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# Complete Nucleotide Sequence and Properties of the Major Species of Glycine Transfer RNA from Wheat Germ†

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ABSTRACT: The complete nucleotide sequence of wheat germ $tRNA_1^{Gly}$ has been determined. It is a small tRNA, containing only 74 nucleotides of which 10 are methylated or otherwise modified. Several important features of this tRNA are: (a) a complete absence of ribothymidine which is the most common methylated nucleoside in tRNA; (b) a methylated sugar in the acceptor stem which is a region almost always free of modified residues; and (c) unusually weak base pairing in the dihydrouridine stem region consisting of only one standard A-U base pair in addition to GΨ, AC, and GU. This tRNA functions in a wheat germ cell free protein synthesizing system directed by either natural mRNAs or poly(G,U). The complete nucleotide sequence of wheat germ $tRNA_1^{Gly}$ is: pG-C-A-Cm-C-A-G-U-m$^1$G-G-U-C-Ψ-A-G-D-G-G-U-A-G-A-A-U-A-G-U-A-C-C-C-U-G-C-C-A-m$^5$C-G-G-U-A-C-A-G-A-m$^5$C-m$^5$C-m$^5$C-G-G-G-U-Ψ-C-G-m$^1$A-U-U-C-C-C-G-G-C-U-G-G-U-G-C-A-C-C-$A_{OH}$.

As our knowledge of transfer RNA broadens, it is becoming increasingly apparent that tRNAs are highly sensitive to changes in their primary structure. Although the primary structures of many tRNAs are now known, only very few of these are from higher organisms, and even fewer are from higher plants (Nishimura, 1974). While chromatographing crude wheat germ tRNA on BD-cellulose[1] columns, we became aware of the unusually early elution which was characteristic of the glycine acceptor tRNAs. Since unusual chromatographic properties often mirror unusual structural features, we examined these tRNAs further. We report elsewhere the isolation of each of the glycine tRNAs of wheat germ, their codon response, and their absence of ribothymidine (K. Marcu, D. Marcu, and B. Dudock, in preparation). We report here the isolation and complete nucleotide sequence of the major glycine tRNA of wheat germ and discuss several of its structural features.

## Materials and Methods

Crude wheat germ tRNA containing about 10% ribosomal RNA and 5% 5S RNA was prepared as previously described (Dudock et al., 1969). Wheat germ tRNAs were fractionated by chromatography on BD-cellulose (Gillam et al., 1967) and on RPC-5 columns (Pearson et al., 1971) both run at neutral and acidic pH (Roe et al., 1973).

The preparation of crude aminoacyl-tRNA synthetases from

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[1] Abbreviations used: $A_{260}$ unit, the quantity of material contained in 1 ml of solution which has an absorbance of 1 at 260 nm when measured in a 1-cm light-path cell; m$^1$A, 1-methyladenosine; m$^6$A, 6-methyladenosine; m$^5$C, 5-methylcytidine; Cm, *O*-methylcytidine; m$^1$G, 1-methylguanosine; m$^1$G>, cyclic 1-methylguanosine phosphate; D, dihydrouridine; T or rT, ribothymidine; BD-cellulose, benzoylated DEAE-cellulose; TLC, thin-layer chromatography; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SAM, *S*-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BAP, alkaline phosphatase; SVD, snake venom phosphodiesterase; NMR, nuclear magnetic resonance.

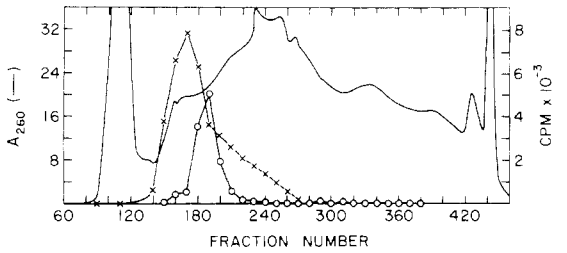


FIGURE 1: Crude wheat germ tRNA (7.5 g; 144 000 $A_{260}$ units) was dissolved in 760 ml of buffer A (10 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM $Na_2S_2O_3$) containing 0.48 M NaCl and applied to a 2.1-l. BD-cellulose column. The RNA was eluted with a linear gradient consisting of 3 l. each of 0.48 M NaCl and 0.60 M NaCl both in buffer A. At tube 200 the remainder of the 0.60 M NaCl solution was removed and replaced by 1100 ml of 0.80 M NaCl in buffer A. When the gradient was complete (tube 300), a high salt wash (2.0 M NaCl, 10% ethanol in buffer A) was used to elute the remainder of the tRNA. The flow rate was 2 ml/min and the fraction size was 18 ml. The RNA eluting in tubes 90–125 was primarily 5S RNA. Glycine was the earliest eluting tRNA found and the glycine containing fractions (tubes 145–205) were pooled. (—) $A_{260}$ per ml; (X) glycine acceptor activity per 25 $\mu$l of column effluent; (O) methionine acceptor activity per 50 $\mu$l of column effluent.

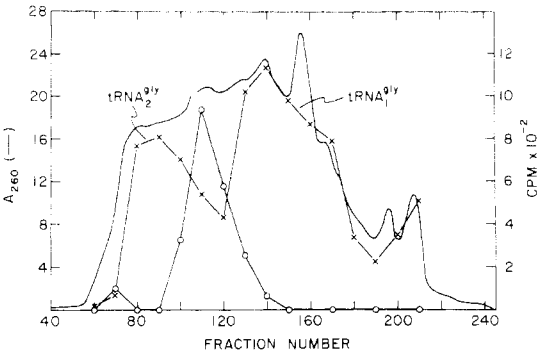


FIGURE 2: Purified wheat germ glycine tRNA (2.0 g; 39 000 $A_{260}$ units) (from Figure 1 and similar columns) was dissolved in 150 ml of buffer B (10 mM Tris (pH 7.0)–10 mM $MgCl_2$–1 mM $Na_2S_2O_3$) containing 0.30 M NaCl and applied to a 1.2 l RPC-5 column. The column was eluted at a flow rate of 3.4 ml/min using a concave gradient consisting of 1.8 l. of 0.30 M NaCl and 1.2 l. of 1.0 M NaCl, both solutions made in buffer B. The fraction size was 17 ml. (—) $A_{260}$ per ml; (X) glycine acceptor activity per 10 $\mu$l of column effluent; (O) methionine acceptor activity per 10 $\mu$l of column effluent.

wheat germ has been described in detail elsewhere (Roe et al., 1973; Marcu and Dudock, 1974).

Wheat germ cell-free protein synthesis extracts were prepared as described (Marcu and Dudock, 1974).

Aminoacylation assays were performed in a final volume of 1 ml and incubated for 30 min at 30 °C with: 50 mM Tris (pH 7.6), 0.5 mM EDTA, 20 mM $MgCl_2$; 2.5 mM ATP (disodium salt), 10 $\mu$M $^{14}$C-labeled amino acid (10 $\mu$Ci/$\mu$mol), 0.01–0.3 $A_{260}$ unit of tRNA and a saturating amount of crude synthetase. Aminoacylation assays were terminated by the addition of 1 to 2 ml of cold 10% $Cl_3CCOOH$. The precipitates were collected on 2.5-cm Whatman GF/A filters (premoistened with 2% $Cl_3CCOOH$), washed five times with 5-ml portions of cold 2% $Cl_3CCOOH$, dried at 150 °C for 15 min, and counted in Omnifluor–toluene.

Precharged wheat germ $tRNA_1^{Gly}$ was prepared for protein synthesis studies in the following manner: 0.02 $A_{260}$ unit of $tRNA_1^{Gly}$ was aminoacylated with [$^3$H]glycine (43 Ci/mmol) essentially as described for the conditions of aminoacylation assays. However, a preparation of crude synthetases free of ribosomes and other tRNAs was used (Marcus, 1972; Allende, 1969) and the amino acid concentration was 0.4 $\mu$M. After 30 min of reaction, essentially all the tRNA was aminoacylated. Crude wheat germ tRNA (200 $\mu$g), 0.5 $\mu$mol of [$^{12}$C]glycine (20-fold excess over [$^3$H]glycine), and 15 $\mu$l of 2 M potassium acetate (pH 4.5) were added to the reaction and immediately followed by phenol extraction. Potassium acetate (300 $\mu$l of 0.1 M) (pH 4.5) saturated phenol was added to the reaction and the mixture shaken for 30 s and centrifuged in a clinical table top centrifuge for 3–5 min. The aqueous phase was removed and reextracted with phenol. The aqueous phase was then precipitated with 2 volumes of cold 95% ethanol. RNA was allowed to precipitate overnight at −20 °C and subsequently collected by centrifugation. The pellet was washed three to five times with cold 95% ethanol and finally stored at −80 °C until needed. The charged tRNA was resuspended in 2 mM potassium acetate (pH 4.5) to maintain stability.

Protein synthesis assays were performed with ~50 ng of [$^3$H]Gly-$tRNA_1^{Gly}$ essentially as described (Marcu and Dudock, 1974) in the presence of various synthetic mRNAs in a wheat germ cell-free protein-synthesizing system (Marcu and Dudock, 1976). The assay was performed in 50 $\mu$l for 60 min at 30 °C, with 7.5 mM MgAc and a 200-fold excess of unlabeled free glycine to prevent deacylation and reacylation of [$^3$H]glycine onto other glycine tRNAs present in the crude protein synthesis extract.

Complete RNase $T_1$ digestions were performed in 50 mM Tris-Cl (pH 7.5) or 50 mM $(NH_4)_2CO_3$ (pH 8.7) with 250 units of RNase $T_1$ per 1 mg of RNA and incubated at 37 °C for 6–8 h. The digestion products were chromatographed on DEAE-cellulose columns run with sodium chloride gradients in the presence of 7 M urea or in ammonium carbonate gradients in the absence of urea as previously described (Katz and Dudock, 1969).

Complete pancreatic RNase digestions were either performed on 0.1 M Tris (pH 7.5) or in 50 mM $(NH_4)_2CO_3$ (pH 8.7) with 50 $\mu$g of pancreatic RNase per mg of RNA and incubated at 37 °C for 8–12 h. When complete, the digestion was diluted by a factor of 2 and then applied to a DEAE-cellulose column equilibrated in $(NH_4)_2CO_3$ buffer.

Wheat germ $tRNA_1^{Gly}$ was prepared for partial $T_1$ RNase digestion either as previously described (Dudock and Katz, 1969) or by exhaustive dialysis. The former procedure involves three successive resuspensions and precipitations of the tRNA sample in an EDTA buffer (50 mM EDTA–0.2 M NaCl). The EDTA was removed in a similar manner with another buffer (50 mM Tris-Cl (pH 7.5)–0.2 M NaCl) and the tRNA finally resuspended in 50 mM Tris-Cl (pH 7.5) for partial digestion. For the dialysis method 20 mg of pure $tRNA_1^{Gly}$ was suspended in 5 ml of a solution of 0.1 M NaCl and 50 mM Tris-Cl (pH 7.5) and dialyzed first against 1 l. of 50 mM EDTA–50 mM Tris-Cl (pH 7.5) and then against 1 l. of 50 mM Tris-Cl (pH 7.5) for 2.5 h, respectively. Subsequently, the tRNA was removed from dialysis and precipitated with 2 parts of cold 100% ethanol. These procedures were required to remove traces of divalent cations possibly bound to $tRNA_1^{Gly}$ which may result in inadequate cleavage by RNase $T_1$ during short incubations at 0 °C. The remainder of the methodology of partial $T_1$ digestion has been described in detail elsewhere (Dudock and Katz, 1969). For chromatography on RPC-5, the sample was added directly to a stainless steel column (equilibrated in 50 mM NaCl–50 mM Tris-Cl (pH 7.5)) through a Milton Roy minipump as previously described (Roe et al., 1973). For fractionation on DEAE-cellulose, 8 M urea was added to the sample to achieve a final concentration of 7 M urea and then it was applied to a glass–DEAE-cellulose column (equilibrated in 50 mM Tris-Cl (pH 7.5)–7 M urea) in portions

with air pressure generated by a peristaltic pump or applied directly through a Milton-Roy minipump.

RNase $T_2$ digestions were performed with 1-2 $A_{260}$ units of an oligonucleotide fragment in 25-50 $\mu$l of 20 mM $NH_4Ac$ (pH 4.5) with 10-20 units of RNase $T_2$ for 8 h at 37 °C. Digestion products were separated by thin-layer chromatography (TLC) on Eastman cellulose chromogram sheets (no. 6064 without fluorescent indicator). Development consisted of isobutyric acid-1 M $NH_4OH$ (5:3, v/v) (solvent A) in the first dimension and 2-propanol-concentrated HCl-$H_2O$ (68:17.1:14.4, v/v/v) (solvent C) for the second dimension as described previously (Katz and Dudock, 1969). Spots were detected with an ultraviolet lamp and were scraped off with a razor blade and eluted with 10 mM HCl for subsequent spectral analysis.

Snake venom phosphodiesterase (SVD) digestions were performed on 1-2 $A_{260}$ units of an oligonucleotide in a 50-$\mu$l reaction containing 40 $\mu$g of SVD, 10 mM $MgCl_2$, 50 mM $(NH_4)_2CO_3$ (pH 8.7) which was incubated at 37 °C for 4 h. Thin-layer chromatography was used to separate the nucleoside from the nucleotides. The solvent system was 1-propanol-concentrated $NH_4OH$-$H_2O$ (7:1:4, v/v/v) (solvent B) in the first dimension and isobutyric acid-1 M $NH_4OH$ (5:3, v/v) (solvent A) in the second dimension as described previously (Katz and Dudock, 1969). Polynucleotide phosphorylase digestions were routinely performed on 3-8 $A_{260}$ units of an RNase $T_1$ fragment. The oligonucleotide was dissolved in 200 $\mu$l of 0.1 M Tris-Cl (pH 8.0) and incubated with 5 $\mu$g of *E. coli* alkaline phosphatase (BAP) at 37 °C. After 1 h of incubation, 200 $\mu$l of 0.1 M potassium phosphate buffer (pH 7-7.2), 50 $\mu$l of 10 mM $MgCl_2$, and 80 $\mu$g of polynucleotide phosphorylase were added followed by an additional incubation of 4-6 h at 37 °C.

Acid rechromatography of oligonucleotide fragments was performed on DEAE-cellulose or RPC-5 columns (Roe et al., 1973) at room temperature unless otherwise noted. DEAE-cellulose buffers consisted of triethylammonium acetate (pH 3.8), ammonium formate (pH 3-3.5), or sodium chloride-formic acid-7 M urea (pH 3-3.5). Nonvolatile buffers were removed by passage through a Bio-Gel P-2 column equilibrated in 100 mM $(NH_4)_2CO_3$ (pH 8.7).

In addition to RNase $T_2$ analysis followed by TLC, base composition studies were also occasionally performed on a stainless steel, jacketed, Aminex A-25 column (Singhal and Cohn, 1973). A digestion of 1-2 $A_{260}$ units of an oligonucleotide was performed in 75 $\mu$l with a combination of SVD, pancreatic RNase, and BAP (20 $\mu$g:5 $\mu$g:10 $\mu$g) in 50 mM Tris-Cl (pH 7.5)-7.5 mM $MgCl_2$ for 3-4 h at 37 °C. A portion of the reaction was applied under pressure with a 10- or 100-$\mu$l syringe through a septum connector mounted on top of the column. The $A_{260}$ profile of the eluate was monitored with a dual wavelength detector.

Dihydrouridine was detected by first spraying a TLC plate with 0.5 M NaOH and then with *p*-dimethylaminobenzaldehyde. The amount of dihydrouridine present was quantitated by the decrease in absorbance observed at 235 nm in 0.1 M NaOH (Molinaro et al., 1968).

Ribonucleases $T_1$ and $T_2$ were purchased from Calbiochem, and pancreatic ribonuclease (RNase A), SVD, and BAP were all obtained from Worthington Biochemical Corp.

[$^3$H]Glycine (43 Ci/mmol), [$^{14}$C]glycine (50 $\mu$Ci/$\mu$mol), and BD-cellulose (50-100 mesh) were purchased from Schwarz/Mann. Poly(G), poly(G,U), and polynucleotide phosphorylase were purchased from Miles.

Plaskon (polychlorotrifluoroethylene) CTFE 2300 grade powder was obtained from Allied Chemical Corp. and Adogen 464 from Ashland Chemical Co.

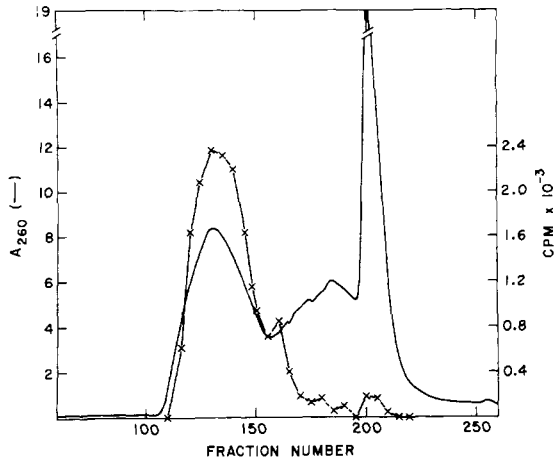


FIGURE 3. Purified $tRNA_1^{Gly}$ (500 mg) (from Figure 2 and similar columns) was dissolved in 100 ml of buffer C (10 mM NaAc (pH 4.5), 10 mM $MgCl_2$, 1 mM $Na_2S_2O_3$) containing 0.35 M NaCl and applied to a 350-ml BD-cellulose column (2 × 110 cm). The column was eluted with a concave gradient of 2.5 l. of 0.35 M NaCl and 1.5 l. of 0.90 M NaCl (both in buffer C). The flow rate was 2.2 ml/min with a fraction size of 17 ml. (—) $A_{260}$ per ml; (X) glycine acceptor activity per 10 $\mu$l of column effluent.

Thin-layer cellulose sheets (20 × 20 cm), no. 6064 without fluorescent indicator, were purchased from Eastman Kodak Co.

Aminex A-25 and Bio-Gel P-2 were obtained from Bio-Rad laboratories.

DEAE-cellulose (Schleicher and Schuell, Inc., Keene, N.H., No. 70 DEAE Standard) was heat treated at 60-70 °C in 0.1 M NaOH for 1 h, washed successively with distilled water, 0.2 M acetic acid, and distilled water and then defined by decantation from distilled water and suspended in column buffer. Columns were packed under pressure with a Milton-Roy minipump and equilibrated with start buffer before use.

Isobutyric acid was purchased from Eastman Organic Chemicals and all other chemicals were obtained from J.T. Baker Chemical Co. and Fisher Scientific Co.

## Results

*Isolation of Highly Purified Wheat Germ $tRNA_1^{Gly}$.* Crude wheat germ tRNA (7.5 g) was chromatographed on a 2.1-l. BD-cellulose column as shown in Figure 1. The major glycine acceptor activity eluted after 5S RNA and was the earliest eluting tRNA found. The purified glycine fractions from this and similar columns were pooled and rechromatographed on RPC-5 at pH 7.0 as shown in Figure 2. This procedure separated $tRNA_1^{Gly}$ from the other major glycine isoacceptor and from most of the methionine acceptance. The $tRNA_1^{Gly}$ from this and similar columns were pooled and a sample was further purified on BD-cellulose at pH 4.5 as shown in Figure 3. This led to highly purified $tRNA_1^{Gly}$ with an acceptor activity of 1.5 nmol per $A_{260}$ unit, which was used for this sequence analysis. The yield was approximately 200 mg of $tRNA_1^{Gly}$ from 7.5 g of crude wheat germ tRNA.

*Complete RNase $T_1$ Digestion.* Wheat germ $tRNA_1^{Gly}$ (>95% pure) was digested to completion with RNase $T_1$ as described in Materials and Methods and applied to a DEAE-cellulose column. The fragments were eluted either with a NaCl gradient containing 7 M urea (Figure 4A) or with two linear gradients of $(NH_4)_2CO_3$ as shown in Figure 4B. Both procedures resulted in similar but not identical resolution, al-

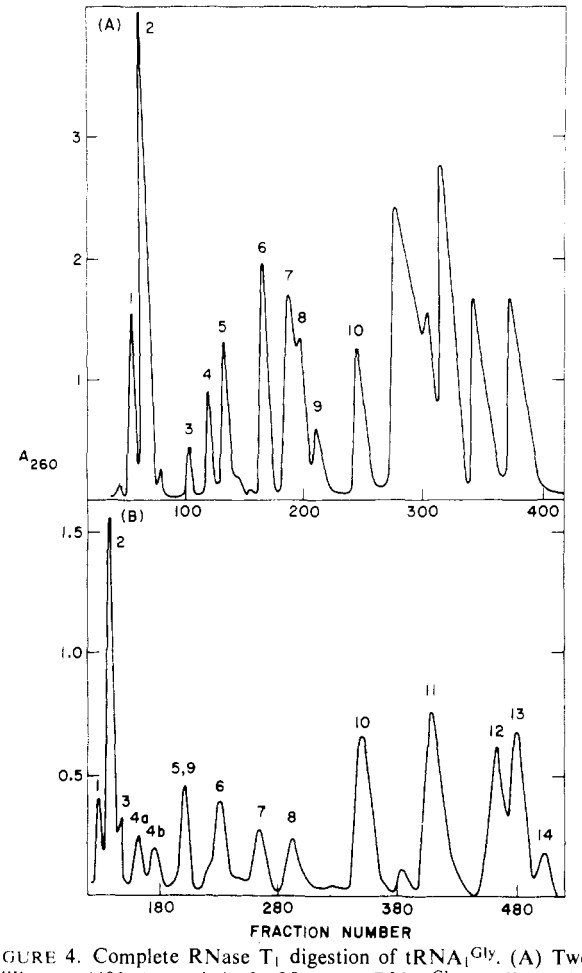


FIGURE 4. Complete RNase $T_1$ digestion of $tRNA_1^{Gly}$. (A) Twenty milligrams (400 $A_{260}$ units) of >95% pure $tRNA_1^{Gly}$ was digested with RNase $T_1$ as described in Materials and Methods and applied to a DEAE-cellulose column (0.3 × 100 cm) after a threefold dilution with start buffer (10 mM Tris (pH 8.0), 7 M urea). The column was eluted with a linear gradient (1000 ml each chamber) of start buffer to 0.3 M NaCl-10 mM Tris (pH 8.0)-7 M urea. The flow rate was 1.3 ml per min with 1.7 ml per tube. (B) Similar to A, except that the DEAE-cellulose column (0.4 × 110 cm) was eluted with two consecutive linear gradients; gradient 1 consisted of 0.015 M $(NH_4)_2CO_3$ to 0.25 M $(NH_4)_2CO_3$ (600 ml each) and gradient 2 consisted of 0.20 M $(NH_4)_2CO_3$ to 0.80 M $(NH_4)_2CO_3$ (400 ml each). The flow rate was 0.9 ml per min with 4 ml per tube.

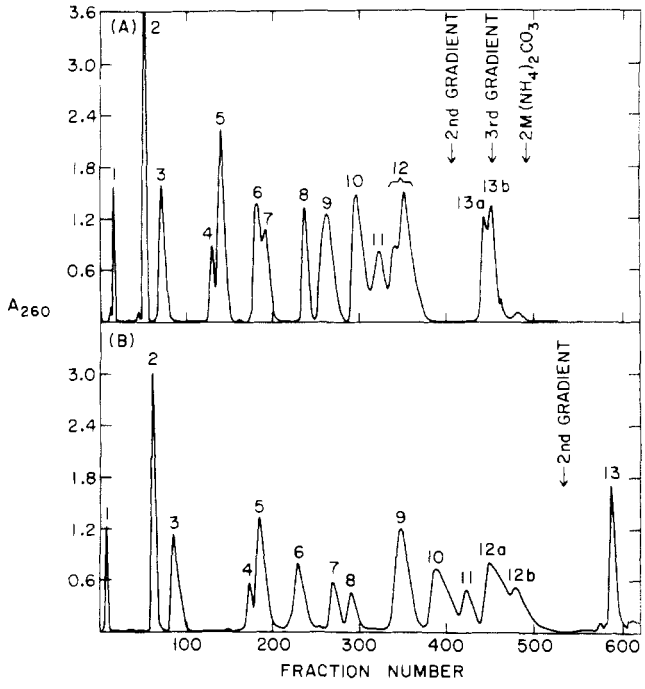


FIGURE 5. Complete pancreatic RNase digestion of $tRNA_1^{Gly}$. (A) Twenty milligrams (400 $A_{260}$ units) of >95% pure $tRNA_1^{Gly}$ was digested with pancreatic RNase as described in Materials and Methods and applied to a DEAE-cellulose column (0.5 × 137 cm) equilibrated with 0.015 M $(NH_4)_2CO_3$ (pH 8.7). A linear gradient of 0.015 to 0.25 M $(NH_4)_2CO_3$ (pH 8.7) (500 ml each chamber) was generated. The column was run with a monostaltic pump at 1.3 ml/min with 2.5 ml/fraction. A second gradient of 0.25 to 0.60 M $(NH_4)_2CO_3$ (50 ml each chamber) was started at fraction 405 and a third gradient of 0.60 to 1.0 M $(NH_4)_2CO_3$ (50 ml each chamber) was begun at fraction 450. A 2 M $(NH_4)_2CO_3$ wash resulted in no recoverable absorbance. The sample recovery was greater than 95%. (B). Another 20 mg of $tRNA_1^{Gly}$ was digested with pancreatic RNase and applied to a DEAE-cellulose column (0.4 × 100 cm) equilibrated with 0.015 M $(NH_4)_2CO_3$. A linear gradient of 0.015 to 0.25 M $(NH_4)_2CO_3$ (1000 ml each chamber) was generated. The column was run with a Milton Roy minipump (100–150 psi) at 1 ml/min and 3.2 ml/fraction. A second gradient of 0.25 to 0.8 M $(NH_4)_2CO_3$ was started at fraction 533.

though the $(NH_4)_2CO_3$ technique was somewhat more amenable to further analysis due to its volatility. Each peak was further purified either by preparative TLC in solvent A or acid rechromatography on DEAE-cellulose as described in Materials and Methods. The identities of the individual nucleotides were determined by their mobilities in solvents A and C and by their acid and base UV spectra. The identities of the individual oligonucleotides and the methods used to determine their sequence are shown in Table I (for additional information on sequence analysis of complete RNase digestion products, see paragraph at the end of this paper concerning supplementary material).

*Complete Pancreatic Digestion.* Wheat gern $tRNA_1^{Gly}$ (>95% pure) was routinely digested for 8–12 h at 37 °C with pancreatic RNase as described in Materials and Methods and applied to a DEAE-cellulose column, and the products were eluted with $(NH_4)_2CO_3$ gradients as shown in Figure 5. Slightly better resolution of particular peaks is noted in each of these digestion patterns and this is probably due to minor changes in the volume and concentration of $(NH_4)_2CO_3$ buffer and to changes in column dimensions. Individual peaks were further purified either by preparative TLC in solvent A or acid rechromatography on DEAE-cellulose as described in Materials and Methods. The identities of the individual oligonucleotides and the methods for their sequence determinations are shown in Table II. Nucleotide analyses were performed via two-dimensional TLC and by UV spectra.

*Parital $T_1$ RNase Analysis of $tRNA_1^{Gly}$.* Wheat germ $tRNA_1^{Gly}$ (>95% pure) was partially digested with RNase $T_1$ for various times at 0 °C and the digestion products were chromatographed either on an RPC-5 column at neutral pH with a NaCl gradient or on a DEAE-cellulose column in the presence of 7 M urea at neutral pH with a NaCl gradient. The results obtained with RPC-5 chromatography of a 27-min partial $T_1$ digestion of 19 mg of $tRNA_1^{Gly}$ have been described previously (Figure 7 of Roe et al., 1973). Specific large oligonucleotides purified by acid rechromatography on RPC-5 were selected for complete $T_1$ digestion analysis and their complete sequences elucidated as shown in Figure 6. In order to obtain additional quantities of other large oligonucleotides, partial $T_1$ digestions of 7- and 1-min duration, respectively, were performed and digestion products fractionated on DEAE-cellulose columns at pressures of 250 psi at neutral pH with NaCl gradients in the presence of 7 M urea as shown in Figure 7. Several large oligonucleotides were selected for further purification at pH 3.5 on DEAE-cellulose in the

TABLE I: Products of Complete RNase $T_1$ Digestion of $tRNA_1^{Gly}$.

| Peak (see Figure 4) | | Analysis[a] | | | | | Molar Yield | |
|---|---|---|---|---|---|---|---|---|
| Column A | Column B | $T_2$ | P | SVD | PP | Sequence | Found | Calcd |
| 1 | 1 | X | | | | U-m$^1$G> | 0.88 | 1 |
| 2 | 2 | X | | | | Gp | 6.9 | 7 |
| 3 | 3 | X | | | | C-[A,C-]$C_{OH}$ | 0.11[b] | 0 |
| 4 | 4 | X | | | | D-Gp | 1.3 | 1 |
| 5 | 5 | X | | | | U-Gp | 1.1 | 1 |
| 6 | 6 | X | | X | | C-[A,C-]C-$A_{OH}$ | 0.81[b] | 1 |
| 7 | 7 | X | | X | | C-U-Gp | 1.0 | 1 |
| 8 | 8 | X | X | | | U-A-Gp | 1.0 | 1 |
| 9 | 9 | X | | | | pGp | 1.3 | 1 |
| 10 | 10 | X | | X | X | U-Ψ-C-Gp | 1.1 | 1 |
| | 10 | X | X | X | X | C-C-A-m$^5$C-Gp | 0.80 | 1 |
| | 10 | X | | X | | A-m$^5$C-m$^5$C-m$^5$C-Gp | 0.80 | 1 |
| | 11 | X | X | X | | U-A-C-A-Gp | 0.92 | 1 |
| | 11 | X | X | X | | C-A-Cm-C-A-Gp | 1.1 | 1 |
| | 11 | X | X | X | X | U-C-Ψ-A-Gp | 0.91 | 1 |
| | 12 | X | X | | | A-A-U-A-Gp | 1.0 | 1 |
| | 12 | X | | | | m$^1$A-U-U-C-C-C-Gp | *c* | 1 |
| | 13 | X | X | X | X | U-A-C-C-C-U-Gp[d] | 1.0 | 1 |
| | 14 | X | | X | X | m$^6$A-U-U-C-C-C-Gp | *c* | 0 |

[a] $T_2$ refers to RNase $T_2$ digestion; P refers to pancreatic RNase digestion; SVD refers to snake venom phosphodiesterase digestion; PP refers to polynucleotide phosphorylase digestion. [b] Presumably peak 3 was observed because of the partial absence of the terminal nucleotide from the 3′ end of the tRNA. The complete sequence of peaks of 3 and 6 was not determined. Since all tRNAs end in C-C-$A_{OH}$, which can be enzymatically added, the sequence of peak 6 is assumed to be C-A-C-C-$A_{OH}$. [c] The ratio of this oligonucleotide containing m$^1$A or m$^6$A varied from one digestion to another, although their combined molar yield was 1.0. [d] To complete the sequence of this fragment, a partial snake venom digestion was performed as previously described (Katz and Dudock, 1969) and the oligonucleotide U-A-C-C-C-$U_{OH}$ was isolated.

Analysis of Partial RNase $T_1$ Fragments of Wheat Germ $tRNA_1^{Gly}$

| Fragment | Purification Figure (Peak) | Complete $T_1$ Products | Sequence |
|---|---|---|---|
| A | 8A (2) | m$^1$A-U-U-C-C-C-Gp + m$^6$A-U-U-C-C-C-Gp (1.0 total)<br>Gp (2.2), U-Gp (1.0), C-A-C-C-$A_{OH}$ (0.9)<br>C-U-Gp (0.9) | m$^1$A-U-U-C-C-C-G-(G-C-U-G-G-U)G-C-A-C-C-$A_{OH}$ |
| B** | 8b (3)* | Gp (2.6),<br>U-Ψ-C-Gp + A-m$^5$C-m$^5$C-m$^5$C-Gp (1.0) | A-m$^5$C-m$^5$C-m$^5$C-G-G-G-U-Ψ-C-Gp |
| C** | 8c (2) | Gp (4.0), U-A-C-A-Gp (0.7), U-Gp (1.3),<br>C-A-C-C-$A_{OH}$ (1.0), C-U-Gp (1.3), U-Ψ-C-Gp (0.7),<br>A-m$^5$C-m$^5$C-m$^5$C-Gp (0.7), m$^1$A-U-U-C-C-C-Gp (0.7) | U-A-C-A-G-A-m$^5$C-m$^5$C-m$^5$C-G-G-G-U-Ψ-C-G-m$^1$A-U-U-C-C-C-G-<br>(G-C-U-G-G-U)-G-C-A-C-C-$A_{OH}$ |
| D | 8A (3) | U-m$^1$G> (1.0), Gp (1.2), pGp (1.2),<br>C-A-Cm-C-A-Gp + U-C-Ψ-A-Gp (0.8 of each) | pG-C-A-$C_m$-C-A-G-U-m$^1$G-G-U-C-Ψ-A-Gp |
| E | 7A (11a)‡* | U-m$^1$G> (.73), Gp (2.3), D-Gp (.76),<br>pGp (1.0), U-A-Gp (.74), U-C-Ψ-A-Gp<br>+ C-A-Cm-C-A-Gp (.73 of each) | pG-C-A-$C_m$-C-A-G-U-m$^1$G-G-U-C-Ψ-A-G-D-G-G-U-A-Gp |
| F | 8B (2a) | U-m$^1$G> (0.8), Gp (2.0), D-Gp (0.8),<br>pGp (1.0), U-A-Gp (1.0), C-A-Cm-C-A-Gp<br>+U-C-Ψ-A-Gp (0.9 of each) A-A-U-A-Gp (0.8) | pG-C-A-$C_m$-C-A-G-U-m$^1$G-G-U-C-Ψ-A-G-D-G-G-U-A-G-A-A-U-A-Gp |
| G | 7B (8) | C-C-A-m$^5$C-Gp (1.0), U-A-C-C-C-U-Gp (1.0) | U-A-C-C-C-U-G-C-C-A-m$^5$C-Gp |
| H | 7B (9) | Gp (1.0), C-C-A-m$^5$C-Gp (1.0), U-A-C-C-C-U-Gp (1.0) | U-A-C-C-C-U-G-C-C-A-m$^5$C-G-Gp |

FIGURE 6. Analysis of partial RNase $T_1$ fragments of wheat germ $tRNA_1^{Gly}$. (‡) This peak was purified by acid rechromatography of Figure 7A (peak 11a) as described in Materials and Methods. (*) The slightly high Gp content is the result of oligonucleotides of defined sequence which are present as minor contaminants in partial molar yield. (**) The purification of fragments B and C have been previously shown. Fragment B is peak 3 of Figure 8b of Roe et al. (1973) and fragment C is peak 2 of Figure 8c of Roe et al. (1973).

presence of 7 M urea. Two such acid rechromatographs are shown in Figure 8 (A and B) of peak 9 (Figure 7A) and peak 11a (Figure 7B). Several peaks located in the rear of the initial neutral, 7 M urea DEAE-cellulose columns (Figure 7A and B) proved to be complex mixtures of partial digestion products upon acid rechromatography and consequently were not studied further (i.e., peaks 10, 12, and 13 of Figure 7A and peaks 11b and 12 of Figure 7B). Peaks 8 and 9 of Figure 7B proved to be homogeneous fragments as judged by complete $T_1$ digestion (see Figure 6 and Figure 9d). Peak 10 of Figure 7B was found to contain the 3′ and 5′ quarters of the tRNA molecule which were also isolated and analyzed from peak 9 in Figure 7A.

The complete nucleotide sequences of these specific large oligonucleotides and others obtained in a similar manner are shown in Figure 6. Large oligonucleotide sequences were determined by complete RNase $T_1$ digestion and individual RNase $T_1$ products were ordered by referring to the data obtained from the complete pancreatic RNase analysis of $tRNA_1^{Gly}$ (Table II), through the information available from

TABLE II: Products of Complete Pancreatic RNase Digestion of $tRNA_1^{Gly}$.

| Peak | Figure | Analysis[a] | | | Sequence | Molar Yield | |
|---|---|---|---|---|---|---|---|
| | | $T_2$ | $T_1$ | SVD | | Found | Calcd |
| 1 | 5A | | | | $A_{OH}$ | 1.0 | 1 |
| 2[b] | 5A & B | | | | Cp | 9.2 | 9 |
| 2[b] | 5A & B | | | | $m^5Cp$ | 1.8 | 2 |
| 3[b] | 5A | | | | Up | 3.0 | 3 |
| 3[b] | 5A | | | | Ψp | 1.6 | 2 |
| 4 | 5A & B | X | | | A-$m^5$Cp | 1.0 | 1 |
| 5 | 5A & B | X | | | A-Cp | 3.2 | 3 |
| 6[c] | 5A & B | X | | | G-Cp | 2.3 | 2 |
| 7[c] | 5A | X | | X | A-Cm-Cp | 1.0 | 1 |
| 8[d] | 5A | X | | X | pG-Cp | 1.0 | 1 |
| 8[d] | 5A | X | | X | G-$m^1$A-Up | 0.9 | 1 |
| 9[c] | 5A | X | | X | $m^1$G-G-Up | 1.1 | 1 |
| 9[c] | 5A | X | | X | A-G-Dp | 1.1 | 1 |
| 10[c] | 5A | X | | X | A-G-Up | 2.0 | 2 |
| 11[c] | 5A | X | | X | G- G-Cp | 1.0 | 1 |
| 12[c] | 5A | | | X | G-G-Up | 2.8 | 3 |
| 12b[e] | 5B | X | X | | A-G-A-$m^5$Cp | 0.8 | 1 |
| 13b[c] | 5A | *f* | | | G-G-G-Up | 0.7 | 1 |
| 13[c] | 5B | X | X | | A-G-A-A-Up | 1.0 | 1 |

[a] $T_2$ refers to RNase $T_2$ digestion, $T_1$ refers to RNase $T_1$, and SVD refers to snake venom phosphodiesterase digestion. [b] This peak was further purified by TLC in solvent A followed by solvent C. [c] This peak was further purified by preparative TLC in solvent A. [d] The two components of peak 8 were separated by acid rechromatography on a DEAE-cellulose column with a 0.04–4.4 M triethylammonium acetate (pH 3.8) gradient. [e] This peak was further purified by acid rechromatography on a DEAE-cellulose column with a 0.1–0.25 M ammonium formate (pH 3.0) gradient. [f] Because of the high G content this oligonucleotide was resistant to RNase $T_2$ digestion and was therefore digested with 0.3 M KOH at 37 °C for 18 h.

previously determined partial RNase $T_1$ fragments and via complete snake venom phosphodiesterase digestion of the original large oligonucleotide where indicated. Complete RNase $T_1$ digestion products were fractionated on a DEAE-cellulose column with an $(NH_4)_2CO_3$ gradient. The identities of complete RNase $T_1$ products were determined in part by their position of elution on the DEAE-cellulose column in $(NH_4)_2CO_3$ buffer which was found to give highly reproducible elution profiles and by either their nucleotide or nucleoside base analysis determined by either RNase $T_2$ treatment followed by two-dimensional TLC or via complete digestion with a mixture of pancreatic RNase, SVD, and BAP (see Materials and Methods) followed by high-pressure Aminex A-25 chromatography at pH 3.75, respectively. Four characteristic RNase $T_1$ digestion profiles are shown for partial $T_1$ digestion fragments A, D, F, and G in Figure 9 (a–d).

The complete nucleotide sequences of partial RNase $T_1$ fragments A–H (see Figure 6) were determined with the following rationale.

Fragment A. The RNase $T_1$ fragment C-A-C-C-$A_{OH}$ was placed at the 3′ terminus since it possesses the 3′ terminal base of the tRNA molecule. The $m^1$A containing fragment was positioned at the 5′ terminus of the fragment due to the release of $m^1A_{OH}$ following SVD digestion of the entire oligonucleotide. Consequently, the nucleotide sequence of fragment A is $m^1$A-U-U-C-C-C-G-(G-C-U-G-G-U-)G-C-A-C-C-$A_{OH}$. The sequence appearing in parentheses cannot be ordered with the available pancreatic RNase data since two other possible internal sequences, namely G-U-G-G-C-U and C-U-G-G-U-G, besides the one shown in parentheses are completely consistent with the presence of the identical pancreatic fragments (G-Cp, G-G-Cp, Up, G-G-Up) and $T_1$ fragments (U-Gp, 2 Gp, C-U-Gp). An analysis of the plausibility of these three sequences in light of the nucleotide sequences of the remaining partial $T_1$ fragments from the tRNA molecule will be discussed later.

Fragment B. Upon methylation of $tRNA_1^{Gly}$ with a ribothymidine forming uracil methyltransferase isolated from *E. coli* MRE 600 and labeled *S*-adenosyl-L-methionine as a methyl donor, two labeled oligonucleotides, T-Ψ-C-Gp and G-G-G-Tp, are isolated following complete $T_1$ and pancreatic RNase digestion of the tRNA molecule, respectively, as previously described (Marcu et al., 1973). From this evidence it is clear that the sequence G-G-G-U-Ψ-C-Gp must exist in $tRNA_1^{Gly}$. Since the fragment G-A-$m^5$Cp is not found in the complete pancreatic RNase analysis and only 1 mol of UΨCGp is isolated upon complete $T_1$ digestion of the tRNA the nucleotide sequence of fragment B is A-$m^5$C-$m^5$C-$m^5$C-G-G-G-U-Ψ-C-Gp.

Fragment C. This fragment contains partial $T_1$ fragments A and B plus U-A-C-A-Gp. Since G-Up is not present in the complete pancreatic RNase analysis while the fragments A-G-A-$m^5$Cp and G-$m^1$-A-Up are present (see Table II), the only possible sequence for fragment C is: U-A-C-A-G-A-$m^5$C-$m^5$C-$m^5$C-G-G-G-U-Ψ-C-G-$m^1$A-U-U-C-C-C-G-(G-C-U-G-G-U-)G-C-A-C-C-$A_{OH}$.

Fragment D. pGp must be at the 5′ terminus of the fragment since it contains the 5′-phosphate moiety of the tRNA molecule and it must be followed by C-A-Cm-C-A-Gp since pG-Cp is present in the pancreatic digestion products and no G-Up is found in the pancreatic analysis (see Table II). Fragment D must also contain $m^1$G in the sequence G-U-$m^1$G-G-U since $m^1$G is present in the fragments U-$m^1$Gp and $m^1$-G-G-Up isolated upon $T_1$ and pancreatic RNase digestion, respectively (Tables I and II). Therefore, the $T_1$ fragments in fragment D can be ordered in the following manner: pG-C-A-Cm-C-A-G-(U-$m^1$G-G-, U-C-Ψ-A-Gp).

Fragment E. This is clearly partial $T_1$ fragment D with the

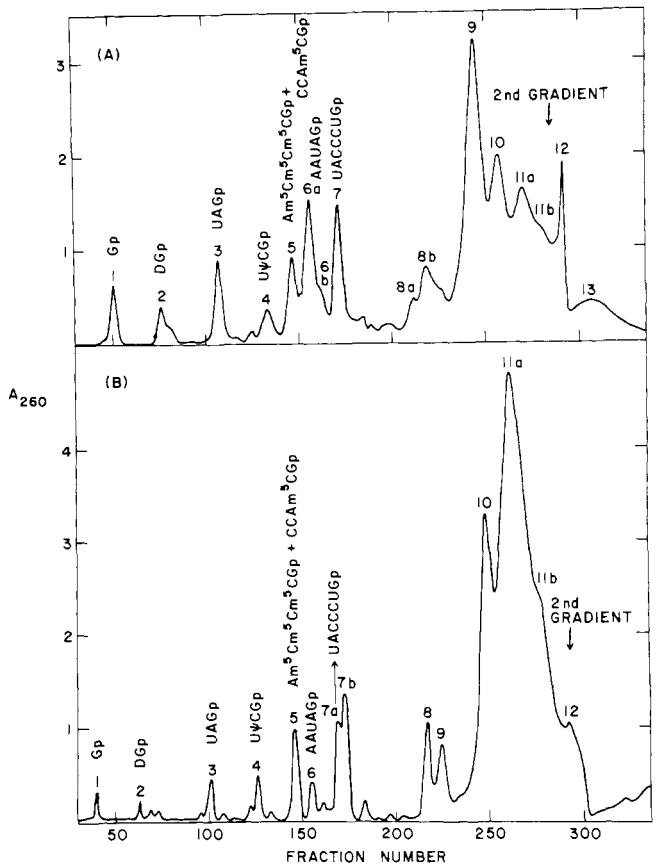


FIGURE 7. Partial $T_1$ RNase digestions of $tRNA_1^{Gly}$ of 7 and 1 min duration. (A) $tRNA_1^{Gly}$ (400 $A_{260}$ units) was subjected to partial $T_1$ RNase treatment for 7 min as described in Materials and Methods. The digested material was applied to a DEAE-cellulose column (0.4 × 105 cm) and two gradients were generated in succession: gradient 1 consisted of buffer D (20 mM Tris (pH 7.6), 7 M urea) with no salt to buffer D containing 0.40 M NaCl, 300 ml each; gradient 2 consisted of buffer D containing 0.40 M NaCl to buffer D containing 0.80 M NaCl, 200 ml each. Gradient 2 started at fraction 286 as indicated. A flow rate of 0.8 ml/min was maintained with a Milton Roy minipump with an overall operating pressure of 250 psi. The fraction size was 2 ml. (B) $tRNA_1^{Gly}$ (480 $A_{260}$ units) was partially digested with $T_1$ RNase for only 1 min at 0 °C. The $T_1$ fragments were fractionated under essentially the same conditions as in A with the following exceptions: 0.42 M NaCl was the upper limit of the first gradient; a second gradient of 0.38 to 0.60 M NaCl both in buffer D was started at fraction 294 as indicated; and the flow rate was 0.74 ml/min.

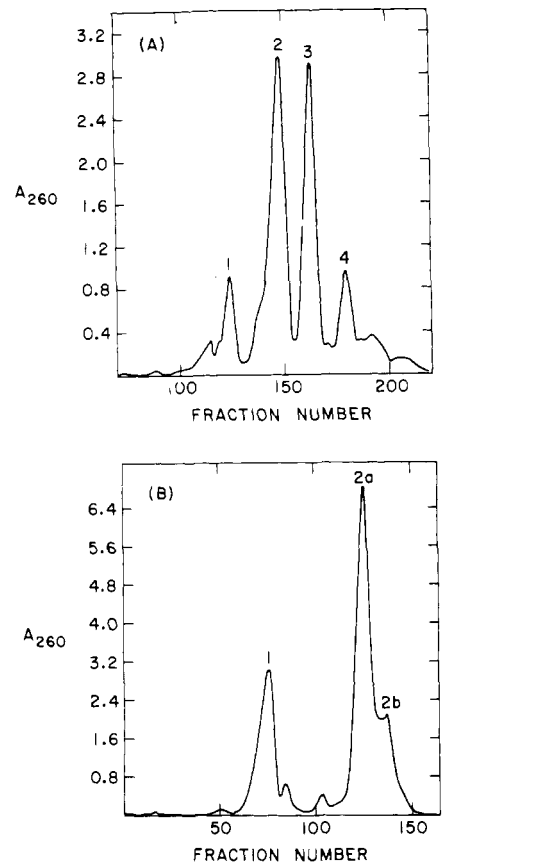


FIGURE 8: Acid rechromatography of large oligonucleotide fragments resulting from partial $T_1$ RNase digestion of $tRNA_1^{Gly}$. (A) Peak 9 (74 $A_{260}$ units) (Figure 7A) was diluted threefold with neutral 7 M urea buffer and loaded onto a neutral 7 M urea DEAE-cellulose column. Following sample application, the column was washed with 20 ml of neutral 7 M urea buffer to remove residual NaCl and then equilibrated with acidic 7 M urea buffer (pH 3.5 HCOOH). A gradient of 7 M urea (pH 3.5 HCOOH) to 0.3 M NaCl + 7 M urea (pH 3.5 HCOOH) (100 ml each) was generated with a Milton Roy minipump at 250 psi. The column was run at 0.6 ml/min with 1 ml/fraction. (B) Peak 11a (Figure 7B) (140 $A_{260}$ units) was fractionated on a 7 M urea (pH 3.5 HCOOH) DEAE-cellulose column essentially as described above with the following changes: a gradient of 0.1 M NaCl + 7 M urea (pH 3.5 HCOOH) to 0.37 M NaCl + acidic 7 M urea buffer (100 ml each) was generated with a flow rate of 0.4 ml/min. Peak 1 was found to contain the same RNase $T_1$ digestion products as peak 2 of Figure 8A. The appropriate peaks were desalted by gel filtration chromatography (see Materials and Methods) for subsequent $T_1$ RNase analysis.

addition of D-Gp, Gp, and U-A-Gp. Therefore, the sequence of fragment E is pG-C-A-Cm-C-A-G-(U-m$^1$G-G-, U-C-Ψ-A-G-) (D-G-, G-, U-A-Gp).

Fragment F. This is obviously fragment E with the addition of A-A-U-A-Gp. Fragment E must end in U-A-Gp in order to generate the pancreatic fragment A-G-A-A-Up (see Table II) since no other pancreatic fragment ends in A-A-Up. The m$^1$G must be in G-U-m$^1$G-G-U- (see discussion of fragment D) and so the sequence of fragment E can now be arranged as pG-C-A-Cm-C-A-G-U-m$^1$G-G-U-C-Ψ-A-G-(D-G-,G-) U-A-Gp and the sequence of fragment F must be pG-C-A-Cm-C-A-G-U-m$^1$G-G-U-C-Ψ-A-G-(D-G-,G-) U-A-G-A-A-U-A-Gp. Since pyrimidine A-G-D-Gp must also be present from the other available complete $T_1$ and pancreatic RNase data on $tRNA_1^{Gly}$, partial $T_1$ fragment F can only be arranged in the following way: pG-C-A-Cm-C-A-G-U-m$^1$G-G-U-C-Ψ-A-G-D-G-G-U-A-G-A-A-U-A-Gp.

Fragments G and H. The only remaining complete $T_1$ fragments are U-A-C-C-C-U-Gp, C-C-A-m$^5$C-Gp, and 1 mol of Gp and these are all found in partial $T_1$ fragments G and H. Since no G-Up is found in the pancreatic analysis, the sequence of fragment G is U-A-C-C-C-U-G-C-C-A-m$^5$C-Gp. This sequence must exist between partial $T_1$ digestion fragments C and F which both entirely encompass the remaining $T_1$ and pancreatic RNase fragments of the tRNA molecule except for 1 mol of Gp. This extra Gp is found in fragment H. The remainder of which is fragment G. Since no A-G-G-Up is found in the pancreatic RNase analysis but 3 mol of G-G-Up are present along with 2 mol of A-G-Up, the sequence of fragment H is U-A-C-C-C-U-G-C-C-A-m$^5$C-G-Gp. This completes the sequence of $tRNA_1^{Gly}$ (i.e., 5′ fragment F-fragment H-fragment C$^{3'}$) as shown in Figures 10 and 11.

*Protein Synthetic Activity of $tRNA_1^{Gly}$ with Synthetic Polymers.* $tRNA_1^{Gly}$ was found to give a 14-fold stimulation in the presence of 10 μg of poly(G,U) in a wheat germ cell-free protein synthesizing system as described in Materials and Methods. No activity was observed with 10 μg of poly(G).

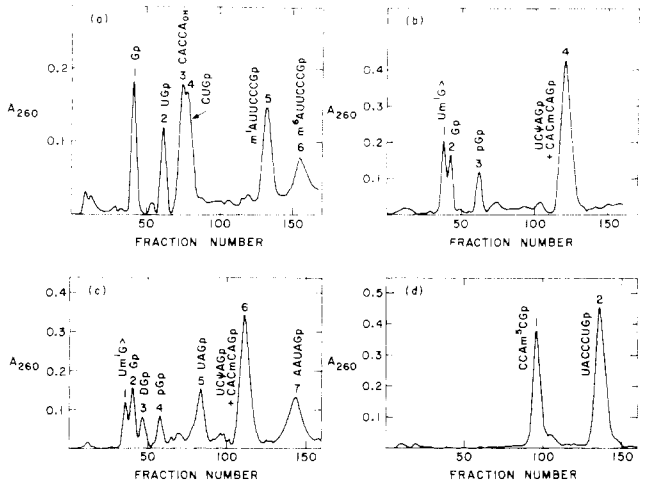


FIGURE 9: Complete $T_1$RNase patterns of partial $T_1$ Fragments A, D, F, and G. (a) Partial $T_1$ fragment A: 5 $A_{260}$ units of peak 2 (Figure 8A) were digested with $T_1$ RNase and applied to a DEAE-cellulose column (0.4 × 55 cm). A linear gradient of 0.05 to 0.5 M $(NH_4)_2CO_3$ (100 ml per chamber) was generated, and the column was run with a Milton Roy minipump (50 psi) at 1 ml min$^{-1}$ fraction$^{-1}$. The following partial $T_1$ fragments D, F, and G were analyzed in the same manner. (b) RNase $T_1$ digestion profile of 8.5 $A_{260}$ units of fragment D. (c) RNase $T_1$ digestion profile of 7 $A_{260}$ units of fragment F. (d) RNase $T_1$ digestion pattern of 7 $A_{260}$ units of fragment G. The identities of the individual peaks are indicated. Fragments were identified as described in Materials and Methods and Results and their molar ratios are shown in Figure 6.

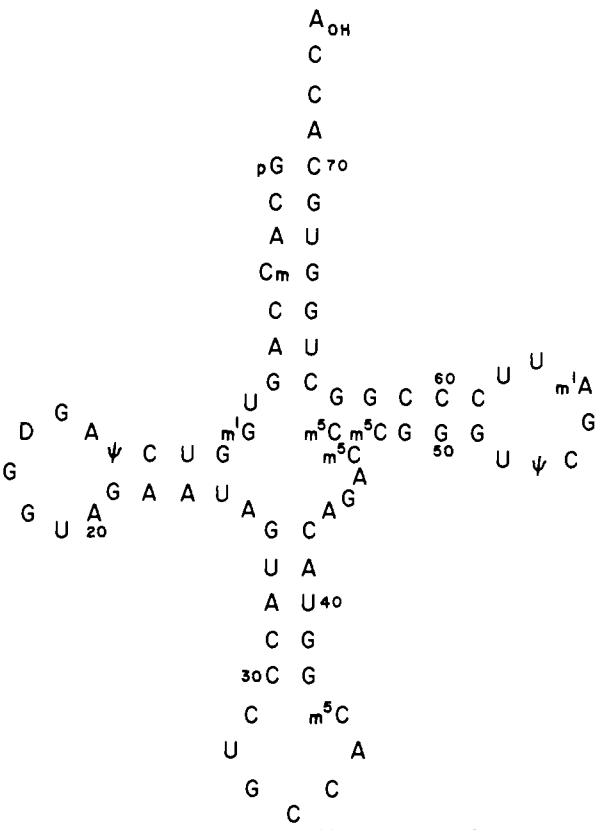

FIGURE 10: The complete nucleotide sequence of wheat germ $tRNA_1^{Gly}$.

## Discussion

The nucleotide sequence of the major glycine tRNA of wheat germ, $tRNA_1^{Gly}$ has been determined. This is a very small tRNA containing only 74 nucleotides, of which ten are methylated or otherwise modified. There is a 2′-*O*-methylcytidine in the acceptor stem which is a region almost always free of base or sugar methylations. This particular modification, 2′-*O*-methylcytidine is also present in the same site in yeast $tRNA_1^{Gly}$ (Yoshida, 1973) There are four 5-methylcytidines, including one in the anticodon loop. Two especially interesting features of this tRNA are the complete absence of ribothymidine and the presence of an unmodified uridine in its place and the extremely poor base pairing in the dihydrouridine stem region.

Wheat germ $tRNA_1^{Gly}$ is an acceptable substrate for *E. coli* glycine tRNA synthetase. The level of heterologous aminoacylation is the same as the level of homologous aminoacylation with crude wheat germ glycine tRNA synthetase, although the kinetics of the aminoacylation reactions have not been studied. The structure of five other glycine tRNAs are known which are acceptable substrates for *E. coli* glycine synthetase: *E. coli* $tRNA_{1,2,3}^{Gly}$, $T_4$ $tRNA^{Gly}$, and *Staphylococcus epidermidis* $tRNA_{1a\&1b}^{Gly}$ (Stahl et al., 1973). Unlike wheat germ $tRNA_1^{Gly}$ these other glycine tRNAs exhibit excellent base pairing in their dihydrouridine stem regions. The only regions in common between these six glycine tRNAs are the first two base pairs of the acceptor stem ($^1$G-C$^{70}$, $^2$C-G$^{69}$) and the two cytidines in the anticodon (see Figure 10). These residues would appear to be part of the *E. coli* glycine tRNA synthetase recognition site.

Part of the nucleotide sequence of the 3′ side of the acceptor stem of $tRNA_1^{Gly}$ cannot be unamibiguously ordered from the available nucleotide sequence information. Two additional possibilities (structures I and II of Figure 12) are consistent with the complete $T_1$ and pancreatic RNase analysis as well as the structure in Figure 10. Two mismatches and one G-U match are present in I and four mismatches and one G-U pair are present in II. Both of these structures would result in a highly distorted acceptor stem for $tRNA_1^{Gly}$, and no tRNA analyzed to date has displayed such excessive loss of base pairs in the acceptor stem region (Nishimura, 1974). The structure shown in Figure 10 has perfect base pairing in the acceptor stem region. In view of this we feel that it is extremely unlikely that either of the alternative structures depicted in Figure 12 is valid for wheat germ $tRNA_1^{Gly}$.

The previous observation (Marcu et al., 1973) that pseudouridine at position 22 from the 3′ end is partially replaced by guanine was not confirmed by further analysis. This observation was based in part on an analysis of the RNase $T_1$ digestion of methylated wheat germ $tRNA_1^{Gly}$. $tRNA_1^{Gly}$ can be quantitatively methylated with *E. coli* rT forming uracil methylase using [$^{14}$C]SAM or [$^3$H]SAM as the methyl donor. Nucleotide sequence analysis showed that the only methylation product was rT and that all of the rT was located at the 23rd position from the 3′ end, which is the normal position occupied by rT in tRNA (Marcu et al., 1973). When this methylated $tRNA_1^{Gly}$ was digested with RNase $T_1$, the expected tetranucleotide T-Ψ-C-Gp was found, and in addition a second product referred to as a "dinucleotide" because of its elution position on DEAE-cellulose, although its actual structure was unknown, was also found (see Figure 2 of Marcu et al., 1973). This "dinucleotide" is routinely found in an RNase $T_1$ digestion of methylated $tRNA_1^{Gly}$ and, under the usual conditions of $T_1$ digestion (see Materials and Methods), corresponds to a molar yield of about 30%. We have studied this observation in some detail and have now found that this dinucleotide is an RNase $T_1$ catalyzed "overdigestion" product of T-Ψ-C-Gp. When methylated $tRNA_1^{Gly}$ is digested with RNase $T_1$, the amount of this overdigestion product was found to vary linearly

Sequence Analysis of Wheat Germ $tRNA_1^{Gly}$

I. $_pGCACmCAGUm^1GGUC\Psi AGDGGUAGAAUAGUACCCUGCCAm^5CGGUACAGAm^5Cm^5Cm^5CGGGU\Psi CGm^1AUUCCCGGCUGGUGCACCA_{OH}$

II. $_pGCACmCAGUm^1GGUC\Psi AG.....UACCCUGCCAm^5CG.....$ . . $.....m^1AUUCCCGGCUGGUGCACCA_{OH}$

$_pGCACmCAGUm^1GGUC\Psi AGDGGUAG.....UACCCUGCCAm^5CGG.....Am^5Cm^5Cm^5CGGGU\Psi CG$ .....

$_pGCACmCAGUm^1GGUC\Psi AGDGGUAGAAUAG.....$ $..GUACAGAm^5Cm^5Cm^5CGGGU\Psi CGm^1AUUCCCGGCUGGUGCACCA_{OH}$

III. $_pG... ...Um^1G.UC\Psi AG..G...AAUAG.....CCAm^5CG.UACAG.$ . . $..G.U\Psi CG$ $.....G...G..CACCA_{OH}$

$.CACmCAG.$ $.G.....DG.UAG.....UACCCUG...$ $..G.....Am^5Cm^5Cm^5CG.G....m^1AUUCCCG.CUG.UG.....$

IV. $_pGC..$ $.AGU$ $...C.AGD...AGAAU...AC.C.GC.Am^5C...AC...$ $.m^5C$ $.GGGU.C.$ $..U.C.GGC.GGU..AC.A_{OH}$

$..ACmC...m^1GGU.\Psi...GGU.....AGU..C.U..C.$ $.GGU..AGAm^5C$ $.m^5C....\Psi.Gm^1AU.C.C...U...GC..C.$

FIGURE 11: (I) The primary structure of wheat germ $tRNA_1^{Gly}$ (74 nucleotides in length) as determined from the nucleotide sequences shown in II, III, and IV. (II) Partial RNase $T_1$ fragments of $tRNA_1^{Gly}$. (III) Complete RNase $T_1$ fragments of $tRNA_1^{Gly}$. (IV) Complete pancreatic RNase fragments of $tRNA_1^{Gly}$.

with digestion time. For short digestions (1 h) the amount of the overdigestion product corresponds to about 5%, the rest of the rT being present in T-Ψ-C-Gp, while in long digestions (24 h) 90% of the rT is present in the overdigestion product and only 10% of the rT remained as T-Ψ-C-Gp. Intermediate digestion times gave intermediate values. Further evidence that the "dinucleotide" is an overdigestion or breakdown product of T-Ψ-C-Gp was obtained by isolating radioactive T-Ψ-C-Gp from an RNase $T_1$ digestion of methylated $tRNA_1^{Gly}$. $^3H$-labeled T-Ψ-C-Gp was divided in half and one part was digested with RNase $T_1$ in $(NH_4)_2CO_3$ as usual (see Materials and Methods) and the other part was subjected to a sham digestion under identical conditions except that no RNase $T_1$ was added. After 6 h of incubation the products were analyzed on a DEAE-cellulose column with an $(NH_4)_2CO_3$ gradient. No overdigestion product was detected in the sham RNase $T_1$ digest while 28% of the rT was found as the overdigestion product in the RNase $T_1$ digest, the remaining rT being present as undegraded T-Ψ-C-Gp.

This overdigestion reaction with T-Ψ-C-Gp was observed with several (at least 5) different lots of RNase $T_1$ enzyme and in the presence of several different buffers including Hepes (pH 7.5), Tris (pH 7.5), and $(NH_4)_2CO_3$ (pH 8.6). Several other wheat germ glycine tRNAs were also methylated as described for $tRNA_1^{Gly}$ (Marcu et al., 1973) and digested with RNase $T_1$ and the products separated on DEAE-cellulose and were also found to give the overdigestion product, although at a considerably slower rate than $tRNA_1^{Gly}$, probably due to the inherent sensitivity of the tRNA to RNase $T_1$ digestion. The chemical nature of the "overdigestion" or breakdown product is unknown.

Wheat germ $tRNA_1^{Gly}$ is active in protein synthesis and responds to poly(G,U) but not to poly(G) which is expected from its $5'GCC3'$ anticodon sequence. Consequently, the absence of ribothymidine (rT) at the 23rd position from the 3′ terminus of the tRNA molecule had no absolute effect on the ability of wheat germ $tRNA_1^{Gly}$ to function in protein synthesis. Indeed, this particular tRNA was found to function more efficiently without rT than with it (Marcu and Dudock, 1976). Wheat germ $tRNA_1^{Gly}$ was methylated with an *E. coli* rT forming uracil methyltransferase and shown to be specifically methylated at the 23rd position from the 3′ terminus resulting in the formation of rT (Marcu et al., 1973). A diminished level of protein synthetic activity was observed for methylated $tRNA_1^{Gly}$ compared with an appropriate sham

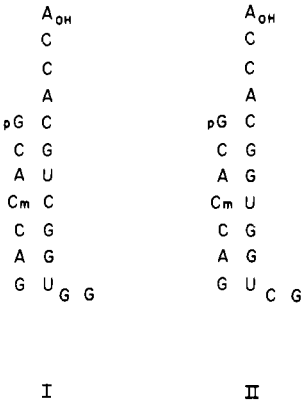


FIGURE 12: Two alternative structures of the acceptor stem region of wheat germ $tRNA_1^{Gly}$.

methylated $tRNA_1^{Gly}$ with natural mRNA in a wheat germ cell-free protein-synthesizing system (Marcu and Dudock, 1976). In addition, this inhibitory phenomenon has been found to occur at the level of the ribosome, after aminoacylation, and is completely reversible by the polyamine, spermine (Marcu and Dudock, 1976). It appears that some specific feature(s) in the structure of $tRNA_1^{Gly}$ allows it to function significantly more efficiently when lacking rT. One possibility is the high 5-methylcytidine content and particularly the 5-methylcytidine in the anticodon loop. Consistent with this is the structure of mouse myeloma $tRNA^{Val}$ (Piper and Clark, 1974) which is the only other eukarotic elongator tRNA of known sequence containing a uridine in place of ribothymidine. This tRNA is also rich in 5-methylcytidine, containing three such residues, including one in the anticodon loop in the same location as is found in wheat germ $tRNA_1^{Gly}$.

Another unusual feature of this tRNA is the poorly base paired dihydrouridine stem region. The absence of base pairing in the dihydrouridine stem of yeast $tRNA^{Asp}$ has been confirmed by NMR studies (Robillard et al., 1976). Additional evidence for the absence of base pairing in the dihydrouridine stem of $tRNA_1^{Gly}$ comes from the partial RNase $T_1$ analysis. For the most part the dihydrouridine loop and stem region are completely degraded even after a short 7-min partial RNase $T_1$ digestion. The amount of U-A-Gp and A-A-U-A-Gp in the 7-min and 1-min partial RNase $T_1$ digestion pattern (Figure 7A and B) indicates the instability of this region to limited RNase $T_1$ digestion.

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Supplementary Material Available

Sequence analysis of complete RNase $T_1$ and pancreatic RNase digestion products of wheat germ $tRNA_1^{Gly}$ (11 pages). Ordering information is available on any current masthead page.

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# Circular Dichroism Studies of Angiotensin II and Analogues: Effects of Primary Sequence, Solvent, and pH on the Side-Chain Conformation†

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ABSTRACT: Conformational aspects of the pressor hormone angiotensin II and 11 of its structural analogues were studied by circular dichroism. Each position of the peptide was singly substituted with an aliphatic residue and alterations of the CD spectra of the resulting analogues in the peptide and aromatic spectral regions (320–250 nm, 250–190 nm) were examined. The spectra of these peptides in 2,2,2-trifluoroethanol solution permit estimation of the relative importance of the various side chains in maintaining the backbone conformation of the hormone. The evolution of the CD spectra in both spectral regions of the peptides in aqueous solution during a titration from pH 1 to pH 12 makes it possible to elucidate further the role of ionizable groups and their interaction with aromatic amino acids such as tyrosine. The results obtained indicate that substitutions in aspartic acid 1, proline 7, and phenylalanine 8 of angiotensin II entail changes in the backbone conformation. On the other hand, the side chains of valine 3, isoleucine 5, and the biologically essential histidine 6 serve mainly to correctly align the phenolic ring of tyrosine in position 4.

In previous papers from our laboratory (Fermandjian et al., 1971a,b, 1976; Greff et al., 1976) we have pointed out the usefulness of circular dichroism measurements for obtaining an overall picture of the conformation of the peptide hormone angiotensin II, its truncated peptides and several of its analogues. Moreover, a recent study showed that CD can give even more detailed information about the relative importance and the function of the side chains in the peptide (Lintner et al., 1975).

Angiotensin II (Asp-Arg-Val-Tyr-(Val or Ile)-His-Pro-Phe) is an extremely potent pressor peptide and has been studied

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