Large Oligonucleotide Sequences in Wheat Germ Phenylalanine Transfer Ribonucleic Acid

DERIVATION OF THE TOTAL PRIMARY STRUCTURE*

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SUMMARY

Large oligonucleotide fragments obtained by partial digestion of wheat germ phenylalanine transfer RNA (tRNA\(^{\text{Phe}}\)) with ribonuclease T\(_1\) and pancreatic ribonuclease have been separated and characterized. The information provided by these analyses and those described in the preceding paper has permitted the derivation of the complete primary structure of tRNA\(^{\text{Phe}}\).

The nucleotide sequence analysis of tRNA has required (a) identification of fragments isolated from complete digestion with pancreatic RNase and RNase T\(_1\) and, in addition, (b) the analysis of large oligonucleotide fragments produced by partial digestion with RNase T\(_1\) (1-4) and, in some cases, partial digestion with pancreatic RNase (5). In continuation of the work reported in the preceding paper (6), this paper reports in detail the conditions of controlled cleavage of tRNA\(^{\text{Phe}}\) with RNase T\(_1\) and pancreatic RNase and the separation and analysis of the large oligonucleotide fragments. The nucleotide sequence of tRNA\(^{\text{Phe}}\) is deduced from overlaps among the large oligonucleotides produced.

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The abbreviations used are: tRNA\(^{\text{Phe}}\), wheat germ phenylalanine transfer RNA; diHU, 5,6-dihydrouridine; 2'OMeG, 2'-O-methylguanosine; 2MeG, N\(_2\)-methylguanosine; diMeG, N\(_2\)-dimethylguanosine; 7MeG, 7-methylguanosine; lMeA, 1-methyladenosine; BMeA, g-methylalanine transfer RNA; diHU, 5,6-dihydrouridine; 2'OMeC, 2'-O-methylcytidine; 2'0MeG, 2'-O-methylguanosine; 2MeG, N\(_2\)-methylguanosine; diMeG, N\(_2\)-dimethylguanosine; 7MeG, 7-methylguanosine; lMeA, 1-methyladenosine; BMeA, 8-methyladenosine; Yw, a fluorescent nucleoside of undetermined structure; p and -, used interchangeably for a phosphate residue.

A detailed comparison of the sequence of wheat germ tRNA\(^{\text{Phe}}\) and yeast tRNA\(^{\text{Phe}}\) (4) has been presented elsewhere (7).

MATERIALS AND METHODS

General Methods—Isolation of wheat germ tRNA\(^{\text{Phe}}\) and the methods of separation and identification of oligonucleotides have been described in the preceding paper (6).

Fluorescence was measured at room temperature in an Aminco-Bowman spectrophotofluorometer with a mercury-xenon arc lamp and an RCA IP-28 photodetector. Fluorescence emission was measured at 440 \(\mu\)m upon excitation at 310 \(\mu\)m. The fluorescence of tRNA\(^{\text{Phe}}\) and its significance are discussed elsewhere (8).

Digesting of Oligonucleotides—Urea and salt were separated from the pooled oligonucleotide peaks by passage through columns (2.6 x 75 cm) of polyacrylamide gel (Bio-Gel P-2) (9), in a buffer of 0.01 M ammonium carbonate. The effluents from chromatography performed at acidic pH were neutralized before passage through the Bio-Gel columns. The recovery of nucleotide material was nearly quantitative in all cases. The effluent from the Bio-Gel column containing the nucleotidic material in 0.01 M ammonium carbonate was frozen and evaporated to dryness. The residue was taken up in a small volume of water, frozen, and reevaporated to dryness. If any residue still remained it was dissolved in a few drops of 1 M acetic acid and evaporated to dryness. This last step was repeated if necessary.

Partial Digestion of tRNA\(^{\text{Phe}}\) with RNase T\(_1\)—In preliminary experiments, approximate conditions for degradation of tRNA\(^{\text{Phe}}\) to yield a high proportion of large fragments were established. A typical degradation of tRNA\(^{\text{Phe}}\) carried out on a large scale and based upon these preliminary findings is as follows. tRNA\(^{\text{Phe}}\) (1200 O.D. units, 1 O.D. unit being defined as that amount of material per ml of solution which produces an absorbance of 1 in a 1 cm light path cell at 260 nm) was dissolved in 20 ml of a solution of 0.05 M Tris pH 7.5, and kept at 0°C. To this solution were added 20,000 units of RNase T\(_1\), 20,000 units of RNase T\(_1\), and 1.000 units of RNase T\(_1\), and permitted to stand for 1 hour in the freezer, and then centrifuged. The residue was redissolved and reprecipitated once more, then allowed to stand for 1 hour in the freezer, and then centrifuged. The residue was redissolved and reprecipitated once more, then allowed to stand for 1 hour in the freezer, and then centrifuged. The solution was then passed through a Bio-Gel filter (2.6 x 75 cm) of polyacrylamide gel (Bio-Gel P-2) (9), in a buffer of 0.01 M ammonium carbonate. The effluents from chromatography performed at acidic pH were neutralized before passage through the Bio-Gel columns. The recovery of nucleotide material was nearly quantitative in all cases. The effluent from the Bio-Gel column containing the nucleotidic material in 0.01 M ammonium carbonate was frozen and evaporated to dryness. The residue was taken up in a small volume of water, frozen, and reevaporated to dryness. If any residue still remained it was dissolved in a few drops of 1 M acetic acid and evaporated to dryness. This last step was repeated if necessary.
Structure of Wheat Germ Phenylalanine tRNA

Large Oligonucleotide Fragments

\[
\begin{align*}
\text{2Me} & \quad \text{DiMe} & \quad \text{OMe} & \quad \text{OMe} & \quad \text{Ime} \\
\text{pGCGGGAUAGCUCAGUGGAGAGCGUCGAAGCUAACGUGCUGGTAACAGAACUCACCGCUCACCCGACCAOH} & \\
\end{align*}
\]

2Me OMe OMe DiMe (33)

GAGAGGCGCACUGAGAGCUAGGUGCUGUGI (34)

OMe DiMe OMe DiMe 7Me H (30)

GAGAGGCGCACUGAGAGCUAGGUGCUGUGI (34)

GAGAGGCGCACUGAGAGCUAGGUGCUGUGI (36)

Peak 29, Fig. 2. . . . 3
Peak 30, Fig. 2. . . . 5
Peak 33, Fig. 2. . . . 12
Peak 34, Fig. 2. . . . 7
Peak 36, Fig. 2. . . . 10

and the mixture incubated for 40 min at 0°. The reaction was stopped by the addition of 2 ml of phenol, which had been previously saturated with 0.05 M Tris pH 7.5. The phenol-water solution was vigorously mixed with a Pasteur pipette and centrifuged, and the phenol layer was discarded. The aqueous layer was similarly extracted four more times with phenol and then freed of phenol by three extractions with water-saturated ether. Remaining ether was removed with a stream of filtered air. Solid urea was added to a final concentration of 7 M, and the solution was applied to a DEAE-cellulose column.

Partial Digestion of tRNA\textsubscript{Phe} with Pancreatic RNase—tRNA\textsubscript{Phe} (22.5 mg) was dissolved in 3.0 ml of 0.02 M magnesium acetate-0.1 M Tris, pH 7.5. The solution was brought to 0° and digested with 50 µg of pancreatic RNase for 1 hour at 0°. The digest was then extracted four times with phenol, followed by four extractions with water-saturated ether. After removing the last traces of ether with a stream of filtered air, solid urea was

Table I

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Elution pattern shown in Fig.</th>
<th>Range of sodium chloride concentration used</th>
<th>Total gradient volume</th>
<th>Fraction volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 29, Fig. 2. . . .</td>
<td>3</td>
<td>0-0.32</td>
<td>400</td>
<td>2.9</td>
</tr>
<tr>
<td>Peak 30, Fig. 2. . . .</td>
<td>5</td>
<td>0.05-0.32</td>
<td>400</td>
<td>2.9</td>
</tr>
<tr>
<td>Peak 33, Fig. 2. . . .</td>
<td>12</td>
<td>0-0.34</td>
<td>700</td>
<td>2.3</td>
</tr>
<tr>
<td>Peak 34, Fig. 2. . . .</td>
<td>7</td>
<td>0.06-0.24</td>
<td>600</td>
<td>2.5</td>
</tr>
<tr>
<td>Peak 36, Fig. 2. . . .</td>
<td>10</td>
<td>0.09-0.25</td>
<td>700</td>
<td>3.0</td>
</tr>
</tbody>
</table>
added to a final concentration of 7 M and the solution was applied to a DEAE-cellulose column.

Separation of Large Oligonucleotide Fragments on DEAE-cellulose Columns—All chromatography on DEAE-cellulose columns, (chloride form) was carried out in the presence of 7 M urea (10) at neutral or acidic pH with a linear gradient of sodium chloride. Columns were packed as a thin slurry with carefully washed and defined DEAE-cellulose (Schleicher and Schuell, No. 70) as previously described (6).

**Large Oligonucleotides from Partial Digestion with RNase T1**

![Graph 1](image1)

**TABLE II**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Identity</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C-A-C-C-A</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>1MeApC-C-A-C-C-G</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>C-U-C-A-C-C-G</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*The values used for molar extinction coefficients have been reported (9).*

![Graph 2](image2)

**TABLE III**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Identity</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-A-G</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>C-diMeG-C-A-G</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Cleavage of the phosphodiester bond between diMeG and $\psi$ with RNase T1 did not occur with the small amounts of enzyme (40 units) used in this study (6, 11).

* Peak 4 differs from Peak 3 by excision of the base of Yw. During chromatography at acidic pH of any fragment containing Yw, some or all of the base of Yw is excised from the fragment by mild acid hydrolysis (6, 12). The resulting oligonucleotide differs from starting material in chromatographic mobility and its lack of fluorescence.*

![Graph 3](image3)

**FIG. 7 (top left).** Elution pattern obtained upon rechromatography of Peak 34, Fig. 2, at pH 3.0 (details in Table I). Peak 34b differed from Peak 34a only in the loss of the base of Yw from the fragment. See Footnote b to Table III. The latter part of the gradient is not shown.

**FIG. 8 (top right).** DEAE-cellulose column (0.3 x 50 cm) chromatography of complete RNase T1 digest of Fragment 34, Fig. 7 (6 O.D. units). Elution was with a linear gradient (total volume 350 ml) of sodium chloride (0 to 0.30 M) in 0.02 M Tris (pH 7.5)-7 M urea.
This is Fragment 34 in Fig. 1. The central part of this fragment, from C-diMeG-... to A-A-G has already been sequenced (see Fragment 30). The isolation in a complete pancreatic RNase digest of tRNA\textsubscript{Phe} of an oligonucleotide G-G-A-G-A-G-A-G-C places the G-A-G-A-G-sequence at the 5' end of Fragment 34. Similarly, the isolation of the complete pancreatic RNase fragment G-A-G-A-G-TMeG-diHU-C-G-C-G-U determines the position of the oligonucleotide TMeG-diHU-C-G in Fragment 34. The remaining two peaks, the dinucleotides C-G- and U-G- were placed at the 3' end of Fragment 34 to the right of 2'0MeG, only after analysis of the partial pancreatic RNase fragment (a) (Fig. 1), which is discussed in detail below. To determine the relative positions of the dinucleotides C-G- and U-G- at the 5' end of Fragment 34, the following experiment was performed. It was reasoned that a partial RNase T\textsubscript{1} digest of Fragment 34 must necessarily yield more of the terminal dinucleotide than penultimate dinucleotide (the terminal dinucleotide can be released without also releasing the penultimate dinucleotide, but the reverse is not true; i.e., the penultimate dinucleotide can not be released without also releasing the terminal dinucleotide). Thus Fragment 34 was subjected to partial RNase T\textsubscript{1} digest and the amount of C-G- and U-G- (both cyclic and noncyclic) were carefully determined. The exact digest conditions are as follows: 13 to 16 O.D. units of Fragment 34 were dissolved in 0.3 ml of 0.01 M magnesium chloride, 0.02 M Tris, pH 7.5. The incubation mixture was then diluted to 0.2 ml and digested with 160 units of RNase T\textsubscript{1} for 5 min. The reaction mixture was then extracted three times with 0.2 ml of ether. Solid urea was brought to 0.02 M, and digested with 160 units of RNase T\textsubscript{1} for 5 min. The reaction mixture was then extracted three times with 0.2 ml of ether, and the last traces of ether were removed by a stream of filtered air. Solid urea was brought to 0.02 M, and the solution was chromatographed on a DEAE-cellulose column (Fig. 9). Peak 1, Fig. 9, was a mixture of cyclic dinucleotides, and Peak 2, Fig. 9, contained a mixture of noncyclic dinucleotides. In each case the ratio of U-G- to C-G- was determined. The data, as summarized in Table V, indicate that U-G- is the 3'-terminal dinucleotide of Fragment 34, and C-G- is the 3'-penultimate dinucleotide.

When column chromatography was performed at acidic pH, the 7 M urea solution was adjusted to pH 3.0 with concentrated hydrochloric acid. Urea solutions were used within 1 week of their preparation.

**Fig. 10 (left).** Elution pattern obtained upon rechromatography of Peak 36, Fig. 2, at pH 3.0 (details in Table I). The latter part of the gradient is not shown.

**Fig. 11 (right).** DEAE cellulose column (0.3 X 50 cm) chromatography of complete RNase T\textsubscript{1} digest of Fragment 36, Fig. 10 (0.0 M units). Elution was with a linear gradient (total volume, 250 ml) of sodium chloride (0 to 0.50 M) in 0.02 M Tris (pH 7.5)-7 M urea.

**Table IV**

Chromatographic analysis of RNase T\textsubscript{1} digest of Fragment 34 (Fig. 8)

<table>
<thead>
<tr>
<th>Run 1</th>
<th>Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 G-</td>
<td>1.0</td>
</tr>
<tr>
<td>1 C-G-</td>
<td>1.0</td>
</tr>
<tr>
<td>2 U-G-</td>
<td>1.0</td>
</tr>
<tr>
<td>3 A-G-</td>
<td>2.1</td>
</tr>
<tr>
<td>4 A-A-G</td>
<td>1.0</td>
</tr>
<tr>
<td>5 7MeG-diHU-C-G-</td>
<td>0.8</td>
</tr>
<tr>
<td>6 C-diMeG-ψ-C-A-G-</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Differs from Peak 8 by excision of the base of Yw (see Table III).

**Table V**

Dinucleotide ratio (U-G-:C-G-) from partial RNase T\textsubscript{1} digest of Fragment 34 (Fig. 8)

<table>
<thead>
<tr>
<th>Run 1</th>
<th>Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of cyclic dinucleotides, U-G-:C-G-</td>
<td>1.95 1.60</td>
</tr>
<tr>
<td>Ratio of noncyclic dinucleotides, U-G-:C-G-</td>
<td>1.79 1.50</td>
</tr>
</tbody>
</table>

* Differs from Peak 12 by excision of the base of Yw (see Table III).
RESULTS AND DISCUSSION

The nucleotide sequence of tRNA^phe^ and the large oligonucleotide fragments essential for its elucidation are shown in Fig. 1.

Fig. 2 shows the pattern obtained when a partial RNase Tₐ digest was chromatographed on a column of DEAE-cellulose with a gradient of sodium chloride in 7 M urea. Since tRNA^pha^ contains the fluorescent residue Yw (6) it was possible to locate fragments containing Yw by reading the fluorescence of column fractions. Comparison of the absorption and fluorescence patterns aided in deciding which fractions were to be pooled.

Pooled peaks were rechromatographed on DEAE-cellulose at pH 3.0, which usually gave fragments homogenous enough for further analysis. The conditions for rechromatography of pooled peaks from partial RNase Tₐ digestion are summarized in Table I.

**Large Oligonucleotide Fragments from Partial Pancreatic RNase Digestion**

The elution pattern obtained upon chromatography of a partial pancreatic RNase digest of tRNAPhe on DEAE-cellulose is shown in Fig. 14. The brackets show those fractions which were pooled for rechromatography at acidic pH. Fig. 15 shows the rechromatography of the partial pancreatic RNase digest on DEAE-cellulose at pH 3.0 (details in Table I).

**Table VII**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Identity</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>G-</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>C-G-</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>pG</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>A-U-A-2MeG-C-</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>C-U-C-A-G-</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Fig. 14 (left).** Chromatography of a partial pancreatic RNase digest of tRNA^pha^ on column (0.4 × 90 cm) of DEAE-cellulose. Elution was performed with a linear gradient of sodium chloride; the mixing chamber contained 200 ml of 7 M urea-0.02 M Tris (pH 7.3)-0.15 M sodium chloride; the reservoir chamber contained 200 ml of 7 M urea-0.02 M Tris (pH 7.3)-0.50 M sodium chloride; 2-ml fractions were collected.

**Fig. 15 (right).** Elution pattern obtained upon rechromatography of the pooled fraction (see Fig. 14) from the partial pancreatic RNase digest of tRNA^pha^, Peak a (Fig. 15) was digested with RNase Tₐ and chromatographed on a DEAE-cellulose column (8-OD, units). Elution was with a linear gradient (total volume 200 ml) of sodium chloride (0 to 0.3 M) in 0.02 M Tris (pH 7.5)-7 M urea. Fraction size was 2.1 ml.

DEAE-cellulose at pH 3.0. Peak a (Fig. 15) was digested with RNase Tₐ and chromatographed on a DEAE-cellulose column (Fig. 16). Table VIII lists the products identified from Peak a, Fig. 15.

**General Comments on Structure of tRNA^pha^**

The nucleotide sequence deduced above for tRNA^pha^ can be accommodated in the cloverleaf model (Fig. 17) (1), as can all other known tRNA sequences. The cloverleaf structures have the following important common features. (a) Terminal sequences are complementary, with base pairing starting at the fifth base of the acceptor end and the first base of the 5' end. (b) Three major identifiable loops containing unpaired nucleotide residues are present; these are from the 5' to the 3' end, a loop containing dHU residues, a loop containing the anticodon, and a loop containing the common sequence G-T-ψ-C-. A detailed discussion of the cloverleaf model can be found elsewhere (13). Wheat germ tRNA^pha^ is unusual in that it contains two purines, guanine, and adenine as opposing bases in a base-paired (stem) region.
This is Fragment a in Fig. 1. The 3’ end of this fragment must be T-$-C-G.. . to C-C-G-C-A-C- from an analysis of Fragments 29 and 36 (Fig. 1). The nucleotide sequence 2’0MeG-A-A-. . . to A-A-G-7MeG-diHU-C-G- has already been determined (see Fragments 30 and 34, Fig. 1) and can be placed at the 5’ end of Fragment a. The remaining two dinucleotides C-G- (Peak 1, Table VIII) and U-G- (Peak 2, Table VIII) must therefore be placed in the middle of Fragment a in the position shown above.

A detailed comparison of yeast tRNA\textsuperscript{Phe} and wheat germ tRNA\textsuperscript{Phe} has been presented elsewhere (7).

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