developed in second dimension by homochromatography on thin layer DEAE-cellulose plate, T4 material as in Fig. 7; D, diagram identifying nucleotides in C (Note: The pH 3.5 arrow is misplaced, and should be in the same relative position as in C, to indicate origin of second dimension.); E, fingerprint of a T6 tRNA on DEAE-paper. The first dimension was on a short, 55-cm cellulose-acetate strip. P indicates position of pink dye marker (acid fuchsin) in first dimension; B's show position of blue dye (xylene cyanol) in first and second dimensions. F, standard fingerprint of combined U1-bacterial alkaline phosphatase digest of T4 material from RPC2 Peak I. The digestion products are 3'-OH rather than 3'-phosphate as they are in A, B, C, D, and E.
### Table III

Initial analysis of U1 ribonuclease digestion products

U1 RNase digestion products from fingerprints as shown in Fig. 8A or 8P were analyzed by standard alkaline and enzymatic digestions. The term “Nucleotide” of Column 1 refers to any U1 digestion product, either mononucleotide or oligonucleotide. Except as indicated in the heading to the fifth column, U1 nucleotides were from standard fingerprints as in Fig. 8A.

| Nucleotide Products | Pancreatic Ribonuclease Digestion Products | Deduced Composition of Sequence | Products of Alkaline and Snake Venom Digestion of 3’-OH U1 Nucleotides | Deduced Sequence or Partial Sequence |
|---------------------|-------------------------------------|---------------------------------|---------------------------------|-------------------------------------|
| u1                  | G                                   | G                              | G                              | G                                   |
| u2                  | 1.0C + 1.0G                         | CG                             | AIK: 2.0A + 2.3C                | C + U                               |
| u3                  | 1.0A + 1.0G                         | AG                             | SV: 2.1A + 1.2C + 1.0G          | U + G                               |
| u4                  | 2.1A + 2.0C + 1.0G                  | (AC, CI) AG                    | AIK: 1.0A + 2.9C                | AAAG                                |
| u5                  | 1.0A + 2.9C                         | (AC, nC)                       | SV: 2.0A + 2.1C                 | CACAC – OH                           |
| u6                  | U + G                               | UG                             |                                 | UG                                  |
| u7                  | pG                                 | pG                             |                                 | pG                                  |
| u8                  | 1.3C + 1.1U + 1.0G                  | (C, U) G                       | AIK: 2.7A + 1.0U                | AU                                 |
| u9                  | 2.3A + 1.0U + 1.0G                  | 1.0AAU + 1.0G                  | AIK: 1.0C + 1.4U + 1.0G         | AAAU                                |
| u10                 | 3.4A + 1.1D + 1.0G                  | 1.0D + 1.0AAAG                | AIK: 3.2A + 1.0G                | Tp, AG                              |
| u11                 | 1.0C + 1.1(T’ + Ψ) + 1.0G           | [(T’ + Ψ), G]                  | AIK: 1.0C + 1.8C + 4.7U         | AU (2C, 4U) G                       |
| u12                 | 1.1A + 1.1C + 5.0U + 1.0G           | [AU, 2C, 4U] G                 | SV: 2.3C + 5.3U + 0.6G          | AAAG                                |
| u13                 | 2.9A + 1.0C + 2.1U”” + 0.8GmG       | 0.88AAAG + 1.0C + 2.0 “U”” + 0.7GmG | AIK: 2.4A + 1.0C + 2.3 “U”” + 0.8GmG | U (AAA) A, 2 “U”” + 1.1GmG |
| u14                 | 1.2A + 5.0C + 4.1U + 1.0G           | 1.4AC + 5.0C + 4.4U + 1.0G     | AIK: 2.1A + 5.3C + 0.4U         | U (AC, 5C, 3U) G                    |
| u15                 | 3.7A + 2.3C + 2.5U”” + 0.9N” + 1.0G | 0.7AAA A, 1.1AC + 2.2C + 1.8 “U”” + 1.0G | AIK: 3.5A + 0.5A”” + 2.0C + 2.3 “U”” + 0.8N” | C (AAA” A, AC, U, N”) G |

* From considerations of space, all representations of phosphate are omitted except for the 5’-phosphate of nucleotide u7. All bases shown are 3’,phosphate, except as noted below. Relative yields were determined by measuring the radioactivity of the paper containing the nucleotide products in a Beckman low background gas-flow counter. Final analysis of the longest nucleotides required further experiments, which are shown in Tables IV and V.

+ All mononucleotides shown as alkaline digestion products are 3’-phosphate. In u12 the notation (T’ + Ψ) indicates one spot containing both Tp and Ψp. In u14, GmG is the dinucleotide GmpGp. In the snake venom digestion product of u12 shown in the fifth column following “SV”: the notation shown means 0.7 pF.

* All bases shown are 3’ phosphate. All products other than mononucleotides were analyzed by alkaline digestion.

d As explained in text, materials analyzed in this column were from U1-phosphatase fingerprints as in Fig. 8P, or had had their 3’-phosphates removed. U5 is the naturally occurring 3’-OH product from the 3’ end of the molecule. Alkaline digestion products shown following “AIK,” are 3’-phosphate. Snake venom digestion products shown following “SV” are 5’-phosphate.

f In alkaline product “0.8 GmG” is GmpG, and snake venom product “1.1 GmG” in unresolved GmG 5’-phosphates. In other experiments, GmG and G 5’-phosphates were resolved and shown to be in equimolar yields.

* The notation “U,” as explained in the text, is unresolved Up and Dp, or pU and pD. As also noted in the text, however, pancreatic digests with U and D were resolved on DEAE-cellulose, pH 3.5.

g The notation “U,” as explained in the text, is unresolved Up and Dp, or pD. As also noted in the text, however, pancreatic digests with U and D were resolved on DEAE-cellulose, pH 3.5.
The degradation product as discussed in Brownlee and Sanger and z = the distance from the digestion product to the next nucleoside in Table V-A. Alkaline digestion yields 3'-mononucleotides for all except the 3'-terminal nucleotide. Also, identical 5'-end sequences identified as those which, from either digestion, never yield a 5'-mononucleotide. Type A products from silkworm digestion alone yield 3'-monophosphate and 3'-OH.

The 5'-base of a type B product appears as a nucleoside diphosphate pXp after alkaline digestion. The pXp nucleotides have characteristic mobilities, and identification of a given pXp is verified by the appearance of the 5'-mononucleotide pX after snake venom digestion. In this case, type A products in both columns had identical mobilities. Pairs of type B products with the same sequence had different mobilities but are shown on the same line in both columns. A series of pairs of Y-end products with identical mobilities could be identified, and sequences of partial snake venom digestion products which involve pXp nucleotides.

Silkworm digestion products from u13 in column "SW" of Table V-B are shown in order, from the 5'-end to the 3'-end, rather than by mobility. Both columns had identical mobilities. Partial products are listed in order of decreasing mobility for each U1 nucleotide. Numbers followed by periods are arbitrary.

Alkaline and snake venom digestion products were not quantitated, but were determined by visual estimation from the autoradiograph film.

Table IV

| Alkaline digestion products\(^a\) from U1 nucleotides | Snake venom digestion products\(^b\) | Deduced sequence of partial snake venom digestion products\(^a\) | M\(^d\) |
|------------------------------------------------------|-----------------------------------|-------------------------------------------------------------|------|
| u13                                                  |                                   |                                                             |      |
| 1. 1.0 Ap + 1.1 Up                                   | n(pUp)                            | ApUpUpU                                                      | 1.9  |
| 2. 1.0 Ap + 2.0 Up                                   | n(pUp)                            | ApUpUpUpU                                                   | 1.0  |
| 3. 1.0 Ap + 3.0 Up                                   | 1.0 pC + 3.1 pU                    | ApUpUpUpUpU                                                  | 0.8  |
| 4. 0.8 Cp + 1.0 Ap + 3.1 Up                          | 1.5 pC + 3.0 pU                    | ApUpUpUpUpC                                                  | 1.8  |
| 5. 1.9 Cp + 1.0 Ap + 3.2 Up                          | 1.7 pC + 4.0 pU                    |                                                             |      |
| u14                                                  |                                   |                                                             |      |
| 1. 1.2 Cp + 1.0 Up                                   | 1.0 pC + 1.1 pA                    | UpCpA                                                        | 1.5  |
| 2. 0.9 Cp + 1.1 Ap + 1.0 Up                          | 1.0 pC + 1.9 pA                    | UpCpApA                                                      | 1.7  |
| 3. 1.2 Cp + 2.0 Ap + 1.0 Up                          | 1.0 pC + 3.5 pA                    | UpCpApApA                                                   | 1.3  |
| 4. 0.9 Cp + 2.6 Ap + 1.0 Up                          | 1.0 pC + 3.3 pA + 1.1 pU           |                                                             |      |
| u15A                                                 |                                   |                                                             |      |
| 1. 1 Up                                              | 1 pC                               | UpC                                                          |      |
| 2. 2.0 Cp + 1.0 Up                                   | 3 pC                               | UpCpC                                                        |      |
| 3. 3.1 Cp + 1.0 Up                                   | 2.5 pC + 1.0 pA                    | UpCpCpC                                                      |      |
| 4. 3.1 Cp + 1.0 Ap + 1.0 Up                          | 3.7 pC + 1.0 pA                    | UpCpCpCpCpC                                                  |      |
| 5. [3.0 Cp + 1.1 Ap + 1.0 Up\(^f\)]                  | 3.9 pC + 1.2 pA + 1.0 pU           | UpCpCpCpCpCpUpU                                              |      |
| 6. [4 Cp + 1 Ap + 2 Up\(^f\)]                       | [4 pC + 1 pA + 2 pU]               | UpCpCpCpCpCpCpUpCpUpC                                        |      |
| 7. [4 Cp + 1 Ap + 3 Up\(^f\)]                       | [5 pC + 1 pA + 2 pU]               |                                                             |      |

\(^a\) Products identified and yields determined as in Table I. Alkaline digestion yields 3'-mononucleotides for all except the 3'-terminal nucleoside of the partial product.

\(^b\) Products identified and yields determined as in Table I. Snake venom phosphodiesterase digestion yields 5'-mononucleotides for all except the 5'-terminal nucleoside of the partial products.

\(^c\) Partial products are 3'-OH.

\(^d\) M = x/y where y = distance from origin to the digestion product and x = the distance from the digestion product to the next smaller degradation product as discussed in Brownlee and Sanger. M does not fall in the ranges for terminal nucleotides as determined by Brownlee and Sanger (C 0.6 to 1.2, A 2.1 to 2.9, U 1.7 to 1.9, and G 2.6 to 4.4 (42)) for all of the products listed. However, it is obvious from the data presented that the sequence determinations are correct. Others have observed that the M values for the terminal nucleotides do not always fall within the expected ranges (41).

\(^f\) Partial products are listed in order of decreasing mobility for each U1 nucleotide. Numbers followed by periods are arbitrary.

\(^g\) Data shown in brackets do not support the deduced sequence shown.

\(^h\) Alkaline and snake venom digestion products were not quantitated, but were determined by visual estimation from the autoradiograph film.
TABLE V
Silkworm nuclease digestion products of U1 nucleotides
3'-Dephosphorylated U1 nucleotides from fingerprints as in Fig. 8F were digested with silkworm nuclease and combined silkworm nuclease-bacterial alkaline phosphatase; products were then separated as described under “Materials and Methods.” Products were analyzed and sequenced by alkaline and snake venom phosphodiesterase digestion.

| Product | Deduced Sequence |
|---------|-----------------|
| A. u13  | A-U-C-U-U-U-U-G |
| B. u14  | U-C-A-A-A-D-D- |
| C. u15A | C-A-C-U-(N)-A-A- |
| D. u15C | U-C-C-C-A-C- |

*SW = products of digestion with silkworm nuclease alone. Products are listed in order from 5'-end to 3'-end of the U1 nucleotide, rather than by mobility.

*SWP = products of combined silkworm-phosphatase digestion. The digest and a plain silkworm nuclease digest were run side by side at pH 3.5 on DEAE-paper. The 5' end products in both columns had identical mobilities. Other products with the same base sequence in both columns had different mobilities (see text).

*Alkaline and/or snake venom digestion products could not be accurately quantitated.

*A U2 ribonuclease digestion product with the same base sequence as these products was isolated from 3'-phosphate U1 nucleotide U14 (see text).

*As noted in text, the 3'-end sequence depends on these products.

Followed by separation of the products on DEAE-paper, pH 3.5, showed the ratio of pyrimidines to be 80% D to 20% U, indicating that both U nucleotides can be modified to D in any given molecule. In addition, some pancreatic digest of U14 followed by separation of the products on DEAE-paper, pH 3.5, showed free U to be in approximately 30% greater yield than free D, indicating 85% modification of the pyrimidine adjacent to GmG. When the product AAAD was further analyzed by alkaline digestion and product identification on Whatman No. 52 paper, the ratio of pyrimidines was 75% D and 25% U. Thus it seems that either or both of the U nucleotides on the 5'-side adjacent to GmG can exist in the modified or unmodified state with the former predominant.

Identification of GmG was accomplished as stated in “Materials and Methods,” and the mobilities for GmG and degradation products obtained from it are given for several electrophoretic and chromatographic systems as described in Table VIII. In addition, Gm and G 5'-phosphates were obtained when dephosphorylated U14 was digested by snake venom phosphodiesterase and the products separated by electrophoresis on Whatman No. 52 paper at pH 3.5.

Sequence Analysis of u15A—Partial snake venom digestions of
u15A were carried out using an incubation time of 30 min at 37°. The results are shown in Table IV. Sufficient products were obtained to sequence the first 9 bases of the 5'-end, although the quantitative results from the alkaline digestion of partial product number 5, shown in brackets, do not fit the deduced sequence shown, and the data for partial products 6 and 7 are visual estimations from autoradiograph films.

Silkworm and silkworm-phosphatase digestions of u15A, shown in Table V-C, generated a series of 5'-end products which could be recognized as such and sequenced. Internal products could be fitted to a consistent over-all sequence. The 3'-end sequence of u15A depends on silkworm-phosphatase products alone. The last silkworm phosphatase product, UpCpG, could be sequenced by alkaline and snake venom digestions. This permitted the sequencing of the next longest product, CpUpCpG. Although the 5'-phosphate analog of the latter, pCpUpCpG was found, it could only have been sequenced as pCpUp(q)G without the information from the smaller silkworm phosphatase product. The final sequence of u15A is as shown, UCCACUUCUCGG.

Sequence analysis of u15C—In addition to problems with variable mobility and yield, nucleotide u15C also proved unamenable to various enzymatic digestions. As seen in Table IV, no partial snake venom digest products were obtained from u15C in sufficient quantity to analyze, although the nucleotide could be digested under conditions of total snake venom digestion.

Digestion of dephosphorylated u15C with U2 ribonuclease generated the products CA, ¥pG, (C,U)A in 0.3 molar yield, and (C,U,N(X))A. ¥pG gave ¥pA as the 3' digestion product and a snake venom digestion product of pG. The other U2 oligonucleotides were also analyzed by T2 digestion. Separation of the T2 digestion products of (C,U,N(X))A by electrophoresis on Whatman No. 52 paper, pH 3.5, gave molar yields of C, U, and A and half-molar yields of slow C and fast C as well as some unidentified products in low yield with various mobilities. Alkaline hydrolysis of the same U2 oligonucleotide gave the same products but with even smaller yields of the unidentified products. It seems likely that the slow and fast C products are different stages of modification of nucleotide N and that the fast C is the same as nucleotide X obtained in the silkworm phosphatase product shown in Table V-D. Furthermore, the presence of the U2 product, (C,U)A suggests that N is indeed a modified U. It should be noted that (C,U,N(X))A ran on DEAE-paper in 7% formic acid with a mobility that is of an oligonucleotide containing only C, U, and A (i.e. only 1 U). It seems plausible that N is basic enough to cause oligonucleotides containing it to run faster on DEAE-paper.

Results from the silkworm and silkworm-phosphatase digestions of u15C were somewhat meager, as seen in Table V-D. Two 5'-end products were obtained, however, which establish the sequence of the first four bases, CACU. A silkworm-phosphatase product was obtained for which alkaline digestion yielded A*Ap, Ap, and ¥pA, and snake venom digestion gave ¥pA, bp, and pG. This implies the sequence A*ApA¥pG and establishes the position of A* within AAA*A. Another silkworm-phosphatase product is shown in Table V-D as XpA. Here base Xp in the alkaline digestion had the “fast C” mobility observed before in the U2 product (C,U,N(X))A.

The sequence data on u15C establish the 3'-end as CACU and the 5'-end as AAA*A¥G. The sequence of the U2 product containing N must then be CUANA and the final sequence for u15C must be CACUNAA*A¥G.

X refers to a possible alternate form of modified nucleotide N and has a mobility of a “fast C” as discussed in the text.

### Table VI

| Nucleotide | Sequence | Molar Yields |
|------------|----------|--------------|
| u1         | Gp       | 1.1          |
| u2         | Gp       | 0.9          |
| u3         | A-Gp     | 2.6          |
| u4         | A-C-A-A-A-Ap | 0.6          |
| u5         | C-A-C-A-A-Gp | 0.7          |
| u6         | U-Gp     | 0.7          |
| u7         | pGp      | 0.6          |
| u8         | U-U-U-Gp | 0.8          |
| u9         | A-A-U-Gp | 1.3          |
| u10        | D-A-A-A-A-Gp | 0.8      |
| u11        | T-U-C-Gp | 1.2          |
| u12        | A-U-U-U-U-C-U-U-Gp | 1.3      |
| u13        | U-C-A-A-A-D-D-D-Gp | 1.1      |
| u14        | U-C-C-C-A-A-C-U-U-C-C-Gp | 1.1      |
| u15C       | C-A-C-U-U-A-A-A-A-A-A-Gp | 0.9      |

* Values were determined by measuring the radioactivity of the paper containing the nucleotides in a Beckman low-background gas-flow counter.

Experimentally determined molar yields of the U1 ribonuclease digestion products are shown in Table VI. The figures are based on a compilation of data from both U1 and U1-phosphatase experiments. The yield shown for u15C (0.9) is deceptively high as it was based largely on results from a series of U1-phosphatase experiments such as in Fig. 8F, in which yields of u15C were relatively good. Yields of u5 and u7 were based on three such determinations. The yield of u15C was exceptionally good in those U1-phosphatase fingerprints (Fig. 8F) for which quantitative data were obtained.

Partial Enzymatic Digestion Products and Over-all Sequence of T4 tRNA<sub>79</sub>

Partial digestions of T4<sup>[32P]rRNA</sup><sub>79</sub>, using U1, pancreatic, and modified ε-carboxymethyllysine-41-pancreatic RNases, were carried out and the products fractionated as described under “Materials and Methods.” Table VII shows a series of such partial digestion products. Many other partial products were characterized than those shown in Table VII which shows more than sufficient examples to establish the over-all sequence of the molecule.

Each product was analyzed by total digestion with U1 and
Table VII

Partial enzymatic digestion products of T₄ [³²P]RNA²⁴a

Partial digestions of T₄[³²P]RNA²⁴a were carried out using U1, pancreatic, and modified ε-carboxymethyllysine-4' pancreatic RNases, and the products were fractionated as described in "Materials and Methods."

In product nomenclature the first three numbers refer to a given experiment, U, P, and MP indicate the enzyme used, and the next number refers to a specific partial product within a given experiment. For U1 RNAse, enzyme to substrate ratios were 1:100 for Experiment 131, 1:100 for Experiments 140 and 263, and 1:300 for Experiment 606. For pancreatic RNAse, enzyme to substrate ratios were 1:500 for Experiments 381 and 411. For modified pancreatic RNAse the ratios were 1:50 for Experiment 571 and 1:20 for Experiment 605.

| Experiment | Enzyme | Ratio |
|------------|--------|-------|
| 131 U1     | U1     | 1:100 |
| 131 U10    | U1     | 1:100 |
| 605 MP9    | MP9    | 1:500 |
| 605 MP1    | U1     | 1:100 |
| 605 MP11   | U1     | 1:100 |
| 605 MP12   | U1     | 1:100 |
| 381 P12    | P12    | 1:500 |
| 606 U7     | U1     | 1:100 |
| 263 U30    | U1     | 1:100 |
| 140 U14    | U1     | 1:100 |

*Note: Additional details are provided in the supplementary materials.*
pancreatic RNases. Mobilities of the total digestion products in 7% formic acid could be compared with known mobilities of total digestion products of the entire molecule, run as markers on the same paper. In addition, the total digestion products were analyzed by further digestion with the opposite enzyme, or with alkali, or both. The combined data usually, but not always, led to a definitive sequence for the partial product. Where a partial product cannot be independently sequenced, however, the results from other partials can be applied to order it. Partial product 606-U12, for instance, is CACUNAAA*AWGCUG. It consists of u15C and u8. Total U1 digestion yields these two products, which are completely identifiable. Inspection will show however that the pancreatic digestion products of order u15C-u8 are identical with those of u8-u15C. The two U1 nucleotides cannot be ordered. However, several partial products in Table VII show definitively that u15C is immediately preceded by u4, CACAG. Furthermore, product 605-MP11, AAA*AAGCUG, shows the 3' end of u15C linked to CUG. Therefore the order of 606-U12 must be as shown.

Examples of partial products which can be independently sequenced are 605-MP20, AAAGGCACAGC, and 571-MP9, AAAGGCAAGCAGC(N).

Total digestion of 605-MP20 with pancreatic RNase yielded AAAGG, AC, and AGC, standard products p10, p19, and p3. They were identifiable by mobility in 7% formic acid. However, the identity of AAAGGC was verified by both alkaline and U1 RNase digestion, AC was verified by alkaline digestion, and AGC was verified by U1 digestion. Theoretically the three pancreatic nucleotides can be arranged in six possible orders. Total digestion of 605-MP20 with U1 RNase yielded products AAAG, G, CACAG, and C. Once these were identified by mobility and alkaline or pancreatic digestion, it was clear that AAAG and C are not normal U1 nucleotides from the intact molecule; they must therefore be the terminals of the modified pancreatic partial. It follows at once that C must be the 3'-terminal since the enzyme cleaves following pyrimidines, and, therefore, AAAG is the 5'-terminal. This leads to AAAG(G, CACAGC), which on the basis of the pancreatic digest leads to the sequence as shown.

571-MP9 is a less trivial example. Here total pancreatic digestion yields AAAGGC, nAC, AGC, and nU. Total U1 digestion yields AAAG, G, CACAG and CACU(N). Pancreatic digestion shows (CACU(N)) to contain C, AC, and nU, and it follows that the 571-MP9 must be AAAG(G, CACAGC), ACA, and nU). The known pancreatic products lead further to AAAGGCACAGC(AC, nU). At this point information about the 3'-end of u15C from silkworm digestions (Table V-D) must be invoked to arrive at AAAGGCAAGCAGC(nU). By the time the experiment has reached this stage, not enough radioactivity remains to determine how many 3'-U nucleotides are present. Since Up and Up are indistinguishable in this digest, the partial product may or may not include N. Hence N is shown in Table VII in parentheses.

Other long U1 and pancreatic partial products such as 381-P12, 263-U30, and 263-U31 could not be completely ordered on the basis of the original data. Information obtained from later partial digests made it possible to establish the sequences as shown.

Partial product 571-MP1, AAADDGmGD, yielded separate pancreatic digestion products in 7% formic acid which alkaline digestion showed to be AAAU, AAAD, U, D, and GmGD. U1 digestion yielded one long product plus D. The long U1 product again gave, on pancreatic digestion, the products AAAU, AAAD, U, D, and GmG. These data again indicate that both the "U" residues preceding GmG in U1 nucleotide u14 can be

**Bacteriophage T4 tRNA**

**FIG. 9.** Cloverleaf pattern arrangement of T4 tRNA**

Identification and Characterization of Modified Nucleotides

Modified nucleotides were identified and further characterized as described under "Materials and Methods." The mobilities of the modified and unmodified nucleotides in the various systems employed are summarized in Table VIII.

All modified nucleotides in the molecule with the exception of N are identified and their mobilities in the various systems agree with published values (29). The identity of nucleotide N remains unknown. It is a basic modification of uridylic acid.

**DISCUSSION**

We believe that the data presented above unambiguously prove the nucleotide sequence presented in Table VII and Fig. 9. The uncertainties in the structure all involve the identification of the modified nucleosides. This is always a problem in sequence determinations in which only radiochemically pure tRNA is used. It is impossible to obtain spectra of the nucleosides and one must rely on chromatographic and electrophoretic mobilities for identification. This is of no use when the nucleoside has not already been well described. The problem is compounded when, as in our case, the tRNA is isolated from phage-infected cells in which modification may often be incomplete (35).
The modified nucleotides D, T, V, and GmG were identified by their mobilities in electrophoresis at pH 3.5 and by chromatography in several systems. Their familiar positions in the cloverleaf model support these identifications.

We suggest that the nucleoside A* in the anticodon loop is 2-methylthio-N-4(γ,γ-dimethylallyl) adenosine. A* has the same electrophoretic mobility on both Whatman No. 3MM and DEAE-paper at pH 3.5 as A in E. coli tyrosine tRNA (34, 35, 41). It is also found in the same position next to the anticodon. This nucleotide has been detected in the position next to the anticodon of several tRNAs recognizing codons beginning with U. It is this nucleotide alone that is responsible for the hydrophobic nature of tRNA\textsuperscript{Tyf} in E. coli (35) and thus presumably of all tRNAs recognizing codons beginning with U (with the possible exception of tRNA\textsuperscript{Phy}). T4 tRNA\textsuperscript{Leu} recognizes UUA (43) and elutes in the hydrophobic fraction on BD-cellulose. Heterogeneity in the modification of A* in tRNA\textsuperscript{Tyf} (41) led to problems in the mobility of oligonucleotides containing A*.

Similar difficulties have been encountered in this study.

Np is another modified nucleotide in the anticodon loop. The fact that the NpA bond is cleaved by pancreatic RNase indicates that N is a pyrimidine. In alkaline digestions of u15CN is present as a nucleotide with one-third the mobility of C at pH 3.5 on Whatman No. 540 paper. In the separation of pancreatic RNase digestion products on DEAE-paper at pH 3.5, Np must have the same mobility as Up. Thus and the fact that both CUUA and CUNA are obtained as U2 products from u15C suggests that N is a modified form of U. N is in the "wobble position" of the anticodon. Scherberg and Weiss (43) have shown that T4 tRNA\textsuperscript{Leu} recognizes only the codon UUA. This is a violation of the wobble hypothesis (44) that has been observed before in cases where there is a 2-thiouridine derivative in the wobble position of the anticodon (45, 46). In E. coli the 2-thiouridine derivative that is found is 2-thio-5-methylaminomethyl uridine (30). Dr. J. Carbon has provided us with an authentic sample of 2-thio-5-methylaminomethyl uridylic acid. Np turned out to have different mobilities in several different chromatographic systems than did the standard. Thus we must leave Np as an unknown with the suspicion that it could be a 2-thiouridine derivative of some sort.

In their original discovery of T4 tRNA, Hsu et al. (6) reported that the T4 tRNA could be labeled with \textsuperscript{35}SO\textsubscript{4}. If the above speculation is correct both A* and N contain a thio modification. In most E. coli tRNAs where there is a U in position 8, it is a 4-thiouridine. T4 tRNA\textsuperscript{Leu} has a U in position 8 but none of the experiments we have done would be capable of telling whether or not this U is a 4-thiouridine or not.

In Fig. 9 we have compared the T4 tRNA\textsuperscript{Leu} sequence with those of E. coli tRNA\textsuperscript{Leu} 1 and 11 (47, 48). The residues that all three molecules share uniquely have been indicated. It can be seen that the three molecules are quite dissimilar. It is unlikely that E. coli contains a tRNA\textsuperscript{Leu} which is any closer in sequence to T4 tRNA\textsuperscript{Leu} than to the other two. T4 tRNA\textsuperscript{Leu} has a U in position 8 but none of the experiments we have done would be capable of telling whether or not this U is a 4-thiouridine or not.

Although quantitative rate measurements have not been performed, it is clear from the work of several groups that T4 tRNA\textsuperscript{Leu} is acylated by the E. coli leucyl-tRNA synthetase (8, 9). Thus all three tRNA sequences which are compared in Fig. 9 are recognized by the same enzyme. Although these comparisons do not allow us to define a synthetase recognition site uniquely, we note that the dihydrouridine loop is nearly identical in all three molecules. Four nucleotides in the 3'-5' stem region are also the same. This region has been implicated as important in the recognition of E. coli tyrosine tRNA by its synthetase (49, 50).

It is also of interest that all three molecules are exactly the
same length (87 nucleotides). The length of the tRNA molecule seems to be a factor in the recognition of tRNA by the yeast phenylalanyl-tRNA synthetase (51).

T4 is closely related to two other bacteriophages, T2 and T6. In Fig. 8F it can be seen that the U1 fingerprint of T6 band 4 RNA is identical in sequence with T4 tRNALeU. T2 and T6 also produce a tRNALCU and that the sequences of this molecule are identical in sequence with T4 tRNALeU. T2 and T6 also produce a tRNALCU and that the sequences of all three molecules are very closely related, if not identical.

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