The Nucleotide Sequence of a Precursor to the Glycine- and Threonine-specific Transfer Ribonucleic Acids of Escherichia coli*

(Received for publication, January 27, 1975)

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The primary nucleotide sequence of an Escherichia coli tRNA precursor molecule has been determined. This precursor RNA, specified by the transducing phage λh80dglyTsuA36 thrT tyrT, accumulates in a mutant strain temperature-sensitive for RNase P activity. The 170-nucleotide precursor RNA is processed by E. coli extracts to form mature tRNAGlu and tRNAAsp. The sequence of the precursor is pG-U-U-C-C-A-G-G-A-U-G-C-G-G-G-C-A-U-C-G-U-A-U-A-G-G-G-C-U-C-A-A-C-A-G-A-U-G-C-G-G-G-T-C-G-A-U-U-C-C-C-G-C-U-G-C-C-C-G-C-C-A-A-G-C-A-C-C-C-C-G-A-U-G-U-G-C-U-G-A-U-A-G-C-U-C-A-G-C-A-C-C-C-U-G-G-U-m7G(A, G)-A-G-G-G-U-G-A-G-m7G-U-C-G-G-C-A-G-T-C-G-A-A-U-C-U-G-C-C-U-A-U-C-A-G-C-A-A-C-C-A-C-A-A-A-G-G-A-G-C-G-C-A-C-C-C-C-G-A-U-G-U-G-C-U-G-A-U-A-G-C-U-C-A-G-C-A-C-C-A-C-U-U
(tRNA sequences are italicized). It contains the entire primary nucleotide sequences of tRNAGlu and tRNAAsp, including the common 3' terminal sequence, CCA. Nineteen additional nucleotides are present, with 10 at the 5' end, 3 at the 3' end, and the remaining 6 in the inter-tRNA spacer region. RNase P cleaves the precursor specifically at the 5' ends of the mature tRNA sequences.

Functional stable ribonucleic acid species, such as mature ribosomal RNA or transfer RNA, are not direct transcriptional products but arise from the cleavage or longer precursor molecules. Recently, precursors to Escherichia coli tyrosine tRNA (1, 2) and to various bacteriophage T4 specified tRNAs (3-5) have been isolated and characterized. An endonuclease (RNase P) which cleaves the tyrosine tRNA precursor specifically at the 5' end of the tRNA sequence was subsequently purified (6). More recently, E. coli mutants defective for tRNA biosynthesis at various steps of the biosynthetic pathway have been isolated and characterized (7, 8). Although our current understanding of tRNA biosynthesis is still incomplete, it is evident that the post-transcriptional processing events include (a) nucleolytic cleavages, (b) nucleotide modifications at specific residues, and, in some cases, (c) enzymatic addition of the terminal C-C-A- sequences.

While much has been learned about post-transcriptional processing of tRNA precursors recently, the fine structure organization of tRNA genes on the chromosome remains unclear. By tRNA-DNA saturation hybridization, it was estimated that there are 40 to 80 tRNA genes per E. coli chromosome (9-12). From genetic data on the relatively few tRNA genes that have been mapped, it is apparent that tRNA genes are scattered throughout the genome (13). On the other hand, increasing evidence has accumulated indicating that bacterial tRNA genes might often occur in clusters containing several closely spaced genes. These clusters can contain identical genes, as at glyV (14, 15) or sup3p (16); or copies of genes specifying different tRNAs, as at the glyT locus (17). Furthermore, Schedl and Primakoff (7) have observed tRNA precursors containing two or more tRNA sequences in E. coli mutant strain A49 (a temperature-sensitive mutant with respect to RNase P activity), indicating that many tRNAs are synthesized via multi-tRNA precursors.

We have investigated the transcription of the glyT thrT tyrT tRNA gene cluster which occurs at 77' on the E. coli chromosome and which specifies the synthesis of tRNAGlu and tRNAAsp respectively (17). Here we report that this tRNA gene cluster is transcribed, at least in part, as a multi-tRNA precursor. In addition, we have determined the total nucleotide sequence of a di-tRNA precursor to the glycine

\* This work was supported by Research Grant CA-11034 from the National Cancer Institute, United States Public Health Service.
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The abbreviations used are: tRNA\textsuperscript{gly}_{\text{Asl}}\text{Asl}, glycine tRNA capable of suppressing the A36 missense mutation in the tryptophan synthetase A protein; tRNA\textsuperscript{Thr}_{\text{Asl}}\text{burst}, threonine tRNA specific for the codons ACU/C; D-5,6-dihydrouridylic acid; m\textsuperscript{5}G, 7-methylguanylic acid; A\textsuperscript{5}, an unidentified derivative of adenylic acid; N\textsuperscript{5}, an unidentified derivative of uridylic acid; mt\textsuperscript{A}, the 3' phosphate of N-[9-β-α-ribofuranosylpurin-6-yl]N-methylcarbamoyl]threonine.
and threonine tRNAs. Preliminary accounts of this work have been presented elsewhere (15, 18).

**EXPERIMENTAL PROCEDURES**

**Bacteriophage and Bacterial Strains**

All of the λ-80 hybrid phage strains employed in this study are heat-inducible and lysogenic-defective, since they contain the temperature-sensitive repressor mutation Clav and the absolute defective mutation Sm'. The helper hybrid phage used is λClavSm'λ80 (hereafter designated λ80). It carries the λ immunity region but possesses the host range of 80. The transducing phage λClavSm', λ80dgIyTsuA36 thrT tyTyT (hereafter designated λ80dgIyTsuA36 thrT tyrT) carries three *Escherichia coli* tRNA genes, termed gTyTsuA36, thrT, and tyrT, which specify the structures of tRNA<sub>lys</sub>, tRNA<sub>tyr</sub>, and tRNA<sub>arg</sub> respectively. Details of the isolation and characterization of these phages have been reported previously (17).

*E. coli* K-12 strain JC100 has the genotype lacZ<sub>mer</sub>, trpA argH and harbors the prophages λ80 and λ80dgIyTsuA36 thrT tyrT. Strain A49 (obtained from P. Schedl, Department of Biochemistry, Stanford University) carries the following genetic markers: lac<sub>com</sub>, trpA36 argH glyS Sm' and in addition, a temperature-sensitive mutation affecting RNase P activity (7). For precursor labeling and preparation, strain A49 was lysogenized with transducing phage λ80dgIyTsuA36 thrT tyrT to produce the lysogenic strain A49 (λ80dgIyTsuA36 thrT tyr7'). Because of the presence of the missense suppressor mutation glyTsuA36 on the prophage, this lysogen is phenotypically Trp'. It was routinely grown and kept in media containing 0.25 mM CaCl₂, 0.001% gelatin, 1 mM MgSO₄, pH 7.2 with 5 mM phosphate. Low phosphate Casamino acids medium (LPC medium) has the same composition except that an equal amount of phosphate-free peptone. LPC medium containing 1 mM phosphate. Three milliliters were transferred to a screw-capped tube and then incubated at 43°C for 20 min to induce the phage. The induced culture was grown at 37°C for 40 min and then at 43°C for 30 min. All operations from this step on were carried out at 43°C. The cells were centrifuged and resuspended in an equal volume of LPC medium containing 0.1 mM phosphate. After shaking for 30 min, the cells were again pelleted and resuspended in 3 ml of LPC medium containing 5 mM of carrier-free [32P]phosphoric acid. Labeling usually was for 30 min. The control sample was prepared from non-lysogenic A49 cells under similar conditions, except that tryptophan (100 μg/ml) was added to the LPC media.

**Extraction of Labeled RNA**

Labeling was terminated by adding 3 ml of redistilled, neutralized phenol to the culture. The mixture was shaken vigorously on a Vortex test tube mixer (Scientific Industries, Inc., model K-500-4) for 30 min at room temperature. The aqueous phase was separated by centrifugation and then transferred to a siliconized tube. To the aqueous phase were added 300 μg of carrier *E. coli* tRNA, 1/5 volume of 2 M sodium acetate, pH 5.0, and 2 volumes of ethanol. The RNA was allowed to precipitate from the extract for 15 min at -75°C, recovered by centrifugation, and dried in vacuo.

**Polyacrylamide Gel Electrophoresis**

Polyacrylamide slab gel electrophoresis was carried out according to the procedure of De Wachter and Fiers (20). The dried RNA sample was dissolved in 15 μl of a solution containing 50% urea, 25 mM NaCl, 1 mM MgSO₄, 0.001% gelatin, 0.25 mM CaCl₂ (H. Inokuchi, quoted in Ref. 19). The pIffer titer was estimated from the optical density reading of the page suspension at 260 nm (A<sub>260</sub> of 1 corresponds to about 4 x 10<sup>1</sup> phage/ml for freshly prepared phage stocks).

**Phage Stocks**

Purified λ80dgIyTsuA36 thrT tyrT phage stocks were obtained by heat induction of JC100, which is lysogenic for the λ80 helper and transducing phages. The transducing phages in the lysate were then purified by two cycles of low and high speed centrifugation followed by banding in a CsCl equilibrium density gradient. The band of lower density (transducing phage) was dialyzed against 80 buffer (0.6 mM Tris HCl, pH 7.5, 1 mM MgSO₄, 0.001% gelatin, 0.25 mM CaCl₂) (H. Inokuchi, quoted in Ref. 19). The phage titer was estimated from the optical density reading of the page suspension at 260 nm (A<sub>260</sub> of 1 corresponds to about 4 x 10<sup>1</sup> phage/ml for freshly prepared phage stocks).

**Low Phosphate Media**

Low phosphate peptone medium (LPP medium) is 0.1 M Tris (pH 7.5) containing (per liter) 1.5 g of KCl, 1 g of NaCl, 1 g of NH₄Cl, 0.2 g of MgSO₄·7H₂O, 0.5 mg of B1 (thiamine hydrochloride), 0.5 ml of Luria broth, and 40 ml of 10% phosphate-free peptone solution (see below). Low phosphate Casamino acids medium (LPC medium) has the same composition except that an equal amount of phosphate-free Casamino acids is used to replace the phosphate-free peptone.

Phosphate-free peptone or Casamino acids were prepared by dissolving 80 g of Difco Bacto-peptone or Casamino acids in 300 ml of water at 45°C, and then adding slowly with stirring 25 ml of 25% MgCl₂·6 H₂O solution and 1.8 ml of concentrated NH₄OH. The inorganic phosphate was allowed to precipitate (as MgNH₄PO₄) for 2 hours at 4°C, and was then removed by centrifugation. The supernatant was brought to pH 7.6 with 5% KOH and any additional precipitate was removed by centrifugation and filtration through Schleicher & Schuell No. 585 filter paper. The filtrate was adjusted to neutral pH, diluted to 800 ml, and autoclaved.

**"P Labeling of RNA**

**Phage Infection Method**—A culture of strain A49 was grown at 30°C to a density of approximately 3 x 10<sup>9</sup> cells/ml, resuspended in 0.2 ml of 80 buffer, and the culture was then infected with purified transducing phage at a multiplicity of 30 phage/cell. Phage adsorption and infection were carried out by incubation for 15 min without shaking. Three milliliters of LPP medium containing 0.1 mM phosphate were added to the infected culture and shaking was resumed to allow phage DNA to replicate. Thirty minutes later, the cells were again harvested, resuspended in 3 ml of LPP medium containing 5 mM of carrier-free [32P]phosphoric acid, and shaking was continued for 15 min. A control sample was prepared under the identical conditions as noted above, except all of the operations were carried out at the permissive temperature (30°C).

**Prophage Induction Method**—The defective lysogen, A49 (λ80dgIyTsuA36 thrT tyrT), was grown at 30°C to 2 x 10<sup>9</sup> cells/ml in LPC medium containing 1 mM phosphate. Three milliliters were transferred to a screw-capped tube and then incubated at 43°C for 20 min to induce the prophage. The induced culture was grown at 37°C for 40 min and then at 43°C for 30 min. (All operations from this step on were carried out at 43°C.) The cells were centrifuged and resuspended in an equal volume of LPC medium containing 0.1 mM phosphate. After shaking for 30 min, the cells were again pelleted and resuspended in 3 ml of LPP medium containing 5 mM of carrier-free [32P]phosphoric acid. Labeling usually was for 30 min. The control sample was prepared from non-lysogenic A49 cells under similar conditions, except that tryptophan (100 μg/ml) was added to the LPC media.

**In Vitro Precursor Cleavage**

Digestion of "P-labeled precursor RNA with crude cell extracts prepared from *E. coli* K-12 strain B286 (wild type RNase P strain) was carried out as previously described (7). Reaction mixtures of 0.2 ml contained: 10 μCi/ml of labeled precursor RNA, 1.6 mg/ml of sonicate protein (tryptophan, 0.1 M NaCl, 0.1% mercaptoethanol, 10 mM Tris-chloride (pH 8.0), and 50 μg/ml of carrier tRNA. After incubation for 30 min at 37°C, the reaction was terminated by the addition of 0.2 ml of redistilled, neutralized phenol and agitation on a Vortex mixer. The cleavage products were precipitated with ethanol and separated on a 5% polyacrylamide gel by prolonged electrophoresis (19 hours at 300 volts).

For cleavage of precursor RNA with purified RNase P, the procedure of Robertson et al. (6) was used. RNase P was purified through the DEAE-Sephadex step as previously described (6). Separation of RNase P cleavage products was carried out on a 12% polyacrylamide gel at 400 volts for 16 hours.

**Sequencing Techniques**

The sequencing procedures described by Barrell (22) were followed for the enzymatic digestion and fingerprinting of purified [32P]tRNA<sup>lys</sup>-tRNA<sup>tyr</sup> precursor. These methods were also used for the...
subsequent characterization of the resulting oligonucleotides by alkaline or enzymatic digestion.

The total exogenous digestion reaction was performed according to the procedure of U. Rensing, as described by Brownlee (23). Partial snake venom phosphodiesterase digests were carried out at room temperature for 2 min using 0.1 mg/ml of enzyme in buffer (0.05 M Tris-chloride, pH 8.9, 0.01 M MgCl,

Digestions of oligonucleotides with combined silkworm nuclease (24, 25) and bacterial alkaline phosphatase were carried out as previously described (26).

Partial enzymatic digestions of intact precursors were carried out using T, or modified pancreatic ribonuclease (c-carboxymethyl lysine-41) (27). Partial T, ribonuclease digestion was carried out in 0.01 M MgCl,

Materials

Materials were obtained from the following sources: pancreatic RNase A, snake venom phosphodiesterase, spleen phosphodiesterase, and E. coli alkaline phosphatase (BAP-F) from Worthington Biochemical Corp.; T, RNase from Calbiochem; U, RNase (Ustilago) was a gift from Dr. H. Okazaki of the Sankyo Co.; the silkworm nuclease was donated by J. Mukai, Kyushu University, Fukuoka, Japan; RNase P was purified from E. coli through the DEAE-Sephadex step as described (6); the carbodiimide blocking reagent, N-cyclohexyl-N'-2-(4-morpholinyl)ethyl carbodiimide methyl-p-toluene sulfonate, from Aldrich Chemical Co.; yeast RNA from Sigma Chemical Co.; RP-14 (13½ x 17 inch) medical x-ray film, acrylamide, N,N',N'-tetramethylene bis(acrylamide), N,N',N',N'-tetramethylene diaminodiace, and cellulose thin layer sheets with fluorescent indicator for thin layer chromatography from Eastman Kodak Co.; DE81 DEAE-cellulose paper from Whatman; cellulose acetate strips, No. 2500 (30 x 570 mm) from Schleicher & Schuell, Inc.; Polygram Cel 300 DEAE-thin layer chromatography sheets from Macherey, Nagel, and Co., carrier-free H,3*P0, from International Chemical and Nuclear Corp. The toluene-based scintillation fluid contained 0.4% Omnifluor (New England Nuclear Corp.). Reagents used for making all buffers were analytical grade. Bacterial media were purchased from Difco Laboratories.

RESULTS

Amplification and Isolation of 32P-labeled tRNA

Previous studies have shown that a cluster of three tRNA genes (gly'T thr'T tyr'T) lies in the region between argH and thi on the Escherichia coli chromosome. These genes specify the structures of tRNA

The synthesis of the phage-specified tRNAs was selectively amplified. By using this method to prepare relatively large quantities of labeled tRNAs, the total nucleotide sequences of tRNA

Amplification and Isolation of 32P-labeled tRNA

A polyacrylamide gel electrophoretic separation of 32P-labeled RNA isolated from A49 and the defective lysogen, A49 (Xh80dglyTsuA36 thr'T tyr'T), under various conditions is shown in Fig. 1. Labeled RNA isolated from A49 cells infected with Xh80dglyTsuA36 thr'T tyr'T shows a prominent RNA band (labeled E) when the labeling was carried out at 43° (lane b), but only a trace amount of band E is seen at the permissive temperature (30°, lane a). Similarly, when the lysogen, A49 (Xh80dglyTsuA36 thr'T tyr'T), is induced and labeled at 43°, the gel pattern again shows the presence of band E RNA (lane c). Non-lysogenic A49 cells labeled at 43° do not contain band E RNA (lane d). Thus, the accumulation of band E RNA in strain A49 depends not only on elevated temperatures (presumably for thermo-inactivation of RNase P), but also on a phage infection or prophage induction. These characteristics suggest that band E RNA could be a tRNA precursor molecule. In fact, this RNA contains the complete nucleotide sequences of tRNA

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Fig. 1. Fractionation by 5% polyacrylamide gel electrophoresis of 32P-labeled RNA isolated from Escherichia coli strain A49: lane a, after infection with the transducing phage Xh80dglyTsuA36 thr'T tyr'T at 30°; lane b, after a similar infection at 43°; lane c, after induction at 43° of the lysogenic strain A49(Xh80dglyTsuA36 thr'T tyr'T); and lane d, a control A49 culture not exposed to the transducing phage. The gel electrophoresis was carried out at 300 volts for 16 hours at 4°. Experimental details are described under "Experimental Procedures." Band A contains completely processed tRNA

Band E contains the tRNA

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the growth medium when labeling was carried out by prophage induction ("Prophage Induction Method" under "Experimental Procedures"). The preparation migrated as a single band on acidic urea acrylamide gels, and was estimated to be 85 to 90% radiochemically pure by measuring contaminants on fingerprints of T, RNase digests. Band E RNA has an estimated size of 172 ± 5 nucleotides, based on its electrophoretic mobility on polyacrylamide gels using E. coli tRNA^\text{TY}, 5 S RNA, and 6 S RNA as markers (not shown).

In vitro Processing of Band E RNA—Purified \(^{32}\text{P}\)-labeled band E RNA was incubated with a cell-free extract prepared from sonicated E. coli strain BF266 (wild type RNase P strain) cells, and the digestion products were then fractionated on a 5% polyacrylamide gel. Two RNA bands (S1 and S2) were resolved on the gel as shown in Fig. 2a. Two-dimensional Sanger fingerprints of complete T, RNase digests of S1 and S2 RNAs (Fig. 3, a and c) were compared with authentic fingerprints of mature tRNA^\text{TY}, 5 S RNA, and 6 S RNA as markers (not shown).

Two RNA bands (S1 and S2) were resolved on the gel as shown in Fig. 2a. Two-dimensional Sanger fingerprints of complete T, RNase digests of S1 and S2 RNAs (Fig. 3, a and c) were compared with authentic fingerprints of mature tRNA^\text{TY}, 5 S RNA, and 6 S RNA as markers (not shown). This was further confirmed by sequence analysis of the T, RNase oligonucleotides (see below). Thus, band E RNA is apparently a precursor RNA that can be processed to form mature tRNA^\text{TY}, 5 S RNA, and tRNA^\text{ACUC}.

The 5'-terminal T, fragment (pGp) of both mature glycine and threonine tRNAs was found in nearly molar yield on the fingerprints of S1 and S2 RNAs. However, the 3'-terminal T, oligonucleotides appeared to be somewhat heterogeneous in size. Although in vitro cleavage to generate mature 5' ends occurs by a specific endonucleolytic split, it seems likely that maturation of the 3' ends might occur by exonucleolytic degradation (33).

**T, Ribonuclease Digestion Products of \(^{32}\text{P}\) tRNA^\text{TY}, tRNA^\text{TM} Precursor—**Purified \(^{32}\text{P}\) tRNA^\text{GOY}, tRNA^\text{TM} precursor was digested to completion with T, ribonuclease, and the products were fractionated by two-dimensional ionophoresis as described by Barrel1 (22). The resulting fingerprint is shown in Fig. 4. The sequence of each of the T, RNase oligonucleotides is given in Table I along with the experimental and theoretical yields. For comparison, Table I also lists the theoretical yields of T, oligonucleotides of tRNA^\text{GU}, 5 S RNA, and tRNA^\text{ACUC} calculated from the known sequences (30, 31) as shown in Fig. 7.

The 5'-terminal fragment of the precursor is pGp(66), which is the only fragment with 5'-phosphate found in the digests. The same nuclease is also present in fragment p17(pG-UV) of the pancreatic RNase digests (see below). Since the mature glycine and threonine tRNA 5' ends are both pG-C-...-,-, fragment t6(pGp) does not correspond to the 5'-terminal T, in either one of the mature tRNAs. Also, it may be seen in Table I that fragments with the sequences, C-U-C-C-A_{OH} or C-A-C-C-A_{OH}, which correspond to the 3' ends of tRNA^\text{GU}, 5 S RNA, and tRNA^\text{ACUC}, are absent in T, digests of the precursor. Thus, the precursor contains different terminal sequences from those of the corresponding mature tRNAs.

Every T, oligonucleotide found in digests of the mature tRNAs (except the terminal oligonucleotides discussed above) is also found in T, digests of the precursor. Therefore it was possible to assign a tentative sequence to each of the fragments present in T, digests of both the precursor and the mature tRNAs. These sequences were subsequently verified by alkaline hydrolysis, pancreatic RNase digestion, and, for tRNAs or longer, by venom phosphodiesterase digestion after dephosphorylation. Fragments t19a and t19b were only partially resolved on the T, RNase fingerprints as was previously observed in tRNA^\text{GU}, 5 S RNA (31). They were analyzed as a mixture without further separation. Fragments t2a and t2b, C-U-C-A-G- from tRNA^\text{GU}, 5 S RNA, and C-A-C-A-G- from tRNA^\text{ACUC}, were not separated on the fingerprints, and were analyzed separately from the fractionated cleavage products generated in vitro (see below).

Fragments t20u and t24u were each present in variable and fractional molar yield. They were also found consistently in T, RNase digests of the in vitro generated tRNAs as shown in Fig. 5, a and c. The base composition of oligonucleotide t20u is (U-2A-2G-), as determined by alkaline hydrolysis. Pancreatic ribonuclease digestion gave the products, U- and A-A-G-, establishing the sequence of t20u as U-A-A-G-. Apparently, this oligonucleotide is the unmodified fragment of t20 (U-mtA-A-G-) since their yields were variable and totaled 1 mol.

Fragment t24u has the identical base composition as t24 (C-C-U-N-C-U-A-G-G-) when determined by alkaline hydrolysis, conditions which convert the modified nucleotide Ap to Ap (30). The pancreatic RNase digests of t24u were analyzed and compared with that of t24; the only difference observed was that A-A-G- from t24 was replaced by the unmodified fragment, A-A-G-, in t24u. After complete enzymatic digestion to 3'-mononucleotides followed by paper chromatography in

![Image](https://www.ncbi.nlm.nih.gov)
b) FIG. 3. Standard two-dimensional fingerprints of the complete T$_r$-RNase digests of in vitro precursor cleavage products generated by incubation with crude cell extracts as in Fig. 2. a, band S1 RNA; b, diagram of a T$_r$-RNase fingerprint of mature tRNA$_{Thr}$$^{31}$; c, band S2 RNA; d, diagram of a T$_r$-RNase fingerprint of mature tRNA$_{A\text{-}A\text{-}G}$$^{29,30}$. Conditions of digestion and fractionation were as described by Barrell (22). Ionophoresis was from right to left on cellulose acetate at pH 3.5, and from top to bottom on DEAE-paper in 7% formic acid.

Systems a and b (22), the modified nucleotide Np was found in both t24 and t24u, whereas Ap was only present in t24. Because fragments t24 and t24u have the same base composition and are each present in fractional molar yield totaling 0.8 mol, we conclude that t24u has the sequence C-C-U-N-C-U-A-A-G- and represents an undermodified form of t24 (from tRNA$_{A\text{-}A\text{-}G}$$^{31}$, see Ref. 30).

Sequence analyses of T$_r$-RNase fragments unique to the precursor are summarized in Table II. Base compositions and pancreatic RNase digestions of these precursor-specific T$_r$-RNase oligonucleotides established the sequences of t1, t6, t7, and t11. Further experiments needed to determine the nucleotide sequences of fragments t14, t17, t26, and t27 are described below.

The partial sequence of oligonucleotide t14, as determined by alkaline and pancreatic RNase digestion, is (3C-, U-)A-A-G-. The loss of a cytosine in the venom phosphodiesterase digestion on 3'-dephosphorylated t14 established that this oligonucleotide is C-(2C-, U-)A-A-G- (Table II). The sequence was further investigated by combined silkworm nuclease and bacterial alkaline phosphatase digestion (26). The completely dephosphorylated small oligonucleotide products were fractionated on DEAE-paper at pH 3.5, and then sequenced by alkaline hydrolysis and venom phosphodiesterase digestion. Among the products, oligonucleotides with the sequences, C-U-C$_{on}$ and C-C-A-A-G$_{on}$, were found, thus it is concluded that the sequence of t14 is C-U-C-C-A-A-G-.

Oligonucleotide t17 has the composition (2U-, 2C-, A-)G-. The loss of a uridine in the venom phosphodiesterase digestion and the results of the pancreatic RNase digestion establish that this fragment is U-(U-, 2C-)A-G- (Table II). Carbodiimide blocking followed by pancreatic RNase digestion gave the products U-U-C-, C-, and A-G-, which establishes the complete sequence of t17 as U-U-C-C-A-G-.

The sequences of oligonucleotides t26 and t27 could not be determined by analysis of the products from alkali, venom phosphodiesterase, and pancreatic RNase digestion. These two fragments were digested with U$_{2}$ ribonuclease, the products fractionated on DEAE-paper at pH 3.5, and then sequenced by alkaline hydrolysis and venom phosphodiesterase digestion. As shown in Table III, the results verified that t26 is (C-A-, C-C-A-)C-U-U$_{on}$ and t27 is (C-A-, C-C-A-)C-U$_{on}$. Their
complete nucleotide sequences were obtained by partial digestion with venom phosphodiesterase. Each partial digestion product was further analyzed by U₉ and pancreatic RNase digests, and the products from these digestions were in turn analyzed by alkaline hydrolysis and venom phosphodiesterase digestion. The results are summarized in Table IV. Oligonucleotides t₂₆ (C-A-C-C-A-C-U-U₁₁) and t₂₇ (C-A-C-C-A-C-U₉) are found in fractional molar yield and their combined yield is less than 1 mol (0.7 mol). They both contain a free 3'-hydroxyl group and differ only in the presence of a single 3' terminal nucleotide, indicating that they are derived from the heterogeneous 3' ends of the precursor.

Pancreatic RNase Digestion Products—A fingerprint of the complete pancreatic RNase digestion products of the precursor is shown in Fig. 5. Combined data from alkaline and T₉-RNase digestion and, in some cases, from venom phosphodiesterase digestion established the nucleotide sequences of all of the pancreatic RNase fragments (Table V). All of the pancreatic RNase fragments of glycine and threonine tRNAs except their 5'-terminal oligonucleotides, pG-C-, are present in the precursor. The additional oligonucleotides derived from precursor-specific regions were identified and their sequences determined as summarized in Table II.

In vitro Precursor Cleavage with RNase P—Purified [¹⁰⁵P]tRNA⁹⁰⁴-tRNA⁷⁰⁴ precursor was digested with RNase P according to the procedure of Robertson et al. (6) and the cleavage products were fractionated by electrophoresis on 12% polyacrylamide gels. Three products are formed (Fig. 2b): two major bands migrating in the approximate position of mature tRNA (EP1 and EP2), and a fast moving component containing a relatively low amount of radioactivity (EP3). Labeled RNAs from these bands were eluted, and their sequences determined as described below.

Fragment EP3 was digested with T₉ or pancreatic RNase and the digests were fractionated by standard two-dimensional fingerprinting techniques. Four products were obtained from each digest, and the sequential analysis of each product was carried out as summarized in Table VI. The nucleotide sequences of all of the oligonucleotides except t₃₂ are established by the data given in Table VI. Oligonucleotide t₃₂ has the structure, U-(U-, 2C-)A-G-, which matches the composition of fragment t₁₇ obtained from the complete T₉ RNase digest of the precursor (Table I); thus it is concluded that t₃₂ has the sequence, U-U-C-C-A-G-G-A-U...

Two-dimensional Sanger fingerprints of pancreatic and T₉ RNase digests of EP1 and EP2 RNA (not shown) were compared with those of the mature glycine and threonine tRNAs as well as that of the precursor. The oligonucleotides from these digests were studied by enzymatic and alkaline digestion as described above for the sequential analysis of EP3 RNA. It was found that EP2 RNA contains the complete primary sequence of mature tRNA⁹⁰⁴, including the 5'-terminal pG-C- sequence. The only structural difference observed was the presence of the 3'-terminal sequence C-A-C-A-C₉ in threonine.

Fig. 4. Standard two-dimensional fingerprint of a complete T₉ RNase digest of [³²P]tRNA⁹⁰⁴-tRNA⁷⁰⁴ precursor. Conditions for fingerprinting were as described by Barrell (22). Ionophoresis was from right to left on cellulose acetate at pH 3.5, and from top to bottom on DEAE-paper in 7% formic acid. Left, autoradiograph; right, diagram identifying the spots.
TABLE I

$T_1$RNase catalog

The oligonucleotides derived from $T_1$RNase digests of $[^32P]tRNA^{Thr}$, $tRNA^{Thr}$ precursor are numbered as in Fig. 4. Experimental molar yields were obtained by measuring the radioactivity of the paper containing each oligonucleotide in a liquid scintillation counter, and the results are expressed relative to $t12a + t12b = 2.0$. They are averages of five separate experiments. Theoretical molar yields and the number of residues are deduced from the final sequence of the precursor (see text), and the known primary sequences of $tRNA^{Thr}_{30}$ and $tRNA^{Thr}_{31}$ (30) as shown in Fig. 7.

| Oligonucleotide | Sequence | Experimental molar yield | Theoretical molar yield |
|-----------------|----------|--------------------------|-------------------------|
| t1              | G-       | 11.6                     | 11                      |
| t2              | C-G-     | 3.4                      | 3                       |
| t3              | A-G-     | 2.4                      | 2                       |
| t4              | C-A-G-   | 1.3                      | 1                       |
| t5              | C-C-C-G- | 0.8                      | 1                       |
| t6              | G-       | 0.7                      | 1                       |
| t7              | U-G-     | 2.6                      | 2                       |
| t8              | D-A-G-   | 1.2                      | 1                       |
| t9              | C-U-G-   | 3.4                      | 3                       |
| t10             | m$^7$G-U-C-G- | 0.9  | 1 |
| t11             | A-U-G-   | 4.3                      | 4                       |
| t12a            | C-U-C-A-G- | 2.0  | 1 | 1 |
| t12b            | C-A-U-C-G- | 0.8  | 1 | 1 |
| t14             | C-U-C-A-A-A-G- | 1.4  | 1 | 1 |
| t15             | D-D-G-   | 2.3                      | 2                       |
| t16             | T-G-C-G- | 0.7                      | 1                       |
| t17             | U-U-C-C-A-C- | 0.8  | 1 | 1 |
| t18             | A-U-U-C-C-C-C-G- | 0.8  | 1 | 1 |
| t19a            | C-A-C-C-C-U-U-G- | 2.0  | 1 | 1 |
| t19b            | C-C-U-A-A-U-C-A-G- | 1.1  | 1 | 1 |
| t20             | U-m$^6$A-A-G- | 0.8  | 1 | 1 |
| t20u            | U-A-A-A-G- | 0.2  | 0* |
| t21             | A-A-U-C-U-G- | 0.9  | 1 | 1 |
| t22             | A-U-A-U-A-G- | 0.9  | 1 | 1 |
| t23             | U-A-U-A-A-A-U-G- | 1.0  | 1 | 1 |
| t24             | C-U-N-C-U-A-A-U- | 0.3  | 1 | 1 |
| t24u            | C-C-U-N-C-U-A-A-G- | 0.5  | 0* |
| t25             | C-U-A-U-U-U-A-U-U-U-A-U- | 1.0  | 1 | 1 |
| t26             | C-A-C-C-C-U-U-U-U-OH | 0.4  | 1 | 1 |
| t27             | U-A-C-U-U-U-U-U-U-OH | 0.3  | 0* |
| others          | C-A-C-C-A-OH | 0.0  | 0 | 1 |
|                 | U-U-C-C-A-OH | 0.0  | 0 | 1 |

Total number of residues = 170    75    76

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a Oligonucleotides t20u and t24u are the undermodified forms of t20 and t24, respectively, as explained in the text.

b Oligonucleotides t26 and t27 are both derived from the 3' end of the precursor. The heterogeneity at the 3' terminus is discussed in the text.
TABLE II

Analysis of precursor-specific T1 and pancreatic RNase digestion products

T1 and pancreatic digestion products derived from the precursor-specific regions of the precursor molecule as listed in Tables I and V were analyzed by standard alkaline and enzymatic digestions (22).

| Oligonucleotide | Alkaline hydrolysis products | Complementary T1 or pancreatic RNase digestion products | Venom phosphodiesterase digestion products | Deduced sequence |
|-----------------|-----------------------------|---------------------------------|---------------------------------|-----------------|
|                 | Cp  | Ap  | Gp  | Up  | pGp |                  | pC  | pA  | pG  | pU  |
| t1              | -   | +   | -   | -   | -   | G-                | G-  |     |     |     |
| t6              | -   | -   | +   | -   | -   | G-                | G-  |     |     |     |
| t7              | -   | 1.0 | 0.8 | -   | -   | 0.9 U+ 1.0 G-    | U-G |     |     |     |
| t11             | -   | 1.0 | 0.9 | -   | -   | 1.1 A-U+ 1.0 G-  | A-U-G |
| t14             | 3.1 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 A-A-G+ 0.8 U-+ 2.7 C- | 2.1 | 1.8 | 1.0 | 0.8 |
| t17             | 2.1 | 0.9 | 1.0 | 1.0 | 1.0 | 1.0 A-G+ 2.0 C-+ 1.7 U- | 1.8 | 1.1 | 1.0 | 1.1 |
| t26             | +   | +   | -   | +   | +   | [A-C+ C+C+ U+]  | +   |     |     |     |
| t27             | +   | +   | -   | -   | -   | [A-C+ C+]C-  | +   |     |     |     |

a Products were characterized by paper electrophoresis at pH 3.5. The yields were measured by liquid scintillation counting and were expressed relative to the residue shown in 1.0 amount. Venom phosphodiesterase digestion was carried out on fragments which had been dephosphorylated with bacterial alkaline phosphatase. The signs + and - denote the presence or absence of the product as determined by visual inspection of the radioautograph.

b Digestions were performed using T1-RNase for pancreatic fragments and pancreatic RNase for T1 fragments. The products were fractionated on DEAE paper at pH 3.5, and were further analyzed by alkaline hydrolysis.

c Quantitative data on these two oligonucleotides failed to establish their structures and further experiments were required (Tables III and IV).

Venom was replaced by the sequence C-A-C-C-A-C-U(-U,,,,) in EP2 RNA. Thus, the 3'-terminal heterogeneity found in the precursor molecule is preserved in this RNase P generated fragment.

A similar comparison between tRNA\textsuperscript{gly} and EP1 RNA revealed that EP1 RNA contains the complete primary sequence of tRNA\textsuperscript{gly} with the identical 5'-terminal sequence pG-C-, but with a different 3'-terminal structure. Two additional pancreatic fragments, A-A-G-A-U- and G-U,,,,, were found in the pancreatic digest of EP1 RNA. In the T1 RNase digest, the 3'-terminal C-U-C-C-A-U- sequence in mature glycine tRNA was replaced by C-U-C-C-A-A-G- along with an extra mole of A U G. Thus, the structure of EP1 RNA is pG-C- ... C-U-C-C-A-A-G-A-U-G-U,,,,, the complete tRNA\textsuperscript{gly} sequence with six additional nucleotides on the 3'-end.

The nucleotide sequences of the three RNase P cleavage products are summarized below: EP3: pG-U-U-C-C-A-G-G-A-U,,,,, EP1: pG-C- ... (tRNA\textsuperscript{gly}) ... C-U-C-C-A-A-G-A-U-G-U,,,,, EP2: pG-C- ... (tRNA\textsuperscript{gly}) ... C-C-C-C-A-C-U-U,,,,, the complete tRNA\textsuperscript{gly} sequence with six additional nucleotides on the 3'-end.

The termini of the precursor as obtained from the T1 and pancreatic digests are pG-U- and C-A-C-C-A-C-U-U,,,, (Tables I and V), suggesting that the arrangement of these three segments in the precursor is 5'-EP3-EP1-EP2-3'. Thus, the tRNA sequence in the precursor has the order 5' tRNA\textsuperscript{gly} ... tRNA\textsuperscript{gly} ... 3', with precursor-specific nucleotides distributed at both termini of the molecule as well as in the inter-tRNA spacer region (see Fig. 7c).
were isolated. The deduction of their structures from the end products is illustrated in Table VII. The sequences of partial fragments T1, CMP1, and CMP2 were deduced by making use of the known tRNA sequences in the precursor.

Knowledge of the sequences of E6 RNA, tRNA\(^{\text{Glu}}\), and tRNA\(^{\text{Asp}}\) together with the overlap data establishes a unique sequence for the tRNA\(^{\text{Gly}}\)-tRNA\(^{\text{Thr}}\) precursor as shown in Fig. 6. The final sequence of the precursor molecule is presented in Fig. 7c with the tRNA regions arranged in the cloverleaf pattern. The arrows indicate the cleavage sites for the precursor processing enzyme, RNase P, which cleaves specifically at two locations to generate the mature 5' ends of the tRNAs. The precursor molecule consists of 170 nucleotides; 151 are faithful copies of the mature glycine and threonine tRNA sequences, while 19 are precursor-specific. Six precursor-specific nucleotides occur in the inter-tRNA spacer region, with ten on the 5' terminus, and the remaining three on the 3' terminus of the precursor molecule.

**Nucleotide Modifications**—All T\(_r\)-RNase fragments from a fingerprint of the [\(^{32}\)P\]tRNA\(^{\text{Gly}}\)-tRNA\(^{\text{Thr}}\) precursor were digested to mononucleotides with a combination of pancreatic RNase and spleen phosphodiesterase. The digestion products from each fragment were then fractionated by paper electrophoresis at pH 3.5, eluted, and characterized by descending paper chromatography using Systems a and b of Rorrell (22). A total of 11 modified nucleotides were found, which agrees with the previous finding that there are four modified nucleotides in tRNA\(^{\text{Glu}}\)\(^{\text{Glu}}\) (29, 30) and seven in tRNA\(^{\text{Asp}}\) (31). These modified nucleotides are: (numbered from the 5'-end of the precursor molecule) ribothymidylic acid (Tp) at positions 63 and 145, pseudouridylic acid (\(\text{yp}\)) at positions 64 and 146, 5,6-dihydrouridylic acid (Dp) at positions 107, 108, and 111, N\(^7\)-methyl-N\(^6\)-carbamoylthreonyl-2'(3')-adenylic acid (mtAp) at position 128, N\(^7\)-methylguanylic acid (m\(^7\)Ap) at position 137, an unidentified derivative of Up (Np) at position 44, and an unidentified derivative of Ap (Ap) at position 47.

The presence of modified nucleotides, Dp in fragment t8, m\(^7\)Ap in fragment t10, mtAp in fragment t20, Np in fragment t124, and both Np and Ap in fragment t24 revealed by the screening procedure noted above. T\(_r\)-RNase fragment t15 has the sequence D-D-G- (residues 107 to 109). Analysis of the nucleotide composition of this fragment by descending paper chromatography using Systems a and b indicated that the

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**Table III**

**Analysis of U\(_r\)-RNase digestion products from oligonucleotides t26 and t27**

Oligonucleotides t26 and t27 were digested with U\(_r\)-RNase and the products were fractionated by ionophoresis on DEAE-paper at pH 3.5. Each product was analyzed by alkaline hydrolysis and venom phosphodiesterase digestion without dephosphorylation as described in Table VI. Yields were estimated in a scintillation counter and were expressed relative to 1 mol of the 3'-terminal or trinucleotides.

| Alkaline digestion products | Venom phosphodiesterase digestion products | Deduced sequence of U\(_r\)-RNase digestion products | Molar yield |
|-----------------------------|-------------------------------------------|-------------------------------------------------|------------|
| t26:                        |                                           |                                                 |            |
| (a) 2.3 C- + 1.0 A-        | C-C- + 1.0 A-                             | C-C-A-                                         | 0.7        |
| (b) 1.2 C- + 1.0 A-        | -A- only                                  | C-A-                                          | 0.8        |
| (c) 1.0 C- + 1.0 U-        | -U only                                   | C-U-U-                                         | 1.0        |
| t27:                        |                                           |                                                 |            |
| (a) 2.2 C- + 1.0 A-        | 0.8 C- + 1.0 A-                           | C-C-A-                                         | 0.7        |
| (b) 1.2 C- + 1.0 A-        | -A- only                                  | C-A-                                          | 0.7        |
| (c) C- only                | -U only                                   | C-U-U-                                         | 1.0        |

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**Table IV**

**Analysis of venom phosphodiesterase partial digestion products from oligonucleotides t26 and t27**

Oligonucleotides t26 and t27 (Fig. 4) were partially digested with venom phosphodiesterase as described under "Experimental Procedures" and the products were fractionated by ionophoresis on DEAE-paper at pH 3.5. Each product was characterized by pancreatic and U\(_r\)-RNase digests. The compositions of these products were in turn determined by alkaline and venom phosphodiesterase digestion products. The yields were measured by scintillation counting and normalized to C-A-. All U\(_r\)-RNase products were found in equimolar quantity.

| Venom phosphodiesterase partial digestion products\(^a\) | Enzymatic digestion products | Deduced sequence |
|-------------------------------------------------------|-----------------------------|-----------------|
| t26-c                                                 | C-A-                        | C-A-            |
| t26-d                                                 | C-A-                        | C-A-            |
| t26-c                                                 | C-A-, C-A-                 | C-A-A-C         |
| t26-d                                                 | C-A-, C-A-                 | C-A-A-C         |
| t26-b                                                 | C-A-, C-A-                 | C-A-A-C         |
| t26-a                                                 | C-A-, C-A-                 | C-A-A-C         |
| t27-d                                                 | C-A-                        | C-A-            |
| t27-c                                                 | C-A-                        | C-A-            |
| t27-b                                                 | C-A-                        | C-A-            |
| t27-a                                                 | C-A-                        | C-A-            |

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\(^a\) Oligonucleotides are numbered as in Fig. 4 and the letter designates the partial products obtained in order of increasing mobility.

\(^b\) The yields were measured by scintillation counting and normalized to C-A-. All U\(_r\)-RNase products were found in equimolar quantity.

\(^c\) Since the number of A residues in each partial digestion product can be deduced from the information given in the previous column, the yields of the pancreatic RNase products were normalized assuming a molar yield of 1.0 or 2.0 for dinucleotides A-C- or A-C-\(^\text{OH}\), whichever is appropriate. Products that are underlined were present in 2 molar yield.
FIG. 5. Standard two-dimensional fingerprint of a complete pancreatic RNase digest of [32P]tRNA^{cyt}-tRNA^{Thr} precursor. Conditions for fingerprinting were as described by Barrell (22). Ionophoresis was from right to left on cellulose acetate at pH 3.5, and from top to bottom on DEAE-paper in 7% formic acid. Left, autoradiograph; right, diagram identifying the spots.
The primary sequence of a precursor RNA to *Escherichia coli* tRNA\(^{\text{Gly}}\) and tRNA\(^{\text{Thr}}\) has been determined. This precursor, 170 nucleotides in length, contains the complete sequences of mature glycine and threonine tRNAs, including the 5'-terminal -C-C-A triplets, along with 19 additional nucleotides located at the 5' and 3' ends of the molecule as well as between the tRNA sequences. Fig. 7c shows the sequence of the precursor with the tRNA portions arranged in the cloverleaf pattern. Eight additional base pairs could be made between the central single-stranded region and the 5' and 3' terminal segments, to give a more stable secondary structure according to the rules of Tinoco et al. (34). They are (positioned from the 5' end as indicated in Fig. 6) G-G-A (7 to 9) with U-C-C (82 to 84), A-A-G (85 to 87) with C-U-U (168 to 170), and G-U (90 and 91) with A-C (164 and 165). Similarly, in the case of a precursor to *E. coli* tRNA\(^{\text{Thr}}\) of unknown sequence, two additional base pairs can be formed to extend the double-stranded -CCA stem to nine base pairs (2). The exact secondary structure of these precursor RNAs is still unknown, however.

When incubated with a cell-free extract prepared from sonicated *E. coli* cells, the precursor is converted to mature glycine and threonine tRNAs. Purified RNase P, as would be expected from the known mode of action of this enzyme (6), cleaves the molecule specifically at two sites (as indicated in Fig. 7c by the arrows), to generate the mature 5' end sequences of both tRNAs. Since it has been shown that the nuclease that processes the 3' end of tRNA precursors is different from RNase P (6, 33), the presence of extra 3' nucleotides in precursor RNA isolated from an RNase P mutant is somewhat surprising. We have observed the presence of the 3'-terminal extra nucleotides even after prolonged labeling for 2 hours at nonpermissive temperatures. It seems clear that maturation at the 5' end is a prerequisite for complete processing of the 3' end. Conceivably, the presence of the extra segment on the 5' terminus could render the few extra nucleotides at the 3' end inaccessible to the maturation enzymes(s), perhaps by permitting extra base pairs to form as described above.

Under the experimental conditions used, nucleotides found modified in mature glycine and threonine tRNAs are also modified in the precursor molecule. Although the modification at some residues is incomplete, it is clear that modification enzymes are capable of using the precursor RNA as their substrate. The observed undermodification might reflect a lower rate of enzymatic modification for the precursor due to secondary or tertiary structural differences between mature tRNAs and their precursors, as suggested by Schaefer et al. (35).

The terminal-CCA segments of both tRNA\(^{\text{Gly}}\) and tRNA\(^{\text{Thr}}\) of *E. coli* are internally located in the precursor RNA sequence. Therefore these -CCA sequences appear to be transcribed directly from the DNA, as in the case of the precursor to tRNA\(^{\text{Thr}}\) (2). On the other hand, Barrell et al. (4) have shown that a tRNA\(^{\text{Arg}}\)-tRNA\(^{\text{Tyr}}\) precursor specified by bacteriophage T\(_4\) lacks the -CCA sequences for both tRNAs. In this case, the enzyme, tRNA nucleotidyltransferase, is probably responsible for the addition of the terminal -CCA sequences (36). In another di-tRNA precursor (tRNA\(^{\text{Phe}}\)-tRNA\(^{\text{Leu}}\)) specified by bacteriophage T\(_4\), the tRNA\(^{\text{Phe}}\) at the 5' end of the precursor lacks the -CCA, while the tRNA\(^{\text{Leu}}\) sequence at the 3' end of the precursor ends normally in -CCA. At the moment it is not clear what significance, if any, can be attached to the presence or absence of the -CCA sequence in tRNA precursor molecules.

Genetic and biochemical studies have shown that three tRNA genes (glyT thrT tyrT) are located on the small section of the *E. coli* chromosome which is incorporated onto

### Table V

**Pancreatic RNase catalog**

| Oligonuclease | Sequence | Experimental molar yield | Theoretical molar yield |
|--------------|----------|-------------------------|------------------------|
| p1           | U + Ur   | 19.7                    | 11.1                   |
| p2           | U       | 21.3                    | 13.0                   |
| p3           | A-U     | 3.6                     | 1.1                    |
| p4           | C-G     | 7.0                     | 3.2                    |
| p5           | A-U     | 5.6                     | 2.0                    |
| p6           | A-U+C   | 3.3                     | 1.2                    |
| p7           | A-A-U   | 1.4                     | 0.7                    |
| p8           | G-C     | 2.5                     | 1.0                    |
| p9           | G-C-U   | 2.0                     | 1.0                    |
| p10          | G-A-U   | 1.5                     | 0.6                    |
| p11          | G-G-U   | 1.5                     | 0.6                    |
| p12          | A-C-C   | 0.4                     | 0.1                    |
| p13          | A-A-C-C  | 0.6                    | 0.1                    |
| p14          | C-A-A-C  | 1.1                    | 1.1                    |
| p15          | A-G-A-C  | 1.0                    | 1.0                    |
| p16          | A-G-A-A-U | 1.1               | 1.1                   |
| p17          | G-G-U   | 0.6                    | 0.6                    |
| p18          | G-G-U   | 1.2                    | 1.2                    |
| p19          | G-C-C   | 1.9                    | 1.9                    |
| p20          | G-C-C-U  | 0.4                    | 0.4                    |
| p21          | A-G-C-U  | 0.9                    | 0.9                    |
| p22          | G-G-U    | 0.7                    | 0.7                    |
| p23          | A-A-A-G-G-U | 0.8                  | 0.8                   |
| others       | G-C     | 0                      | 0                      |
| pG          | G-U     | 0                      | 0                      |
| pG          | G-C     | 0                      | 0                      |
| pG          | G-G     | 0                      | 0                      |
| Total number | of residues | 170                  | 75                     |

*a* Oligonucleotide p22a is the unmethylated form of p22 as discussed in the text.

*b* Not detected as it contains no 3'-P.
TABLE VI

Products of T1 and pancreatic RNase digestions of EP3 RNA

The T1 and pancreatic RNase fragments generated from [32P]EP3 RNA were analyzed by alkaline hydrolysis, venom phosphodiesterase, and T1 or pancreatic RNase digestions as described in Table II. The yields of the products were estimated by liquid scintillation counting and were expressed relative to the residue shown in 1.0 amount.

| T1 RNase fragment | Composition | | Products of pancreatic RNase digestion | Structure deduced | Molar yield |
|-------------------|-------------|---------------------------|-------------------|-----------------|-------------|
| t31               | -G-         | -G-                      | 0.9               |
| t32               | 1.8 C-, 2.0 U- | 1.8 U-, 2.0 C-          | 1.0               |
| t33               | G-          | G-                       | 1.0               |
| t34               | A-          | A=-U,-                   | 1.1               |

| Pancreatic RNase fragment | Products of complete ribonuclease digestion | Structure deduced |
|---------------------------|---------------------------------------------|-------------------|
| P31                       | 0.9 -G-, 1.0 U-                             | A-G-G-A-U-         | 0.9               |
| P32                       | U-                                           | A-U-G-C-G-         | 1.0               |
| P33                       | C-                                           | A-G-A-G-U-         | 1.7               |
| P34                       | 2.1 A-, 2.0 C-                             | A-G-G-A-U-         | 1.0               |

TABLE VII

Analysis of products of partial enzyme digestions

Partial digestions of [32P]EP3RNA/CtRNA precursor were performed using T1 or pancreatic RNase (c-carboxymethyl lysine-41), and the products were fractionated as described under "Experimental Procedures." The partial products were eluted, and one-half of each was digested with T1-RNase and the other half with pancreatic RNase. The complete digests were fractionated by standard two-dimensional fingerprinting techniques, and the resulting fingerprints were compared with those of the complete precursor (Figs. 4 and 5) for identification. When necessary, additional enzymatic digestions were used to complete the identification. Molar yields were usually determined by visual estimation.

| Fragment | Products of complete ribonuclease digestion | Structure deduced |
|----------|---------------------------------------------|-------------------|
| T1       | A-A-U-C-C-G-, C-U-G-, C-U-C-C-A-A-G-, C-C-C-G-C- | A-A-U-C-C-C-G-C- |
| T2       | A-A-U-C-C-G-C-, C-C-U-A-A-G-, C-C-C-C-C-C-U- | A-A-U-C-C-C-U- |
| CMP1     | C-A-U-G-C-, A-U-G-, A-U-G-, G-C-U-        | A-G-G-A-U-       |
| CMP2     | A-A-U-G-, A-U-G-, U-G-, A-U--           | A-G-G-A-U-       |

a Fragments T1 and T2 were produced by partial T1-RNase digestion, CMP1 and CMP2 were produced by partial digestion using modified pancreatic RNase (c-carboxymethyl lysine-41).

b Underlined products are present in yields of two or more.

c Except for fragment T2, the sequences of these fragments cannot be deduced uniquely from their degradation products without reference to the known mature tRNA sequences (Fig. 7a and 7b). Underlined sequences are derived from precursor-specific regions in the precursor molecule.
Fig. 6. Overlap between the primary sequences of tRNA^{UCA} and tRNA^{GCG}, the sequence of partial digestion products, and the sequence of the 5'-terminal segment, EP3 RNA. Sequences of partial digestion products were deduced as summarized in Table VII. The sequence of EP3 RNA (residues 1 to 10) was determined as indicated in the text. Precursor-specific nucleotides are indicated by a line above the sequences.

Fig. 7. a, nucleotide sequence of mature tRNA^{Gly} (29, 30). The arrows indicate the changes resulting from the glyTsuA36 mutation. N, an unidentified derivative of uridine, is present in a portion of both species. b, nucleotide sequence of mature tRNA^{ACU}, (31); c, nucleotide sequence of the tRNA^{Gly}, tRNA^{Thr} precursor. The arrows indicate the RNase P cleavage sites.

the transducing phage, λh80glyTsuA36 thrT tyrT (17). Wu et al. (28) have mapped these tRNA genes on the transducing phage DNA by hybridizing ferritin-labeled E. coli tRNA to heteroduplexes of λh80glyTsuA36 thrT tyrT DNA with the DNA of the parental phage and scanning the product in the electron microscope. A cluster of three ferritin binding sites (tRNA genes) was seen near the center of the bacterial DNA segment on the transducing phage, separated by spacings of 260 (± 30) and 140 (± 30) nucleotides. Hybridization competition of the ferritin-tRNA with unlabeled purified tRNA^{Gly}, and partially purified tRNA^{Gly} gave the apparent gene order (proceeding left from B-P'): thrT, tyrT, glyT. This gene order is now uncertain, however, since a tRNA^{Gly} tRNA^{Thr} precursor RNA specified by this same phage has been isolated and sequenced.

Although the sequencing data has shown that glyT and thrT
are adjacent on the E. coli chromosome and are transcribed into a single polynucleotide chain, the relative location of tyrT has not been established. Equimolar synthesis of these three tRNAs in induced wild type E. coli cells lysogenic for the defective plasmid λhi80Δgly+TsclA30 (the tyrT gene is located in this region of the chromosome) has been previously demonstrated (17). Under the conditions used in our labeling experiments with RNase P mutant cells, tRNA\textsubscript{tyr} is also amplified in amounts equal to tRNA\textsubscript{thr1}, and tRNA\textsubscript{thr2} is amplified in amounts equal to tRNA\textsubscript{tyr1} and tRNA\textsubscript{thr2} (37). This RNA migrates on the gel with the electrophoretic mobility expected for mature tyrosine tRNA (band A, Fig. 1), and gives a T\textsubscript{1} RNase fingerprint characteristic of completely processed tRNA\textsubscript{tyr1} (32). It is possible that the initial transcript contains all three tRNAs in tandem and the tRNA\textsubscript{thr1}\textsubscript{tyr} is rapidly removed, even at the nonpermissive temperature. Thus, enzymes other than RNase P might be involved in the post-transcriptional processing of multi-tRNA precursors. Such nuclease activities have been observed by Schedl (33), and by Ghysen and Celis (37). Since the tRNA\textsubscript{thr1}\textsubscript{tyr} precursor lacks a 5' terminal nucleoside triphosphate, this molecule seems to be a partially processed intermediate generated by nuclease cleavage(s) from a still longer primary transcript.

Joint transcription of tRNA genes has been observed for six of the bacteriophage T4-coded tRNAs (3). Recently, evidence has been presented indicating that two tandem tRNA\textsubscript{thr1} genes in E. coli are also transcribed together (37). In addition, a large precursor containing several copies of a tRNA\textsubscript{thr1}\textsubscript{tyr} sequence, and a precursor containing multiple tRNA\textsubscript{thr1} sequences have been identified in E. coli strain A49 (Ref. 15). Thus, biosynthesis of tRNA via multi-tRNA precursors seems to be a common feature of bacterial tRNA gene transcription.

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*J. Biol. Chem.* 1975, 250:5542-5555.

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