Studies on Polynucleotides

I.XXXIX. YEAST PHENYLALANINE TRANSFER RIBONUCLEIC ACID: PRODUCTS OBTAINED BY DEGRADATION WITH PANCREATIC RIBONUCLEASE

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U. L. RAJBHANDARY, R. D. FAULKNER, AND ALEXANDER STUART
From the Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

Purified phenylalanine transfer RNA (tRNA) from yeast has been degraded with pancreatic ribonuclease, and the fragments obtained have been separated and identified. The 3'-terminal nucleoside is cytidine, and the 5'-terminal dinucleotide is pGpC. Fourteen minor nucleosides are present, including 7-methylguanosine, 2'-O-methylcytidine, and a nucleoside the identity of which is not established. The sequences involving some of the minor nucleosides, N2-dimethyl-GpCp, GpTp, and ApGp-dihydro-Up, are common to many other tRNAs. The largest oligonucleotide produced is an octanucleotide with the sequence GpGpGpApGpAp-GpCp.

The nucleotide sequence analysis of yeast alanine tRNA derived by Holley et al. (1) has been followed by the elucidation of the sequences of serine tRNA (2), tyrosine tRNA (X), and phenylalanine tRNA (4) from yeast. The general principles utilized in all these studies were (a) degradation of the tRNA with specific ribonucleases and separation and analysis of the fragments obtained (5-9), and (b) isolation and characterization of larger fragments from partial enzymatic digests (9-11) in order to obtain overlaps of sequences necessary for the derivation of a unique sequence. The present paper describes the detailed separation and qualitative and quantitative analyses of oligonucleotide fragments produced by the action of pancreatic RNase on tRNAPh. The following paper (12) reports on the analyses of fragments produced by digestion of tRNAPh with T1 RNase. Brief reports of these results have been summarized elsewhere (4, 13, 14).

In the preceding paper (15), we reported on the terminal sequence analyses of two peaks (Peak I and Peak II) of yeast tRNAPh purified by countercurrent distribution, and concluded that these two peaks represented a single species of tRNAPh. By the use of radioactive end group-labeling techniques (16, 17), it was found that the nucleotide sequences at the 5' and 3' termini of tRNAPh (Peak I) were pGpC- and -CpApCpC, respectively. These results are now confirmed from the data described in this paper and in the following paper (12).

EXPERIMENTAL PROCEDURE

General Procedures

Chromatography—Paper chromatography was carried out on Whatman No. 1 or Whatman No. 3MM paper. The following solvent systems were used: Solvent A, 2-propanol-concentrated ammonium hydroxide-water (7:1:2, v/v); Solvent B, 1-propanol-concentrated ammonium hydroxide-water (55:10:35, v/v); Solvent C, isobutyric acid-concentrated ammonium hydroxide-water (66:1:33, v/v), pH 3.7; Solvent D, 1-butanol-concentrated ammonium hydroxide-water (86:5:14, v/v); Solvent E, ethanol-1 M ammonium acetate, pH 7.5 (70:30, v/v); Solvent F, ethanol-1 M ammonium acetate, pH 7.5 (70:30, v/v); Solvent G, t-butyl alcohol-benzyl alcohol-water (40:20:10:20, v/v); Solvent H, ethanol-1 M ammonium acetate, pH 7.5 (70:30, v/v).

Electrophoresis—Paper electrophoresis was performed on Whatman No. 3MM paper with a voltage gradient of 40 volts per cm for 1 hour in 0.05 M ammonium formate buffer, pH 2.7. The paper was immersed in Varsol (a high boiling petroleum fraction) kept cold with running water.

Column Chromatography—DEAE-cellulose (Whatman DE-23) was washed successively with 0.2 N sodium hydroxide, water, 0.2 N hydrochloric acid, and water. A fine suspension of the resin in 1 M ammonium carbonate was used for packing the column.
column was then washed thoroughly with 0.01 M ammonium carbonate before use.

For chromatography in the presence of 7 M urea (18), a fine suspension of the resin in 0.5 M sodium chloride containing 7 M urea and 0.02 M Tris buffer, pH 7.3, was used for packing the column.

**Elution from Paper**—Elution of nucleotidic material from paper chromatograms or electrophoretograms was carried out with water; in all cases a corresponding area was also eluted and used as blank. Recovery of ultraviolet-absorbing material was usually in the range of 90%.

**Analytical Methods**—Estimation of inorganic phosphate was carried out with the method of Chen, Toribara, and Warner (19) as modified by Ames and Dubin (20). Inorganic phosphate was detected on paper chromatograms with the ammonium molybdate-perchloric acid spray reagent (21), and dithionite dye either with the sodium metaperiodate-p-rosaniline hydrochloride spray (22) (reagent for vicinal diols) or with the sodium hydroxide-p-dimethylaminobenzaldehyde spray reagent (23).

**Ultraviolet Absorption Spectroscopy**—A Zeiss PMQ II spectrophotometer was used for measurement of absorbance, and a Cary 14 spectrophotometer for recording spectra.2

**Enzymes**—Pancreatic RNase, snake venom phosphodiesterase, micrococcal nuclease, and *Escherichia coli* alkaline phosphatase were purchased from Worthington; prior to its use, alkaline phosphatase was treated as described previously (24). T1 RNase either was a gift from Dr. F. Egami (25) or was obtained from Sankyo Company, Ltd., Tokyo, and was purified by column chromatography. T1 RNase (partially purified) was kindly provided by Dr. K. Takahashi. Calf spleen phosphodiesterase was a preparation purified as described previously (26).

**Methods**

**Paper Chromatography of Incubation Mixtures**—The incubation mixture, containing 2 to 10 O.D. units of nucleotidic material, was evaporated to dryness, and the residue was dissolved in 10 to 50 μl of water and applied as a 3- to 8-cm-wide band for paper chromatography.

**Characterization of Oligonucleotides**—This was basically done by degradation of the oligonucleotide with the various enzymes listed above. Unless otherwise mentioned, all the products were obtained in quantities close to equimolar ratios except pseudouridine, which was usually in the region of 0.6 to 0.8 mole per mole of oligonucleotide. (For a review of the mode of action of these enzymes, see References 25 and 27.)

**General Conditions for Enzymatic Degradations**—All incubations were conducted at 37°. For T1 RNase the mixture, in 0.5 ml, contained the oligonucleotide (4 to 8 O.D. units), Tris buffer, pH 7.5 (5 μmoles), and T1 RNase (20 to 30 units). Incubation time was 5 hours. The mixture for alkaline phosphatase contained, in 0.5 ml, the oligonucleotide (5 to 10 O.D. units), ammonium bicarbonate (10 μmoles), and alkaline phosphatase (10 μg). Incubation was conducted at pH 8.0 to 8.5 for 4 to 6 hours. For degradation with T1 RNase and alkaline phosphatase combined, the oligonucleotide (5 to 10 O.D. units) was incubated with ammonium bicarbonate (10 μmoles), T1 RNase (20 to 30 units), and alkaline phosphatase (5 to 10 μg) in 0.5 ml for 6 to 8 hours. For T2 RNase, the mixture, in 0.5 ml, contained the oligonucleotide (2 to 5 O.D. units), sodium acetate, pH 5.0 (5 μmoles), and the enzyme (50 to 150 μg). Incubation was conducted for 6 to 8 hours. For snake venom phosphodiesterase, the oligonucleotide (2 to 10 O.D. units) was incubated with ammonium bicarbonate (10 μmoles) and enzyme (5 to 10 μg) for 6 to 8 hours. The mixture for micrococcal nuclease, in 0.2 to 0.4 ml, contained the oligonucleotide (2 to 4 O.D. units), calcium chloride (1 to 2 μmoles), glycine-sodium hydroxide buffer, pH 8.6 (5 to 10 μmoles), and the enzyme (50 μg). Incubation time was 5 to 6 hours.

**Hydrolysis with Alkalii**—The oligonucleotide (2 to 5 O.D. units) was dissolved in 1 N sodium hydroxide (0.2 ml) and left at room temperature for 24 hours. Sodium ions were removed with Dowex 50 (pyridinium form) resin, the resin was removed by filtration, and the filtrate and washings were evaporated for chromatography.

**Identification of Nucleosides and Nucleotides**—Nucleosides or mononucleotides were identified by their ultraviolet absorption spectra at pH 1, 7, and 13 and by their mobilities in the various chromatographic systems. For the final identification, authentic markers of the appropriate nucleosides or nucleotides were always present during chromatography.4 Dihydropyrimidine derivatives were identified from their characteristic loss of absorption in alkaline medium (28).

**RESULTS**

**Purification of tRNA**

This included three steps of countercurrent distribution and was essentially the same as described previously (29), except for scaling up in the amount of tRNA used. Thus, 8 g of crude yeast tRNA were subjected to a 200-cycle countercurrent distribution in an EC 580 countercurrent fractionator (total phase volume per tube, 80 ml), and the partially purified tRNA\(^\text{Ph}e\) (16,000 O.D. units and 10 to 20% with respect to phenylalanine acceptor activity) was subjected to an 800-cycle distribution in the same machine. The major peak of tRNA\(^\text{Ph}e\) (15, 29) (2,500 O.D. units, 55 to 65% pure) was finally purified by a 1,200-cycle distribution in the EC 520 fractionator (total phase volume, 20 ml per tube). Overall yield of tRNA\(^\text{Ph}e\) was usually 60 to 80 mg; unless otherwise stated, phenylalanine acceptor activity of the tRNA used in most of these experiments was higher than 90%.

**Digestion of tRNA**

With Pancreatic RNAs and Chromatography on DEAE-cellulose (Carbonate Form)

tRNA\(^\text{Ph}e\) (355 O.D. units in 0.02 M phosphate buffer, pH 7.0) was incubated at 37° with pancreatic RNase (0.9 mg) and phosphate buffer, pH 7.0 (100 μmoles), in a volume of 6 ml. After 10 hours (increase in total O.D. units was 27.8%), the incubation mixture was chromatographed on DEAE-cellulose (carbonate form). The pattern of elution is shown in Fig. 1. The recovery of total nucleotidic material was higher than 92%, and further elution of the column with 1 M sodium chloride did not release any nucleotidic material.

Fraction within a certain peak were pooled, and ammonium carbonate was removed by repeated evaporation from water. Removal of the last traces of ammonium carbonate was effected

1 Throughout this paper, spectrum is meant to indicate ultraviolet absorption spectrum.

2 For degradation of oligonucleotides containing minor nucleoside components, the amount of enzyme used was increased by a factor of 2- to 5-fold.

3 We thank Dr. Ross Hall for supplies of minor nucleosides used as reference samples.
by mixing an aqueous solution of the oligonucleotide with Dowex 50 (pyridinium form) resin. The resin was filtered off and washed with dilute ammonium hydroxide. The filtrate and washings were evaporated to dryness twice from dilute ammonium hydroxide. No significant difference between the absorbance of the pooled peak before and after this treatment was observed.

The identification of the mononucleotides and the oligonucleotides produced and quantitative estimation of these products are summarized in Table I.

Identification of Pancreatic RNase Digestion Products (Fig. 1)

Peak 1 (Cytidine)—This was identified as cytidine from its mobility in Solvents A and D and from its spectrum. The amount of adenosine present was 5 to 10%.

Peak 2 (C-cyclic-p; dIHU-cyclic-p)—The complete spectrum of a small portion indicated the presence of a cytidine derivative and a dihydropyrimidine derivative (28); these were found to be C-cyclic-p and diHU-cyclic-p. The cytidine derivative was characterized by chromatography in Solvent B. The bulk of the peak material was treated further with pancreatic RNase and alkaline phosphatase, and the nucleosides produced were chromatographed in Solvent F against markers of uridine, pseudouridine, and diHU. The area of the chromatogram corresponding to diHU (marker on either side of the chromatogram detected by the spray reagent) was eluted. diHU was present in the eluate as judged by its spectrum.

**FIG. 1.** Chromatography of a pancreatic RNase digest of tRNA^Phe^ (355 O.D. units) on a column (1 X 104 cm) of DEAE-cellulose (carbonate form). Elution was carried out in three stages. Stage I was elution with an increasing gradient produced from 300 ml each of 0.01 M, 0.05 M, 0.08 M, 0.1 M, and 0.16 M ammonium carbonate in five chambers of a Varigrad (30); Stage II was elution with a linear gradient produced from 250 ml each of 0.16 M and 0.2 M ammonium carbonate; and Stage III was elution with a linear gradient produced from 750 ml each of 0.2 M and 0.8 M ammonium carbonate.

**TABLE I**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Composition</th>
<th>molar ratio</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Cytidine</td>
<td>7.6</td>
</tr>
<tr>
<td>2</td>
<td>C-cyclic-p</td>
<td>7.6</td>
</tr>
<tr>
<td>3</td>
<td>dIHU-cyclic-p</td>
<td>7.6</td>
</tr>
<tr>
<td>4</td>
<td>Cp</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Up</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>dMeGpCp</td>
<td>21.6</td>
</tr>
<tr>
<td>7</td>
<td>ApCp</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>GpUp</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>pGpCp</td>
<td>17.8</td>
</tr>
<tr>
<td>10</td>
<td>Ap2MeGpCp</td>
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</tr>
<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>GpGMeApUp</td>
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<tr>
<td>15</td>
<td>GpGpAp2Up</td>
<td>25</td>
</tr>
<tr>
<td>16</td>
<td>GpGpApGpApGpCp</td>
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</tr>
<tr>
<td>17</td>
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<tr>
<td>18</td>
<td>GpGpApGpGpGpGpCp</td>
<td>42.8</td>
</tr>
</tbody>
</table>

a Calculated on the basis of contribution of the individual nucleotides (5, 6) after correction for hypochromicity. Approximate hypochromicity of the oligonucleotides at neutral pH and at 260 m\(\mu\) was measured after hydrolysis of the oligonucleotides either with alkali or with T\(_2\) RNase.

b No quantitative determination was attempted, as this peak consisted of a mixture of other materials.

The yield of this oligonucleotide was low when degradation with pancreatic RNase was carried out for prolonged periods. However, with shorter incubation periods (5 to 7 hours), the yield of this oligonucleotide (Peak 8, Fig. 2) increased to 0.8 to 0.9 mole per mole of tRNA^Phe^.
A gave adenosine and pC, indicating that the dinucleotide in this peak was ApCp.

**Peak 8 (GpTp; GpCp)**—The components of this peak were separated by paper electrophoresis at pH 2.7 and characterized as GpTp and GpCp by procedures identical with those used for dImeGpCp and ApCp.

**Peak 9 (GpUp; pGpCp)**—This peak consisted of a mixture of GpUp and pGpCp, which were easily separated by chromatography in Solvent B. The faster traveling component was characterized as GpUp, in much the same way as the dinucleotides described above. The structure of the slower moving component as pGpCp was deduced from the following considerations: (a) it had a lower mobility in Solvent B compared to GpUp; (b) on dephosphorylation it produced GpCp; (c) on alkaline hydrolysis the products were pGp and Cp (separation in Solvent B).

**Peak 10 (Ap2MeGpCp; Gp1MeApUp)**—The components of this peak were found to be Ap2MeGpCp and Gp1MeApUp. Characterization of the latter compound was complicated by the fact that the amount of this trinucleotide was small (part of this trinucleotide was isolated as Gp6MeApUp; see data on Peak 11); furthermore, during incubations at 37° for enzymatic digestions or chromatography in ammoniacal solvent systems, conversion of 1MeA to 6MeA (32) produced two bands from a single oligonucleotide. The combined yield of Gp1MeApUp and Gp6MeApUp was, however, close to 1 mole per mole of tRNA*εε*, and, since the ready conversion of 1MeA to 6MeA is well established (33), the trinucleotide Gp1MeApUp must be a genuine constituent of tRNA*εε*. The evidence for the structure of the trinucleotides is presented below.

Dephosphorylation and chromatography in Solvent C produced a major component (Band I) and two minor components traveling closely together but separated (Bands II and III). The ratio of nucleotidic material in Band I to that in Bands II and III combined was 2.4. The fastest traveling compound (Band I) was characterized as Ap2MeGpCp by degradation with snake venom phosphodiesterase and chromatography in Solvent A. The products obtained were adenosine, p2MeG, and pC; the mononucleotides could be resolved in Solvent G after dephosphorylation into the corresponding nucleosides and identified by their spectra and mobilities.

The structure of material in Band II was established as Gp6MeApU from the products of snake venom phosphodiesterase, which were guanosine, p6MeA, and pU (1:1:1, separation in Solvent A). The component of Band III was similarly identified as Gp1MeApU; the products in this case were guanosine, pGMeA, and pU (1:1:1, separation in Solvent A). The products were adenosine, p2MeG, and pC; the mononucleotides could be resolved in Solvent G after dephosphorylation into the corresponding nucleosides and identified by their spectra and mobilities.

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alkaline phosphatase, the products were ApG and diHU (ApG identified by degradation to adenosine and pG with snake venom phosphodiesterase, and diHU from its spectrum and mobility in Solvents A and F); (b) treatment with snake venom phosphodiesterase and chromatography in Solvent A yielded adenosine, pdiHU, and pG. pdiHU had the same mobility as pU and was converted to inorganic phosphate and diHU by the action of alkaline phosphatase. Inorganic phosphate was detected by spraying the lower half of the chromatogram (close to the origin) run in Solvent A with the molybdate spray reagent (21), and diHU (present in the upper half) by the periodate spray reagent (22) for glycols. A paper blank eluted from the previous chromatogram and treated similarly gave neither inorganic phosphate nor any diHU.


The sequence analysis of the hexanucleotide 2\'OMEpApApApApApPp was a more difficult problem because of (a) the presence of minor nucleosides at either end, which resulted in a slow rate of degradation with exonuclease; (b) the presence of the minor nucleoside Y, the identity of which is not known; and (c) the absence of any phosphodiester linkages hydrolysable by T1 RNase. The total evidence for the sequence of this hexanucleotide came from a combination of degradative studies, which are summarized in Figs. 3 and 4 and are described in detail below. (In some of the enzymatic digests, small amounts (15 to 20%) of fluorescent material distinct from the major band of fluorescence were observed upon paper chromatography in ammoniacal systems. The possibility, therefore, that Y was a labile nucleoside that could undergo rearrangement or partial degradation to another fluorescent material either during incubation at 37° or during chromatography could not be ruled out.)

The oligonucleotide was not degraded by T1 RNase and hence does not contain guanosine, inosine, 2MeG, or diMeG. The nucleotide composition of the oligonucleotide and the 3'-terminal end group was determined by alkaline hydrolysis and chromatography in Solvent B. The products were pseudouridine (indicating that the 3'-terminal end was pseudouridine), a fluorescent material with mobility similar to that of a mononucleotide, Ap (2 moles), and 2\'OMEpAp (1 mole). 2\'OMEpAp was characterized by digestion with snake venom phosphodiesterase subsequent to dephosphorylation. Digestion of the oligonucleotide with spleen phosphodiesterase proceeded very slowly, with the release of some 2\'OMEpAp and Ap. The slow rate of degradation...
of this oligonucleotide indicated that 2′OMeG might be the 5′-terminal end group of this oligonucleotide. Further evidence that 2′OMeG represented the 5′-terminal end group was obtained by exhaustive treatment of the oligonucleotide with snake venom phosphodiesterase. The only nucleoside product detected was 2′OMeG (20% yield), and, since it was already known that alkaline hydrolysis gives 2′OMeG, the partial sequence of the oligonucleotide is 2′OMeGpAp(λp, λp, Ap)pAp, in which the order of the nucleotides shown within parentheses is not known. The information for the nucleotide sequence came from the isolation of a fluorescent tetranucleoside triphosphate, pA, and pY (chromatographic separation in Solvent B) as the major products from the action of snake venom phosphodiesterase on the oligonucleotide. The fluorescent product was characterized as 2′OMeGpApY by degradation either with micrococcal nuclease to 2′OMeGpAp and ApY (separation in Solvent A, where ApY travels faster and is fluorescent) or with T2 RNase to 2′OMeGpAp, Ap, and Y (separation in Solvent A). On digestion with T2 RNase, the products from ApY were Ap and Y (separation in Solvent A).

Further support for the structure of the oligonucleotide was obtained by treatment with T2 RNase. The products which were completely resolved by paper chromatography (Solvent E) were 2′OMeGpAp, Ap, ApY-cyclic-p, pseudouridine, and also a small amount of Y-cyclic-p. ApY-cyclic-p was identified by degradation with spleen phosphodiesterase to Ap and Y-cyclic-p (separation in Solvent E or A). It should be noted that, whereas ApY was cleaved completely by T2 RNase to Ap and Y, ApY-cyclic-p was largely resistant to the action of this enzyme: furthermore, it appears that ApY-cyclic-p is not further hydrolyzed by T2 RNase to ApYp. Products obtained by the action of micrococcal nuclease on the hexanucleotide (Fig. 4) were in agreement with the structure deduced and also provide direct proof of the presence of the dinucleotide sequence ApYp. After chromatography of such a digest in Solvent B, the oligonucleotides identified were mostly 2′OMeGpAp, ApYp, and ApYp, with some Ap and Yp.

The final supporting evidence for the structure of the hexanucleotide came from a study on large oligonucleotide fragments obtained by partial T2 RNase treatment of tRNA

It was found that, during resolution of these large oligonucleotides by column chromatography on DEAE-cellulose in 7 M urea and at acidic pH, the characteristic bright greenish blue fluorescence of Y (under ultraviolet light) in some of the oligonucleotides was no longer present, and also that the corresponding hexanucleotide isolated from further degradation of these oligonucleotides had a distinctly altered spectrum from that of the hexanucleotide containing Y (Fig. 5). Furthermore, the dephosphorylated hexanucleotide could now be partially degraded with pancreatic RNase to yield 2′OMeGpApApY-cyclic-p and ApY (separation in Solvents A and B). The characterization of 2′OMeGpApApY-cyclic-p, which showed that it was not adenosine or A-cyclic-p but could be either Ap or =IpY-cyclic-p; and, finally, from its lack of susceptibility to alkaline phosphatase, which ruled out the possibility that it was Ap.

Peak 13 (GpAp1pUp; ApGp1p2′OMeCpUp)—The constituents of this peak comprised the bulk of total GpApUp produced and part of ApGp1p2′OMeCpUp, also found in Peak 12. These were dephosphorylated and resolved by chromatography in Solvent C. The faster traveling component was identical as ApGpAp2′OMeCpU as described above. The slower traveling band, on degradation with T1 RNase alone, gave 2 moles of Gp and 1 mole of ApU (separation in Solvent B); with T1 RNase and alkaline phosphatase, the products were 2 moles of guanosine and 1 mole of ApU (separation in Solvent C).

Peak 14 (ApGp1pApUp)—Except for a small amount (5 to 10%) of contaminant, this peak was mostly ApGp1pApUp (part of this pentanucleotide was also present in Peak 15). The oligonucleotide was purified by chromatography in Solvent C and then subjected to combined degradation with T1 RNase and alkaline phosphatase, and the products, ApG and ApApU, were separated in Solvent A. ApApU was characterized either by treatment with snake venom phosphodiesterase (adenosine, pA, and pU were formed in equimolar amounts) or by hydrolysis with alkali (2 moles of Ap and 1 mole of uridine were formed).

Alternatively, the pentanucleotide was first dephosphorylated,
and the isolated pentanucleoside tetraphosphate then was treated with T<sub>1</sub> RNase alone. The products ApGp and ApApUp were then resolved by chromatography in Solvent B.


Earlier analyses (13, 35) by combined degradation with T<sub>1</sub> RNase and alkaline phosphatase indicated that this compound could be a heptanucleotide; on the other hand, its elution from the column close to a pentanucleotide suggested that it was at the most a hexanucleotide. A further difficulty in the analysis of this compound was due to the ring opening of the imidazole moiety of the 7MeG present in this oligonucleotide during chromatography in ammoniolic systems or during prolonged incubation at 37° (36-38), which gave rise to a nucleoside previously designated as X (13). This degradation of one of the constituent nucleosides was kept at a minimum by avoiding the use of ammoniolic solvent systems. Thus (a) combined action of T<sub>1</sub> RNase and alkaline phosphatase produced 2 moles of guanosine, 1 mole of ApG (these were not separated in Solvent C) and 1 mole of 7MeGpU (seen as a dark blue fluorescent spot different from the bright greenish blue fluorescence of Y on the chromatogram). The faster traveling band consisted of guanosine and ApG; these could be separated in Solvent A, and the amounts of guanosine and ApG produced were estimated (moles of guanosine to moles of ApG produced was 2.04:1). 7MeGpU was characterized by degradation with snake venom phosphodiesterase (excess enzyme and prolonged incubation were found necessary for complete digestion) to 7MeG and pU. 7MeG was identified by its mobility in Solvents C and H, its characteristic dark blue fluorescence under ultraviolet light, and its spectrum. (b) The 5'-end group of the hexanucleotide was shown to be guanosine, by dephosphorylation and subsequent treatment with snake venom phosphodiesterase. The only nucleoside produced was guanosine. The above experiments permitted a deduction of the sequence GpGpApGp7MeGpUp for this oligonucleotide. The ordering of Gp and ApGp shown within parentheses was made possible from studies on large oligonucleotide fragments isolated by a limited digestion of tRNA<sup>Ph</sup> with T<sub>1</sub> RNase and identification of ApGp7MeGpU as the 5'-terminal sequence of a large oligonucleotide (34).

**Peak 16 (GpGpGpApApApApGp)—**The neutral spectrum of this oligonucleotide showed a maximum at 253 m<sub>υ</sub> and indicated a high content of guanosine residues. From its position of elution from the column, this oligonucleotide was expected to be free of contaminants. For this reason and in view of the expected difficulty of recovery from paper chromatograms, small aliquots (2 O.D. units) were chromatographed in Solvents B and C over prolonged periods to check for the purity of this oligonucleotide. Only a single sharp band at the origin was present. Combined degradation of the oligonucleotide (5 O.D. units) with T<sub>1</sub> RNase and alkaline phosphatase yielded cytidine, guanosine, and ApG in the molar ratio 1:3.2:2.1 (separation in Solvent A), and indicated that this compound was an octanucleotide with the nucleotide composition 3Gp, 2ApGp, Gp. The characterization of this as GpGpGpApApApGpGp by a method involving dephosphorylation, partial degradation with snake venom phosphodiesterase, and subsequent labeling of the 2',3'-diol end groups in the oligonucleotides produced is described in an accompanying paper (17).

**DISCUSSION**

As a first major step toward the elucidation of the sequence of yeast tRNA<sup>Ph</sup>, this paper has reported on the qualitative and quantitative analyses of mono- and oligonucleotides produced by the action of pancreatic RNase on tRNA<sup>Ph</sup>. Methods commonly used for the separation of oligonucleotides have been either two-dimensional paper chromatography or electrophoresis (or both) (39, 40), or column chromatography on DEAE-cellulose (18, 41, 42). The former technique was used mostly for the small scale analysis of fragments from valine tRNA (43, 44), alanine tRNA (43, 45), and tyrosine tRNA (45) and is normally limited to oligonucleotides up to the pentanucleotide level; recently, modified techniques have made possible the separation of small amounts of a large number of oligonucleotides obtained by degradation of tRNAs (46). The technique of column chromatography was applied (5-7) to the sequence analysis of alanine, serine, and tyrosine tRNAs and, in the present work, proved adequate for resolution of fragments (Fig. 1) obtained from tRNA<sup>Ph</sup>. In cases when more than one component was present within a peak, a single step of paper chromatography usually separated the constituent oligonucleotides.

Sequential analysis of oligonucleotides was carried out by the use of standard techniques (for a review, see Reference 35), and except in the case of oligonucleotides containing some minor nucleosides, was straightforward. The major problems encountered in these oligonucleotides were mainly the interconversion of 1MeA to 6MeA through ring opening and ring closure (32, 33), the labile nature of 7MeG (36-38), and a much slower rate of attack by exonucleases (47).

The isolation of 7MeGpU from T<sub>1</sub> RNase digestion of GpGpApGp7MeGpUp and the isolation of 7MeGpUpCp5MeCpUpGp from T<sub>1</sub> RNase digestion of tRNA<sup>Ph</sup> (12) indicate that 7MeGpX (X = nucleoside) bonds are not susceptible to the action of this enzyme.

Fourteen minor nucleosides were found in tRNA<sup>Ph</sup>, including 2'0MeC and 7MeG, which have been characterized for the first time from a purified RNA. A new minor nucleoside, which has not yet been identified (here designated Y), was also present. This nucleoside exhibited a bright fluorescence in ultraviolet light, and the fluorescence persisted very strongly in large oligonucleotides isolated from tRNA<sup>Ph</sup> (34, 48) and even to a certain extent in the tRNA<sup>Ph</sup> itself. Y displayed a highly characteristic spectrum (Fig. 6), with a pronounced maximum at 210 m<sub>υ</sub> in neutral solutions. Conversion of Y to another unidentified and nonfluorescent nucleoside (Y', see above) caused a distinct change in the spectral characteristics of the hexanucleotide 2'0MeGpApApYpApYp (Fig. 5). Furthermore, the oligonucleotide could now be degraded to 2'0MeGpApApY' cyclic-p and ApY', indicating that Y' (and hence possibly Y) could be a pyrimidine derivative. Some evidence either that Y is cationic at neutral pH or that conversion to Y' generates an anion can be deduced from the observation that oligonucleotides containing Y were eluted earlier from a DEAE-cellulose column than the corresponding oligonucleotides in which Y was changed to Y' (34, 48). The exact chemical nature of Y remains unknown at this time.
The identification of cytidine at the 3' end and pGpCp at the 5' end of tRNA$^{Phe}$ supports the conclusion arrived at (15) by means of end group-labeling techniques. The sequences GpTp, di-MeGpCp, and ApGpdHUp present in tRNA$^{Phe}$ have been found in other tRNAs (5-7). The largest oligonucleotide obtained from pancreatic RNase digests of tRNA$^{Phe}$ was the octanucleotide GpTpGpGpGpGpCp, the characterization of which was described before (17). While closely related oligonucleotides such as GpGpGpGpGpGpGpUp (5) and GpGpGpGpGpCp (6) are present in alanine and tyrosine tRNAs, respectively, the positions of these oligonucleotides in the three tRNAs are different (1, 3, 4).

The yield of the oligonucleotides obtained from tRNA$^{Phe}$ shows that these compounds are genuine constituents of tRNA$^{Phe}$ (Table I). This is also indicated by the data reported in an accompanying paper (12), in which very good correlations between fragments produced by pancreatic RNase and those produced by T$_1$ RNase on tRNA$^{Phe}$ were observed.

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