Evidence for the Occurrence of a Common Pentanucleotide Sequence in the Structures of Transfer Ribonucleic Acids*

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Transfer ribonucleic acids are a mixture of different molecular species of similar size (1). They can be distinguished by the ability of each of them to accept only one amino acid and transfer it to protein (1). The elucidation of the structures of tRNAs is of considerable interest in view of the highly specific nature of these reactions.

It has been shown that the 3' hydroxyl terminal sequence, the amino acid acceptor end, of all functional tRNA molecules is \(-p\text{CpCpCpA}\) (2, 3). The 5' phosphate terminal was found to be predominantly \(p\text{Gp}\text{Gp}\) (4). Aside from these similarities, the structures of different tRNA molecules seem to vary significantly, as indicated by structural studies performed on bulk (1) as well as purified tRNA preparations (5).

The nucleotide composition of tRNA is characterized by the presence of several minor components in addition to the four major nucleotides. The minor nucleotides include pseudouridylic acid (\(\text{G}\)) and ribothymidylic acid (\(\text{T}\)), as well as other methylated derivatives of purine and pyrimidine nucleotides (6, 7). Each of the purified tRNAs studied has a specific content of minor nucleotides, which occupy definite positions along the RNA chain (5). The role of the minor nucleotides in the structure and function of tRNA is still little understood.

This paper presents evidence that strongly suggests that the sequence \(-p\text{CpCpCpA}\), including two minor nucleotides, is part of the structure of all tRNA molecules. This sequence gives the tetranucleotide \(p\text{GpCpGp}\) on digestion of the RNAs with RNase T1. The isolation of this tetranucleotide from digests of purified alanine-, valine-, and tyrosine-tRNAs, as well as from bulk tRNA of yeast, \(E.\ co\text{l}i\), and rat liver, will be described.

EXPERIMENTAL PROCEDURE

Preparation of Transfer Ribonucleic Acid

The preparation of tRNA from pressed baker's yeast by phenol extraction has been carried out according to Holley (8). tRNA from \(E.\ co\text{l}i\) (strain KB) was similarly prepared. Rat liver tRNA was prepared according to Brunngraber (9). Purified alanine-, valine-, and tyrosine-acceptor RNAs from yeast have been obtained by repeated countercurrent distribution as described by Apgar, Holley, and Merrill (10).

Digestion of Transfer Ribonucleic Acid with Ribonuclease T1

The reaction mixture, in a total volume of 1.3 ml, consisted of 25 to 35 mg of RNA in 0.85 ml of \(\text{H}_2\text{O}\), 0.25 ml of 0.1 sodium phosphate buffer at pH 7.5, and 500 units of RNase T1 (purchased from Sankyo Company, Ltd., Tokyo). A drop of chloroform was added to the mixture in order to prevent growth of microorganisms. Incubation was carried out at 37°C for 24 hours.

Separation of RNase T1 Digestion Products by Column Chromatography

Two types of columns were used.

DEAE-Sephadex Column—In a modification of the procedure of Stachelin, Peterson, and Sober (11), a column 0.25 cm was packed with an aqueous suspension of DEAE-Sephadex A-25, medium (Pharmacia Fine Chemicals, Inc.). The sample was diluted to 10 ml with water and the products of the digestion were adsorbed on the column. Elution was carried out with an increasing gradient produced by the following solutions of ammonium carbonate (placed in four chambers of a Varigrad in the specified order): 0.04 M, 240 ml; 0.2 M, 280 ml; 0.4 M, 233 ml; and 1.0 M, 225 ml. (Volumes were corrected according to the specific gravities of the solutions.) When the Varigrad was placed at a height of 30 feet above the top of the column, an average flow rate of 7 ml per hour was obtained. Fractions of 3.0 to 3.5 ml were collected and their absorbances at 260 nm were measured. The fractions of each peak were pooled and freed from ammonium carbonate by repeated evaporation in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide.

DEAE-cellulose-Urea Column—This type of column was developed by Tomlinson and Tener (12) for the separation of oligonucleotides based primarily on their net charge. When a very long column was used, factors other than charge become effective in the separation, resulting in a high resolution of oligonucleotides.

The column (0.6 cm) was packed with a slurry of DEAE-cellulose (Carl Schleicher and Schuell) suspended in 7 M urea. Before the sample was applied to the column, 1.6 g of urea were added to the sample and the volume was brought to 3.8 ml with \(\text{H}_2\text{O}\). A solution of increasing concentration of sodium acetate, pH 7.5, in 7 M urea was used for elution. The
concentration gradient was generated by the following solutions (placed in four chambers of a Varigrad in the specified order): 7 M urea, 340 ml; 0.4 M sodium acetate in 7 M urea, 340 ml; 7 M urea, 340 ml; and 1.0 M sodium acetate in 7 M urea, 340 ml. Fractions of 4 ml were collected at a flow rate adjusted to 22 ml per hour. To improve the reproducibility of the fractionation, the column was maintained at a constant temperature of 33° by means of a water jacket.

**Desalting**

Before analysis of the oligonucleotides, urea and salts were removed from the solutions (cf. 13). The fractions included within a given peak were pooled and diluted 4-fold with H2O. The oligonucleotides in the sample were adsorbed on a column (0.6 x 10.0-cm) of DEAE-cellulose suspended in H2O. Salts and urea were removed by thorough washing with H2O. Oligonucleotides were recovered from the column by elution with 1 M ammonium carbonate (approximately 10 ml were required for a quantitative recovery of the ultraviolet-absorbing material). Ammonium carbonate was removed by evaporation as previously described.

**Separation of Oligonucleotides by Paper Electrophoresis**

Oligonucleotide fractions were subjected to further purification by paper electrophoresis. This was performed with the use of Whatman No. 3HR paper in 20% acetic acid which was adjusted to pH 2.7 with NH4OH (14). A voltage gradient of 47 volts per cm was applied and the temperature was maintained at 10°. Ultraviolet-absorbing bands were cut out and were eluted with H2O.

**Fig. 1.** Elution patterns of RNase T1 digests of alanine-, valine-, and tyrosine-tRNAs from yeast, chromatographed on a DEAE-Sephadex column and eluted with a nonlinear gradient of ammonium carbonate. For further details, see "Experimental Procedure." The different columns (a, b, and c) were run at different times with some variation in volumes of fractions. The highest peak in each pattern is Gp.
**Determination of Base Composition of Oligonucleotides**

The dried oligonucleotide samples were hydrolyzed to mononucleotides with 50 μl of 0.3 N KOH at 37° for 18 hours, or with 25 μl of 1.0 N KOH at 80° for 1 hour. The hydrolysates were applied to Schleicher and Schuell No. 589 paper and were analyzed by two-directional ascending paper chromatography. The solvent system run in the first direction was isopropyl alcohol-water (60:40) with NH₃ in the vapor phase according to Markham and Smith (15). The chromatogram was developed in the second direction with the isopropyl alcohol-HCl system of Wyatt (16) (170 μl of isopropyl alcohol, and 41 ml of concentrated HCl diluted to 250 ml with H₂O). Mononucleotides were identified by their positions on the chromatograms and, after elution, by their characteristic spectra at several pH values (recorded with the Cary model 15 spectrophotometer). Mononucleotides were estimated quantitatively by their absorption at 260 μm at pH 2.0. The molar extinction coefficients used were: Gp, 11.8 × 10⁴; Cp, 8.6 × 10⁴; Up, 10.0 × 10⁴; Ap, 14.2 × 10⁴ (17); Tp, 8.9 × 10⁴ (18); and ψp, 8.6 × 10⁴ (19).

**Determination of Nucleotide Sequence of Tetranucleotides**

Micrococcal nuclease and snake venom phosphodiesterase were used for the degradative reactions by which the sequence was established. (The detailed scheme will be presented under "Results.") A preparation of micrococcal nuclease was kindly provided by Dr. M. Laskowski, Sr. The reaction mixture for this enzyme consisted of the oligonucleotide dissolved in 0.25 ml of water, 0.05 ml of 1 M Tris-HCl buffer at pH 8.0, 0.05 ml of 0.1 M calcium chloride, and 0.1 or 0.2 ml of micrococcal nuclease solution (1.750 viscosity units/0.1 ml). The mixture was incubated at 37° for a period of 30 minutes or 1 hour. At the end of the incubation period, solid urea was added to make a 7 M urea solution, and the volume was brought to 3.0 ml with a 7 M urea solution. The reaction products were separated on a column of DEAE-cellulose in 7 M urea (12). Under the conditions used, the separation depended mostly on the charge of the fragments. A linear gradient of sodium acetate in 7 M urea at pH 7.5 was used for elution. The resulting peaks were desalted and further resolved by electrophoresis at pH 2.7 as previously described.

Snake venom phosphodiesterase was also used in the structural studies. The enzyme was purchased from Worthington Biochemical Corporation and was further purified by Dr. E. B. Keller (20). The reaction mixture included the sample dissolved in 0.05 ml of 1 M Tris-HCl buffer at pH 7.4, 0.05 ml of 0.5 M magnesium chloride, and 0.17 ml of a 2 mg per ml solution of snake venom phosphodiesterase. The mixture was incubated at 37° for 3 hours and the products were separated by electrophoresis. Their characterization was based on their relative mobilities and absorption spectra.

**RESULTS**

**Isolation of (Tp, ψp, Cp)Gp from RNase T1 Digests of Purified Transfer Ribonucleic Acids**

The fractionation of the T1 digestion products of yeast alanine-, valine-, and tyrosine-acceptor RNAs is illustrated in Fig. 1. The elution patterns of the three RNAs differ markedly, indicating the structural diversity of the purified RNAs. Upon further analysis, it has been found that a tetranucleotide having an identical base composition (Tp, ψp, Cp)Gp can be isolated from the three digests.

The full analytical procedure will be illustrated in a typical example of the isolation of (Tp, ψp, Cp)Gp from RNase T1 digests of purified transfer ribonucleic acids from yeast.

**TABLE I**

<table>
<thead>
<tr>
<th>Source of material</th>
<th>Fraction of total digest recovered in peak</th>
<th>TpψpCpGp contents of peak</th>
<th>Fraction of total digest recovered as TpψpCpGp</th>
<th>Amount of TpψpCpGp recovered per mole of RNA</th>
<th>Nucleotide composition of purified TpψpCpGp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 7 of alanine-RNA digest (Fig. 1a)</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>mole</td>
<td>Gp, 1.0; Cp, 1.18; ψp, 0.89; Tp, 1.23</td>
</tr>
<tr>
<td>Peak 11 of valine-RNA digest (Fig. 1b)</td>
<td>6.45</td>
<td>55.06</td>
<td>3.56</td>
<td>0.79</td>
<td>Gp, 1.0; Cp, 0.96; ψp, 1.02; Tp, 1.02; (Ap, 0.1; Up, 0.25)</td>
</tr>
<tr>
<td>Peak 11 of tyrosine-RNA digest (Fig. 1c)</td>
<td>4.42</td>
<td>67.1</td>
<td>2.97</td>
<td>0.66</td>
<td>Gp, 1.0; Cp, 0.92; ψp, 0.90; Tp, 0.98</td>
</tr>
</tbody>
</table>

* The percentage of TpψpCpGp in any of the studied peaks, and hence in the total digest, was calculated on the basis of the ultraviolet absorption peak. This method is subject to errors owing to some diffusion and streaking of the electrophoretic bands. This is thought to be the case for the somewhat low molar yields of TpψpCpGp from the purified RNAs. In the case of tyrosine-RNA, a poor resolution of the tetranucleotide-containing peak in the original column chromatography can also account for the low value obtained.

† A recovery of 4.55% as TpψpCpGp was taken to be the quantitative recovery for 1 TpψpCpGp residue per mole of RNA. This approximate value is based on the following considerations. The contribution of each nucleotide to the total ultraviolet absorption of the RNA molecule can be taken, approximately, as the ratio of the molar extinction coefficient of the given nucleotide to the sum of the molar extinction coefficients of the total number of nucleotides in the molecule ("total" coefficient). In the same way, one can estimate the contribution of a particular sequence to the total ultraviolet absorption by adding up the molar extinction coefficients of the nucleotides in that sequence and relating the sum to the "total" sum of molar extinction coefficients for an "average" molecule of tRNA of a chain length of 75 nucleotides composed of equal amounts of Up, Ap, Cp, and Gp. This "total" extinction coefficient will be equal to 8.3 × 10⁴ (at neutral pH). The combined molar extinction coefficients for TpψpCpGp are 3.78 × 10⁴, corresponding to 4.55% of the total.

‡ The values are expressed relative to Gp.
FIG. 2. Elution patterns of RNase T1 digests of yeast, E. coli, and rat liver bulk tRNA, chromatographed on a DEAE-cellulose-urea column and eluted with a nonlinear gradient of sodium acetate in 7 M urea. For further details, see "Experimental Procedure."

experiment. An RNase T1 digest of alanine-RNA containing 710 \(A_{260}\) units\(^2\) was fractionated. (In order to minimize errors in calculation due to hyperchromicity, readings of absorbance were taken on the digested material rather than on the starting material.) As is shown in Fig. 1a and in Table I, 37.9 \(A_{260}\) were eluted as peak 7 corresponding to 5.3% of the total absorbance at 260 m\(\mu\) originally put on the column. The fractions included within Peak 7 were pooled and evaporated. The material was then dissolved in 50 \(\mu\)l of water and was subjected to electrophoresis for 1½ hours. The electrophoretogram showed the presence of four distinct ultraviolet-absorbing components. These were eluted with water and their absorbance at 260 m\(\mu\) was measured. In the order of increasing mobility, these components were found to contain 3.0, 10.0, 1.56, and 0.3 \(A_{260}\), respectively, or 14.4, 76.6, 7.6, and 1.4% of the total amount of material recovered from the electrophoretogram. These values were taken to be the percentages of the different components of the original peak. It then appeared that the peak contained one major oligonucleotide amounting to 76% of the total ultraviolet-absorbing material together with some minor contaminants.

The nucleotide composition of the major component was found to be: Gp, 1.0; Cp, 1.18; \(\psi_p\), 0.89; and Tp, 1.23. The preliminary structure assigned to this oligonucleotide was that of a tetranucleotide, (Tp, \(\psi_p\), Cp)Gp, with the sequence of nucleotides inside the parentheses not specified. This type of analysis was extended to material contained within Peak 11 of the valine-RNA digest (Fig. 1b) and Peak 11 of the tyrosine-RNA digest (Fig. 1c). The results of these experiments are summarized in Table I. The results show that on digestion with RNase T1 the valine- and tyrosine-RNAs also give a tetranucleotide, (Tp, \(\psi_p\), Cp)Gp, in a yield approximating 1 mole per mole of each of the RNAs studied.

\(^2\) One unit of absorbance at 260 m\(\mu\) is defined as the amount of material giving an absorbance reading of 1.0 at 260 m\(\mu\) when dissolved in 1.0 ml and with a light path of 1.0 cm.
Isolation of (Tp, $p, Cp)Gp from T1 Digests of Bulk Transfer Ribonucleic Acid

The results presented in the preceding section suggested the possibility that the sequence, isolated as a tetranucleotide, (Tp, $p, Cp)Gp, might be common to the structures of all tRNA molecules from yeast and possibly to tRNAs from other species as well. In order to test this hypothesis, T1 digests of total tRNA from yeast, E. coli, and rat liver were analyzed for this sequence. The fractionations of these digests are shown in Fig. 2. The tetranucleotide (Tp, $p, Cp)Gp was further purified by electrophoresis. Fig. 3 shows a tracing of an electrophoretogram of the major component included within Peak 13 of yeast tRNA (Fig. 2a), Peak 14 of E. coli tRNA (Fig. 2b), and the peak containing (Tp, $p, Cp)Gp from purified alanine-RNA. In each case, the material having the same electrophoretic mobility as the major component of the alanine-RNA peak was analyzed. Material similarly obtained from the rat liver tRNA digest (Fig. 2c) was also analyzed. The results are summarized in Table II.

It is evident from the nucleotide composition of the electrophoretically purified material that, in addition to Tp, $p, Cp, and Gp, Up and Ap are present. That Up and Ap arise from contaminating oligonucleotides is shown by two different experiments. By varying the conditions of the column fractionation of the RNase T1 digest of yeast tRNA, a nearly pure tetranucleotide was obtained from the electrophoretogram (21). A second purification, as a result of incubation with micrococcal nuclease, will be described in the following section.

When corrected for impurities, the results indicate that bulk tRNAs isolated from yeast and E. coli yield (Tp, $p, Cp)Gp. The recovery of the tetranucleotide from both sources is comparable to its recovery from the purified tRNA preparations (Table I) and suggests the presence of 1 residue per RNA chain.

The results obtained for the rat liver RNA are less conclusive. The presence of contaminants in the RNA preparation may explain the low recovery of the tetranucleotide from this source.

**Table II**

<table>
<thead>
<tr>
<th>Source of material</th>
<th>Fraction of total digest recovered in peak</th>
<th>(Tp, $p, Cp)Gp contents of peak*</th>
<th>Fraction of total digest recovered as (Tp, $p, Cp)Gp</th>
<th>Amount of (Tp, $p, Cp)Gp recovered per mole of RNA†</th>
<th>Nucleotide composition of (Tp, $p, Cp)Gp containing electrophoresis band‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 13 of yeast tRNA digest (Fig. 2a)</td>
<td>11.1%</td>
<td>30.6%</td>
<td>3.4%</td>
<td>0.76 mole</td>
<td>Gp, 1.07; Cp, 1.07; $p, 0.83; Tp, 1.00; (Ap, 0.12; Up, 0.85)</td>
</tr>
<tr>
<td>Peak 14 of E. coli tRNA digest (Fig. 2b)</td>
<td>10.5%</td>
<td>31.1%</td>
<td>3.3%</td>
<td>0.73 mole</td>
<td>Gp, 1.47; Cp, 1.23; $p, 0.75; Tp, 1.00 (Up, 1.20)</td>
</tr>
<tr>
<td>Peak 13 of rat liver tRNA digest (Fig. 2c)</td>
<td>10.0%</td>
<td>18.5%</td>
<td>1.9%</td>
<td>0.42 mole</td>
<td>Gp, 1.56; Cp, 1.15; $p, 1.74; Tp, 1.00; (Up, 1.85; unidentified nucleotide, 1.24)</td>
</tr>
</tbody>
</table>

* The T1 digestion of bulk tRNA produces a highly complex mixture of oligonucleotides, and the purification of (Tp, $p, Cp)Gp is rendered more difficult by the presence, in the same peak, of oligonucleotides having the same electrophoretic mobility. For the calculation of the amount of pure (Tp, $p, Cp)Gp in the electrophoretic band, the assumption was made that Tp is located exclusively in this sequence and thus could be a measure of the amount of pure tetranucleotide present. This assumption is supported by the presence of only 1 Tp residue in each of the purified RNAs (5).

† See the corresponding footnote to Table I.

‡ The values are expressed relative to Tp.
and, by analysis of the pancreatic RNase digests, it has been shown that Tp occurs in the sequence -GpTp- in all three RNAs. Since the Gp-Tp linkage would be cleaved by RNase T1, this places the Tp at the 5'-hydroxyl end of the tetranucleotide, and the tetranucleotide can be represented as Tp(ψp,Cp)Gp. The problem of establishing the complete structure of the tetranucleotide is, therefore, reduced to the problem of determining the order in which Cp and ψp are arranged in the molecule. The use of micrococcal nuclease for the structural study was suggested to us by Dr. M. Laskowski, Sr.

Upon the degradation of the tetranucleotide by micrococcal nuclease, one of the following sets of products would be expected (22).

\[ \text{TpψpCpGp} \rightarrow \text{Tpψp} + \text{Cp} + \text{Gp} + \text{trace CpGp} \]

or

\[ \text{TpCpψpGp} \rightarrow \text{TpCp} + \text{ψp} + \text{Gp} + \text{trace ψpGp} \]

The composition of the major dinucleotide obtained should establish the sequence of the tetranucleotide. The products formed on the hydrolysis of Tp(ψp,Cp)Gp with micrococcal nuclease were fractionated according to their charge on a DEAE-cellulose-urea column. Fig. 4 illustrates the elution pattern of the degradation products formed from the tetranucleotide isolated from the valine-RNA. The mononucleotide peaks, further resolved by electrophoresis, was found to contain 0.35 μmole of Cp and 0.36 μmole of Gp. The dinucleotide gave on electrophoresis one major component with a faster mobility.
than Up, together with some very faint bands. The major band was eluted, and the spectrum at pH 2.0 and 14.0 (Fig. 5) was characteristic of the composite spectra of equivalent concentrations of Tp and Up. The typical bathochromic shift in alkaline solution could be due only to the presence of Up. This identifies the dinucleotide as TpUp, of which 0.47 Hmole was recovered. The major band digests of the alanine-, valine-, and tyrosine-tRNAs from yeast. The dinucleotide as Tp#p, of which 0.47 Hmole was recovered. of bulk tRNAs support this view. The indications are that Tp and Up. The typical bathochromic shift in alkaline sequence in the three RNAs are extremely small. This suggested the possibility that this sequence occurs in all tRNAs. Analyses of bulk tRNAs support this view. The indications are that Tp is always preceded by Gp, and therefore the common sequence is GpTpUpGp. It is known from results obtained by pancreatic RNase digestion (5) that the sequences to the left of –GpTpUpGp– vary in the three purified RNAs. However, the possibility exists that the common sequence may extend a few nucleotides to the right. The evidence that this pentanucleotide sequence is a common structural feature of all molecules of tRNA suggests that it may have a functional significance in one of the interactions in which tRNA is involved in the course of protein synthesis. Obviously, this interaction cannot be one in which discrimination between the different tRNAs occurs.

SUMMARY

Evidence is presented that the pentanucleotide sequence –GpTpUpGp– occurs in all yeast transfer ribonucleic acids, and may also occur in all Escherichia coli and rat liver transfer ribonucleic acids.

Acknowledgment—We wish to thank Dr. M. Laskowski, Sr., for his advice and for his generous gift of micrococcal nuclease.

REFERENCES

Evidence for the Occurrence of a Common Pentanucleotide Sequence in the
Structures of Transfer Ribonucleic Acids
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