NUCLEOTIDE SEQUENCE OF THE ANTICODON REGION
OF BARLEY EMBRYO PHENYLALANINE TRANSFER RNA*

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Highly purified tRNA^Phe from barley embryos was completely digested with
pancreatic ribonuclease and T1 ribonuclease. The digestion products were separated
using DEAE-cellulose chromatography.

The Y base-containing fragment of the anticodon region of tRNA^Phe has the
following nucleotide sequence: CpmCgwpCpApGpApcmpUpGmpApYpAwp-CpUpGp, i.e. the same as in the anticodon region of wheat germ and pea tRNA^Phe.

The primary structure of a transfer RNA was elucidated first by Holley et al.
in 1965 and, although more than one hundred of these molecules have been se-
quenced so far (for review see Barrell & Clark, 1974), only few of them were from
higher plants. These were: a tRNA specific for glycine from wheat germ (Marcu
et al., 1977) and four phenylalanine tRNAs from wheat germ (Dudock & Katz.
1969), pea (Everett & Madison, 1976) and lupin seeds (Rafalski et al., 1977) and
from chloroplasts of bean leaves (Guillemout & Keith, personal communication).
The evident interest in the plant phenylalanine tRNA species is caused by the relative ease of tRNA^Phe isolation and the possibilities of comparisons with the known
sequences of tRNA^Phe from mycoplasma (Kimball et al., 1974), bacteria (Barrell
& Sanger, 1969; Guerrier-Takada et al., 1975), fungi (RajBhandary et al., 1967)
and mammals (Keith et al., 1973, 1974; Roe et al., 1975). This series of tRNA^Phe
species could provide new insight into the molecular mechanisms of evolution.

The known nucleotide sequences of mammalian phenylalanine-tRNA are identi-
cal, as are those of the cytoplasmic plant tRNA^Phe species. This suggests the
identity of isoaccepting tRNA in the related groups of organisms. To extend our
knowledge on homology of plant tRNAs we have undertaken an investigation of the
nucleotide sequence of the anticodon region of tRNA^Phe from barley embryos.

* This work was supported by the Polish Academy of Sciences within the project 09.7.
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MATERIALS AND METHODS

Chemicals

BD-cellulose was purchased from Serva (Heidelberg, G.F.R.), DEAE-cellulose DE-52 from Whatman (Maidstone, Kent, England), and RPC-5 from Miles Lab. (Elkhart, Ind., U.S.A.). Bio-Gel P-2 (100 - 150 mesh), AG 50W-X4 exchanger and Aminex A-7 were from Bio-Rad Laboratories (Richmont, Calif., U.S.A.). L-[14C]-Phenylalanine (522 mCi/n mole) was from the Radiochemical Centre (Amersham, England). All other chemicals were reagent grade products. The enzymes were obtained from Worthington (Freehold, N.J., U.S.A.) and used without further purification.

Preparation of tRNA\textsuperscript{Phe}

Crude barley embryo tRNA was isolated by the modified procedure of Dudock et al. (1969), as described by Augustyniak et al. (1976). tRNA\textsuperscript{Phe} was isolated from the crude tRNA by chromatography on BD-cellulose (Labuda et al., 1974), except that a salt gradient was used instead of the ethanolic gradient for elution of tRNA\textsuperscript{Phe} from the BD-cellulose column. The tRNA\textsuperscript{Phe}-containing fractions emerged at NaCl concentrations ranging from 1.4 to 1.75 M. The phenylalanine acceptance of tRNA coincided well with the typical fluorescence maximum at 420 - 430 nm when excited at 310 nm. The appropriate fractions were pooled. tRNA\textsuperscript{Phe} was precipitated with ethanol, and purified by RPC-5 chromatography at pH 5.0. The fractions showing fluorescence at 420 nm were collected and rechromatographed on RPC-5 at pH 7.5.

Aminoacylation of tRNA\textsuperscript{Phe} was performed as described by Labuda et al. (1974).

The fluorescence measurements were conducted at room temperature using a Hitachi MPF-4 spectrofluorimeter.

Enzymatic degradation of barley tRNA\textsuperscript{Phe}

Digestion with pancreatic RNase. tRNA\textsuperscript{Phe} (110 A\textsubscript{260} units\textsuperscript{*}) was dissolved in 6.0 ml of 0.1 M-Tris/HCl buffer, pH 7.5, mixed with the enzyme (470 µg) and incubated at 37°C for 70 min. The reaction was stopped by addition of solid urea to 7 M final concentration, and the mixture was fractionated by DEAE-cellulose chromatography.

Digestion with RNase T\textsubscript{1}. tRNA\textsuperscript{Phe} (435 A\textsubscript{260} units\textsuperscript{*}) was dissolved in 7.0 ml of 0.1 M-Tris/HCl buffer, pH 7.5, and incubated with 6000 units of RNase T\textsubscript{1} for 3.5 h at 37°C. The reaction was stopped by addition of solid urea to 7 M final concentration.

\textsuperscript{*} One A\textsubscript{260} unit equals the amount of material that gives absorbance of 1.0 at 260 nm when dissolved in 1 ml of water and measured in a cell of 1 cm path-length.
DEAE-cellulose chromatography of oligonucleotides

Whatman DE-52 DEAE-cellulose was washed successively with 0.2 m-KOH, water, 0.2 m-HCl, water and 1.0 m-NaCl, then suspended in water, degassed (50°C, in vacuum), packed into columns and equilibrated with an appropriate buffer containing 7.0 m-urea. Both enzymatic digests of tRNA\(^\text{P}^{\text{th}}\) were fractionated first at pH 7.2 - 7.8 (for details see legends to the Figures), and the fractions showing fluorescence at 420 nm were desalted and rechromatographed on DEAE-cellulose at pH 4.5. The oligonucleotides were freed from urea and salts by adsorption on a short DEAE-cellulose column (200 μl bed volume per 1 A\(_{260}\) unit of the desalted material). The columns were washed exhaustively with water, and the oligonucleotides were eluted with 2.0 m-ammonium bicarbonate, which was then evaporated under vacuum. Alternatively, the oligonucleotides were desalted by gel filtration on Bio-Gel P-2, 100 - 150 mesh.

All the DEAE-cellulose chromatograms were developed with linear NaCl gradients as indicated in the Figures. Buffers were delivered to the columns either by gravity (column length less than 100 cm, flow rate 6 ml/h) or with a Mikrotechna (Prague, Czechoslovakia) piston-type pump (columns exceeding 100 cm, flow rate 12 - 22 ml/h).

Enzymatic degradation of oligonucleotides

Digestion with pancreatic RNase. The oligonucleotides obtained on digestion with RNase T\(_1\) were further degraded with pancreatic RNase under the following conditions: 1 A\(_{260}\) unit of an oligonucleotide dissolved in 100 μl of 20 mm-Tris/HCl buffer, pH 7.5, was incubated with 5-20 μg of the enzyme for 2 h at 37°C, and the products were fractionated on DEAE-cellulose in the presence of 7 m-urea, pH 7.6.

Digestion with RNase T\(_1\). The oligonucleotides obtained by digestion with pancreatic RNase were further degraded with RNase T\(_1\). The incubation mixture contained 1 A\(_{260}\) unit of an oligonucleotide and 10-20 units of the enzyme in 100 μl of 20 mm-Tris/HCl buffer, pH 7.5. For degradation of the guanosine-rich oligonucleotides, concentration of the enzyme was increased by a factor of 5. The mixture was incubated at 37°C for 5 h and analysed by DEAE-cellulose chromatography.

Digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase. The 5'-end groups of the nucleotides were identified by complete degradation with snake venom phosphodiesterase (Khorana, 1961). Partial digestion with this enzyme was carried out according to Holley et al. (1964).

Stepwise degradation of the oligonucleotides was performed as follows: 1 A\(_{260}\) unit of an oligonucleotide was incubated at 37°C in 100 μl of 0.2 m-sodium acetate, pH 8.0, 0.1 m-MgCl\(_2\), with 15 μg of bacterial alkaline phosphatase. After 30 min, 5 μg of snake venom phosphodiesterase was added to the mixture, and the incubation was continued according to Holley et al. (1964) at 15-25°C (the oligo-
nucleotides free of modified nucleotides were digested at lower temperature). From the incubation mixture 10 μl aliquots were withdrawn every 5 min and applied directly to short (20-30 μl bed volume) DEAE-cellulose columns prewashed with water. The nucleosides were eluted with water; the undigested oligonucleotide residues were eluted with 2 M-ammonium bicarbonate.

Total degradation of the oligonucleotides was performed with snake venom phosphodiesterase and bacterial alkaline phosphatase to determine the nucleotide composition. The reaction mixture contained an oligo- (or mono-) nucleotide (1 A$_{260}$ unit), 10 μg of venom phosphodiesterase and 10 μg of alkaline phosphatase in 100 μl of 0.2 M-sodium acetate, pH 8.8; it was incubated at 37°C for 8 h.

**Identification of nucleosides**

The nucleosides were identified and quantitated by high-pressure liquid chromatography on Aminex A-7 as described by Sen & Ghosh (1974) using a DuPont 830 Liquid Chromatograph. Occasionally, the nucleosides were analysed by thin-layer chromatography (Rogg et al., 1976). Dihydouridine was identified by the sodium hydroxide-p-dimethylaminobenzaldehyde reagent (Fink et al., 1956) and was determined by monitoring the decrease in absorbance at 235 nm in alkaline medium (Molinaro et al., 1968).

**RESULTS AND DISCUSSION**

The asymmetrical peak observed on RPC-5 chromatography was due to the occurrence of two molecular forms of barley tRNA$^{Phe}$ (Labuda et al., 1974). For the sequence determination, the mixture containing both forms was used. The purity of the isolated tRNA$^{Phe}$, calculated from its amino acid acceptor activity (1.45 nmole of phenylalanine per 1 A$_{260}$ unit of tRNA), was over 90%; RPC-5 chromatography suggested an even higher degree of purification. One gram of crude barley tRNA yielded about 100 A$_{260}$ units of the purified tRNA$^{Phe}$.

The preparation of tRNA$^{Phe}$ used for the sequence determination showed a significant fluorescence maximum at 430 nm which indicates the presence of Y base in the tRNA investigated. Since in all the eukaryotic phenylalanine tRNAs sequenced so far Y base was found in the position adjacent to the 3' end of the anticodon, its fluorescence was used in the present paper as a marker of the oligonucleotides derived from the anticodon region of barley tRNA$^{Phe}$.

Oligonucleotides with fluorescence typical for Y base were found both in the pancreatic and in the RNase T$_{1}$ digests of tRNA$^{Phe}$. Among the products of the complete pancreatic digest of tRNA$^{Phe}$, separated by chromatography on DEAE-cellulose in 7 M-urea at pH 7.6 (see Fig. 1), only the fraction denoted as PVII showed the 430 nm fluorescence. This fraction on rechromatography on DEAE-cellulose at pH 4.5 (at lower pH the Y base could split off) yielded three peaks (PVII-1, PVII-2 and PVII-3, see Fig. 2A). Only fractions PVII-2 and PVII-3 exhibited fluorescence with the maxima at 420 and 430 nm, respectively.
Partial digestion of the PVII-3 oligonucleotide with phosphodiesterase was performed according to Holley et al. (1964) and yielded, in addition to mononucleotides, several partial degradation products (Fig. 2B) among which the fragment containing Gmp¹, two Ap and Y was identified. Its 5'-end was Gmp, recovered with about 30% yield. Since the action of phosphodiesterase slows down in the neighbourhood of Y base (RajBhandary et al., 1968), the sequence of this fragment should be GmpApApY. The alkaline hydrolysis (0.3 M-NaOH, 18 h, 40°C) of PVII-3 followed by digestion with alkaline phosphatase, yielded two A, ψ, and GmpA; Y was not detected, probably due to degradation during the hydrolysis. Combined with the known specificity of pancreatic RNase, these results allow the conclusion that the structure GmpApApYpApwp should be ascribed to PVII-3, which is in agreement with the total nucleotide composition of this hexanucleotide shown in Table 1.

The results of nucleotide analysis of PVII-2 suggested its heterogeneity. Therefore, this fraction, after excision of Y base according to Thiebe & Zachau (1968), was submitted to further chromatography at pH 2.9. As shown in Fig. 2C, lowering of the pH and removal of Y base allowed fractionation of PVII-2 into two fractions (peaks PVII-2a and PVII-2b). The nucleotide compositions of these fractions are shown in Table 1. The fraction PVII-2b was analysed in the same way as PVII-3 and yielded the same products. It was concluded, therefore, that the structure of Y base is the only difference between PVII-2b and PVII-3 (the fluorescence maximum of PVII-2 was shifted by 10 nm as compared with PVII-3). The Y base of the eukaryotic tRNA³⁹ (Thiebe et al., 1971) is not stable (Frihart, 1974) and undergoes

¹ Abbreviations used: Gm, 2'-O-methylguanosine; Gm, 2'-O-methylcytidine; γ, pseudouridine; m²G, N²,N²-dimethylguanosine; m⁴FA, 2-methylthio-N⁹-isopentenyladenosine.
Fig. 2. DEAE-cellulose chromatographic analysis of the pancreatic RNase digest of barley tRNA^\text{ps}.

A. Fraction PVII (cf Fig. 1) was rechromatographed on the DEAE-cellulose column (0.4 x 47 cm); elution was with a linear NaCl gradient (0.05 - 0.18 M) in 7 M-urea - 0.02 M-ammonium formate, pH 4.5. The total volume of the gradient was 300 ml; fractions of 3.3 ml were collected at a flow rate of 11 ml/h.

B. Fraction PVII-3 (cf Fig. 2A) was treated with snake venom phosphodiesterase (see Methods); the digest was applied on the DEAE-cellulose column (0.45 x 42 cm) and eluted with a linear NaCl gradient (0.0 - 0.3 M) in 7 M-urea - 0.02 M-Tris/HCl, pH 7.6. The total volume of the gradient was 100 ml; fractions of 1.6 ml were collected at a flow rate of 12 ml/h.

C. Fraction PVII-2 (cf Fig. 2A) was applied to the DEAE-cellulose column (0.3 x 30 cm) and eluted with a linear NaCl gradient (0.05 - 0.12 M) in 7 M-urea - 0.02 M-formic acid, adjusted to pH 2.9 with HCl. The total volume of the gradient was 80 ml; fractions of 1.0 ml were collected at a flow rate of 11 ml/h.
Table 1

Oligonucleotides from the anticodon region of barley tRNA\textsuperscript{Phe}

Methods used for sequence assignment: A, Alkaline hydrolysis; B, pancreatic RNase digestion; C, RNase T\textsubscript{1}, digestion; D, partial digestion with snake venom phosphodiesterase; E, stepwise degradation of oligonucleotide with bacterial alkaline phosphatase and snake venom phosphodiesterase; F, 5'-end determination; G, complete digestion with bacterial alkaline phosphatase and snake venom phosphodiesterase; H, chromatography on DEAE-cellulose in 7 M-urea (pH 7.6); I, chromatography on DEAE-cellulose in 7 M-urea (at acidic pH); J, high-pressure liquid chromatography; K, fluorescence measurements.

In PVII-2b, Y base was excised under mild acidic conditions.

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<th>Peak No.</th>
<th>Composition</th>
<th>Basis for sequence assignment</th>
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changes during storage of tRNA\textsuperscript{Phe} even at low temperature (Hancock et al., 1971), or in alkaline (Yoshikami & Keller, 1971) or acidic (Thiebe & Zachau, 1968) media. Separation of the Y-base-containing oligonucleotides into two fractions indicates that Y base in PVII-3 has a smaller positive charge than in PVII-2a. A similar modification of Y base in the intact barley tRNA\textsuperscript{Phe} might be responsible for its separation into two forms by the RPC-5 chromatography.

Alkaline hydrolysis of PVII-2a followed by the treatment with alkaline phosphatase yielded two A, G, and CmpU. Since its digestion with RNase T\textsubscript{1} resulted in the formation of ApGp and ApCmpUp, and since its 5'-end was Ap, it was concluded that the structure of PVII-2a was ApGpApCmpUp.

Chromatography of the RNase T\textsubscript{1} complete digest of tRNA\textsuperscript{Phe} on DEAE-cellulose in 7 M-urea at pH 7.6 is shown in Fig. 3. The fluorescence of fraction T\textsubscript{1}XI at 430 nm was due to the presence of Y base. Fraction T\textsubscript{1}XI was rechromatographed on DEAE-cellulose at pH 3.4, giving one major (T\textsubscript{1}XI-2) and one minor (T\textsubscript{1}XI-1) peak, both showing fluorescence typical for Y base. Alkaline hydrolysis of T\textsubscript{1}XI-2 yielded CmpUp, GmpAp and the mononucleotides: three Ap, Up, Yp, Cp and Gp (Y was not detectable by high-pressure liquid chromatography) showing that this fragment was a dodecanucleotide (for total composition see Table 1).

Complete digestion of T\textsubscript{1}XI-2 with pancreatic RNase resulted in the formation of mononucleotides Cp, Up, and Gp, trinucleotide ApCmpUp, and hexanucleotide GmpApApYpAypAyp. The sequence of ApCmpUp was unambiguously established from its nucleotide composition, determination of 5'-end, and the presence of
CmpUp in the alkaline hydrolysate. The structure of the hexanucleotide was elucidated as described for PVII-3. The stepwise degradation of T1 XI-2 with alkaline phosphatase and venom phosphodiesterase released successively G, U, and C, indicating that the oligonucleotide had the 3'-sequence ...CpUpGp. The sequential appearance of nucleosides during the stepwise degradation of T1 XI-2 permitted the conclusion that the sequence ApCmpUp was located at the 5'-side of the hexanucleotide. This conclusion was confirmed by identification of Ap at the 5'-end of T1 XI-2. The sequence of the whole dodecanucleotide was thus ApCmpUpGmp-ApApYpApψpCpUpGp.

Fraction T1 XI-1 was treated in the same way as T1 XI-2, yielding identical products as from T1 XI-2. It was concluded therefore that T1 XI-1 differed from T1 XI-2 only by a structural modification of the Y base.

Fraction T1 IX after rechromatography at pH 3.4 gave several minor and one major fraction, the latter denoted as T1 IX-5. Its complete digestion with pancreatic RNase yielded two Cp, m3Gψp, and ApGp. Since digestion of T1 IX-5 with excess RNase T1 resulted in the release of CmpGp, and since the 3'-end of T1 IX-5 was Cp, the 5'-terminal sequence of the oligonucleotide was CmpGpψp... Dinucleotide ApGp obtained from the pancreatic digest of T1 IX-5 derived obviously from the 3'-end; thus the whole oligonucleotide had the sequence: CmpGψpCpApGp.

The analysis of the barley tRNA\textsuperscript{Pas} fragments establishes the sequence of 18 nucleotides, as judged by the overlapping oligonucleotides liberated by pancreatic and T1 RNases (Table 2). The comparison with the anticodon sequences of other tRNA\textsuperscript{Pas} species (Table 2) shows that the anticodon regions of cytoplasmic tRNA\textsuperscript{Pas} from barley and from the other higher plants are identical. Preliminary results of
### Table 2

The anticodon sequences of tRNA\(^{Phe}\) from various sources

The oligonucleotides examined were isolated from cytoplasm (except where otherwise indicated).

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<tr>
<th></th>
<th>PVII-20</th>
<th>PVII-3</th>
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<tr>
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<td>25</td>
<td>30</td>
<td>35</td>
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<td>T,IX-5</td>
<td>T,IX-6</td>
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<tr>
<td>Yeast(^e)</td>
<td>C-m(\overline{G})-G-C-C-A-G-A-A-Cm-U-Gm-A-A-A</td>
<td>Y-A-y-m(\overline{G})C-U-G-</td>
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\(^a\) Dudock & Katz (1969)
\(^b\) Everett & Madison (1976)
\(^c\) Rokasli et al. (1977)
\(^d\) Keith et al. (1976), Rae et al. (1975)
\(^e\) Kistler & Stover (1974)
\(^f\) Guerrier-Takada et al. (1975)
\(^g\) Chong et al. (1976)
\(^h\) Guerrier-Takada & Keith (personal communication)
\(^i\) Kimball et al. (1975)
\(^l\) Kistler & Stover (1974)

the analysis of other fragments of barley tRNA\(^{Phe}\) indicate their great similarities to the fragments of wheat germ tRNA\(^{Phe}\) (Dudock & Katz, 1969). No oligonucleotides were found that could suggest replacement of G-C base pair in TyC stem by A-U pair, as recently described by Rafalski et al. (1977) for lupin tRNA\(^{Phe}\). Thus, it is probable that the primary structure of the barley tRNA\(^{Phe}\) molecule is identical with that of tRNA\(^{Phe}\) from wheat germ, at least, within the anticodon region.

We are grateful to Professor Dr. J. Pawelkiewicz for his interest and helpful discussions. We thank Miss Małgorzata Krotofil for her skillful technical assistance.

**REFERENCES**


SEKWENCJA NUKLEOTYDOWA ANTYKODONOWEGO REGIONU tRNA FENYLOALANINOWEGO Z ZARODKÓW JĘCZMENIA

Streszczenie


Received 10 August, 1977.