Sequence studies of nonradioactive *Mycoplasma* tRNA^{Phe} with the aid of polynucleotide phosphorylase and polynucleotide kinase

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ABSTRACT

The known methods of enzymatic phosphorylation with [³²P]phosphate of the 3'- or 5'-hydroxyl group of an oligonucleotide have been applied to oligonucleotides derived from *Mycoplasma* tRNA^{Phe}. The fingerprints obtained by both methods are very similar to each other and to that of uniformly labelled tRNA. The sequence of some oligonucleotides was determined by partial digestion of the 3'-phosphorylated fragment with spleen phosphodiesterase and of the corresponding 5'-phosphorylated fragment with venom phosphodiesterase.

INTRODUCTION

Although uniformly labelled [³²P]RNAs are most widely used for nucleotide analysis, sequence analysis of non-radioactive RNA using polynucleotide kinase has been very successful (1-3). In this technique the 5'-hydroxyl termini of oligonucleotides are enzymatically phosphorylated with [³²P]phosphate. We have previously described a complementary method of phosphorylating with radioactive phosphate using polynucleotide phosphorylase (4) the 3'-end of oligonucleotides derived by T₁ RNase digestion of unlabelled tRNA (5). In this paper we extend the method to pancreatic RNase fingerprints. The most useful application of the PNPase labelling method is its use in conjunction with the phosphorylation of the 5'-hydroxyl end of oligonucleotides by polynucleotide kinase. As an example we studied nonradioactive *Mycoplasma* tRNA^{Phe}. The T₁ RNase fingerprints obtained by both methods are very similar. The comparison of the partial venom phosphodiesterase digests of the 5'-labelled oligonucleotide with that of the partial spleen phosphodiesterase digest of the corresponding 3'-labelled oligonucleotide allowed complete sequence determination of a penta- and a hexanucleotide selected as examples. The sequence of these T₁ RNase fragments was needed in order to establish the total nucleotide sequence of *Mycoplasma* tRNA^{Phe} (6).

MATERIALS AND METHODS

General. [α - 32 P]GDP (specific activity 5.8 Ci/mmol) and [α - 32 P]UDP (specific activity 6.5 Ci/mmol) were obtained from New England Nuclear Corporation. Purified *Mycoplasma* sp. (Kid) tRNA^{Phe} with a specific phenylalanine acceptor activity of 1.5 nmoles/A₂₆₀ unit was given by Dr. M.E. Kimball (7). T₁ RNase (Sankyo) was purchased from CalBiochem, bacterial alkaline phosphatase and pancreatic RNase were obtained from Worthington Biochemical Corp., and spleen phosphodiesterase and yeast hexokinase were products of Boehringer-Mannheim Corp. Snake venom phosphodiesterase, T₄ polynucleotide kinase and (γ - 32 P]ATP (specific activity 30 Ci/mmol) were kindly provided by Dr. U.L. RajBhandary. Primer dependent *M. luteus* PNPase (8) was a generous gift of Dr. M.F. Singer. The trinucleotides UpCpA and CpUpC were chemically synthesized (9) and a gift of Dr. H.G. Khorana.

End Group Labelling of Oligonucleotides Derived from tRNA by Action of T₁ RNase. The procedure involves the following steps: (i) Degradation of tRNA with T₁ RNase, (ii) Removal of the 3'-phosphomonoester groups of the oligonucleotides by bacterial alkaline phosphatase, (iii) Inactivation of alkaline phosphatase by nitrilotriacetic acid, (iv) Phosphorylation of the 3'-hydroxyl groups by the action of PNPase, [α - 32 P]GDP and T₁ RNase (5); or alternatively phosphorylation of the 5'-hydroxyl groups with [γ - 32 P]ATP and polynucleotide kinase, followed by destruction of the excess ATP by phosphorylation of glucose to glucose-6-phosphate catalyzed by yeast hexokinase (2).

The incubation mixture (100 μ l) contained 0.5 A₂₆₀ units pure tRNA^{Phe} and 2.5 units of T₁ RNase in 50 mM Tris-HCl (pH 7.5). After 3 hr at 37° alkaline phosphatase (1 μ g, 0.026 units) was added and the reaction continued at 37° for 2 hr. Then a solution of nitrilotriacetic acid (40 mM, pH 7.5) was added to a final concentration of 5 mM. After further incubation for 20 min at room temperature, the mixture was held at 100° for 2 min and then quickly cooled. This mixture was frozen and kept as a stock solution for the labelling experiments.

For the labelling of the 3'-hydroxyl groups (5) the incubation mixture (50 μ l) contained 80 mM Tris-HCl (pH 8.2) - 2 mM MgCl₂ - 80 μ g/ml bovine plasma albumin, 5 μ l of the above digest (containing 0.5 nmoles of 3'-hydroxyl termini), 2 nmoles of [α - 32 P]GDP, 0.2 units of primer dependent PNPase and 0.5 units of T₁ RNase. After incubation at 37° for 210 min the mixture was evaporated to dryness and fingerprinted by the standard

procedures.

For the labelling of the 5'-hydroxyl groups (2) the incubation mixture (10 μ l) contained 2.5 μ l of the above digest, 1.5 nmoles [γ - 32 P]ATP, 2 units of T_4 polynucleotide kinase and 10 mM $MgCl_2$ - 15 mM Tris-HCl (pH 8.0) - 15 mM 2-mercaptoethanol. After incubation at 37° for 30 min, 25 nmoles of glucose and 0.1 μ g of yeast hexokinase were added. After 5 min at 37°, 2.5 nmoles of ATP were added and the incubation continued at 37° for 5 min. This step was repeated once. After heating to 100° for 2 min the mixture was ready for fingerprinting.

3'-End Group Labelling of Oligonucleotides Derived from tRNA by the Action of Pancreatic RNase. This procedure is very similar to the one described for T_1 RNase digests (5) and involves the following steps: (i) Degradation of tRNA with pancreatic RNase, (ii) Removal of the 3'-phosphomonoester groups of the oligonucleotides by bacterial alkaline phosphatase, (iii) Inactivation of alkaline phosphatase by nitrilotriacetic acid, (iv) Phosphorylation of the 3'-hydroxyl groups by the action of PNPase, [α - 32 P]-UDP, and pancreatic RNase.

The incubation mixture (100 μ l) contained 0.5 A_{260} units of pure tRNA^{Phe} and 1 μ g of pancreatic RNase in 50 mM Tris-HCl (pH 7.5). After 3 hr at 37° alkaline phosphatase (1 μ g, 0.026 units) was added and the reaction continued for 2 hr. After further incubation for 20 min at room temperature, the mixture was held at 100° for 2 min and then quickly cooled. This mixture was frozen and kept as a stock solution for the labelling experiments.

For labelling the 3'-hydroxyl ends the incubation mixture (50 μ l) contained 80 mM Tris-HCl (pH 8.2) - 2 mM $MgCl_2$ - 80 μ g/ml bovine plasma albumin, 5 μ l of the above digest (containing 1 nmole 3'-hydroxyl termini), 1-2 nmoles of [α - 32 P]UDP, 0.2 units of primer dependent PNPase and 1.5 μ g of pancreatic RNase. After incubation at 37° for 210 min the mixture was evaporated to dryness and fingerprinted.

Secondary Analysis. The labelled fragments were eluted from the fingerprints and either completely hydrolyzed by pancreatic or T_1 RNase, or partially degraded by snake venom or spleen phosphodiesterase and the products separated by electrophoresis on DEAE paper at pH 3.5 or pH 1.9 (10).

Fingerprinting Procedures. The standard procedures described by Barrell (10) were used.

RESULTS

In a previous publication we reported on the enzymatic phosphorylation with [32 P]phosphate of the 3'-hydroxyl group of oligonucleotides derived from T_1 RNase digests. We have extended this technique to label the oligonucleotides from pancreatic RNase digests using [α - 32 P]UDP, PNPase and pancreatic RNase. As an example, the pancreatic fingerprint of *Mycoplasma* tRNA^{Phe} is shown in Figure 1. A comparison of this figure with Figure 1 of the preceding paper (6) shows the fingerprint derived from nonradioactive tRNA^{Phe} and that of uniformly labelled tRNA^{Phe} to be very similar. With the exception of Cp and Up, the number of fragments observed in both fingerprints is the same. However, the molar ratios are lower for the smaller oligonucleotides found in the map of 3'-[32 P]phosphate fragments (Table I). This is not surprising, since the ability of short oligonucleotide to serve as primer for polynucleotide phosphorylase is lower than that of longer oligonucleotides (11). When higher concentrations of UDP were used in the reaction the yields did not improve significantly. The two major spots in Figure 1 are ppU, the starting material, and Up, its conversion product after PNPase and pancreatic RNase action. The relative ratios of ppU and Up indicate that the PNPase reaction proceeds only to 50%. This is in agreement with earlier observations (see e.g. 12) of the equilibrium of the reaction of PNPase.

A useful application of labelling the 3'-hydroxyl ends of oligonucleotides is in conjunction with the enzymatic phosphorylation of the 5'-hydroxyl groups by polynucleotide kinase: RNA is digested with T_1 RNase and the fragments dephosphorylated. Then one aliquot of the mixture is phosphorylated at the 5'-end by polynucleotide kinase and another aliquot phosphorylated at the 3'-end with PNPase. The fingerprints of the two preparations should be very similar (1). The only exception would be the oligonucleotide from the 3'-end of the original RNA, if the terminal base is not G. A complete sequence determination of the oligonucleotides should be possible by comparing one-dimensional electrophoretic separations of the partial venom phosphodiesterase digest of a 5'-labelled oligonucleotide with that of the partial spleen phosphodiesterase digest of the corresponding 3'-labelled oligonucleotide. Observation of the mobility shifts (1,13) of the sequential partial degradation products should make further enzymatic analysis unnecessary. The different set of fragments produced by the action of the two exonucleases (progressing in opposite directions) leads to complementary information and serves as a mutual control. As an example we

Fig. 1. Two-dimensional fractionation of a pancreatic RNase digest (labelled at the 3'-end) of *Mycoplasma* tRNA^{Phe}.

Table I

Molar Yields and Characteristics of Oligonucleotides

Spot Number	Structure of Oligonucleotide ^a	Molar Yield			T ₁ RNase digestion products found
		UDF concentration 20 μ M	40 μ M	Theoretical (from sequence) ^b	
3	A-C-	0.3	0.4	3	A-C-
4	m ⁷ G-U-	0.15	0.2	1	m ⁷ G-U-
5	G-C-	0.3	0.25	1	C-
6	G-A-C-	1	1.2	1	A-C-
7	A-G-C-	2.2	2.3	2	C-
8	A-A-U-	0.9	0.9	1	A-A-U-
9	A-G-A-C	0.9	1.0	1	A-C-
10	G-U-	0.3	0.4	5	U-
11	G-G-C-	1.1	1.3	1	C-
12	A-G-U-	1.0	1.0	1	U-
13	G-A-A-m ¹ G-C-	0.6	0.6	1	C-
14	A-G-A-G-C	0.7	0.6	1	C-
15	G-G-U- + G-G-D-	2.9	2.8	3	U- + D- ^b

^aSequences as determined for uniformly labelled tRNA (6).

^bDetermined by thin layer chromatography (see 6).

Fig. 2. Two-dimensional fractionation of a T_1 RNase digest (labelled at the 3'-end) of *Mycoplasma* tRNA^{Phe}.

Fig. 3. Two-dimensional fractionation of a T_1 RNase digest (labelled at the 5'-end) of *Mycoplasma* tRNA^{Phe}. A is glucose-6- $[^{32}P]$ phosphate.

attempted to determine the unknown base sequence of a penta- and a hexanucleotide of *Mycoplasma* tRNA^{Phe} (6). First, a T₁ RNase fingerprint of the 3'-[³²P] phosphorylated oligonucleotides (Fig. 2) and a corresponding fingerprint of the 5'-[³²P] phosphorylated oligonucleotides (Fig. 3) were made. Both fingerprints are very similar to each other and to the fingerprint of uniformly labelled RNA (Fig. 2 in the preceding paper). The major product in Figure 3 is glucose 6-[³²P]phosphate (A) which is the destruction product of [γ -³²P]ATP, the phosphate donor in the polynucleotide kinase reaction (2). In the fingerprint of the 3'-phosphorylated fragments (Fig. 2) ppG is absent. Confirming our earlier observations (5) this indicates that the equilibrium of the PNPase reaction with GDP lies on the side of the oligonucleotide. The only difference between the two maps is the location of oligonucleotide t6, the 3'-terminal fragment of the tRNA with the sequence A-C-C-A-C-C-A_{OH} (6). In Figure 2 it has the sequence A-C-C-A-C-C-A-Gp, as shown by the appearance of A-Gp as the product of pancreatic RNase digestion (data not shown). The compound is the result of end addition of a G residue to the terminal adenosine of tRNA by PNPase. However, in Figure 3, the expected product is pA-C-C-A-C-C-A_{OH}, which

Fig. 4. Partial spleen phosphodiesterase digest of T₁ RNase end products (3'-phosphorylated) T12, T13, and T14 from Figure 2. Aliquots from each enzymic digest were taken after 0, 2, 5, and 10 min and applied to DEAE paper.

should move in the position indicated (analysis was not performed). The composition of the oligonucleotides was analyzed only in some cases and agrees with those given in Table II of the preceding paper (6).

Since the base sequences of oligonucleotides t12, t13, and t14 were not known (6), these fragments were isolated from both fingerprints, eluted, and subjected to limited digestion with spleen phosphodiesterase or venom phosphodiesterase. Two authentic markers, [32 P]pC-U-C and [32 P]pU-C-A were used as standards. Figures 4 and 5 show the electrophoretic separation of the resulting fragments on DEAE paper at pH 1.9. It is apparent from these results that the sequence of t12 is C-U-C-A-G, of t13 U-C-C-A-C-G, and of t14 U-m⁷G-U-C-G (cf. preceding paper).

Fig. 5. Partial snake venom phosphodiesterase digest of T₁ RNase end products (5'-phosphorylated) t12 and t13 from Figure 3 and of pU-C-A and p-C-U-C. Aliquots from each enzymic digest were taken after 0, 2, 4, 8, and 10 min and applied to the DEAE paper. Since the higher enzyme concentration used gave too much degradation, results of a digest with less enzyme are also shown.

DISCUSSION

The enzymatic phosphorylation with [32 P]phosphate of the 5'-hydroxyl group of deoxyribo- or ribooligonucleotides by polynucleotide kinase has been a useful tool in nucleic acid sequence analysis (1-3). The enzymatic phosphorylation of the 3'-hydroxyl has been more difficult. While terminal nucleotidyl transferase allows in a more complex procedure the phosphorylation with radioactive phosphate at the 3'-end of deoxyoligonucleotides (14), dephosphorylated ribooligonucleotide fragments derived from T₁ RNase

digests of RNA can be readily phosphorylated by the combined action of PNPase and T_1 RNase using $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ (5). This procedure has now been extended to pancreatic RNase digest of tRNA.

A combination of both methods of phosphorylation has distinct advantages for sequence analysis of nonradioactive RNA. The fingerprints obtained from nonradioactive *Mycoplasma* tRNA^{Phe} by enzymatic phosphorylation of either the 5'-ends (2) or of the 3'-ends (5) and from uniformly labelled *Mycoplasma* tRNA^{Phe} are very similar. The only difference was found in the position of the 3'-terminal oligonucleotide which can be easily identified in this way.

The availability of the same oligonucleotide phosphorylated in one case at the 3'-end and in the other at the 5'-end allows rapid sequence analysis of these oligonucleotides by comparing the products of their limited digestion with spleen and venom phosphodiesterase, respectively. The products need not be further analyzed; their sequence can be deduced from the mobility shifts (1,13) on one-dimensional electrophoresis produced by the successive loss of a nucleotide residue. If, in the case of larger oligonucleotides, a clear characterization cannot be made from the 5'-labelled fragment, for example, analysis of the corresponding 3'-labelled fragment will give the answer. However, the presence of modified nucleotides requires further analysis. In this way oligonucleotides (at least up to chain length 6) can be sequenced easily. The analysis of larger oligonucleotides can also be achieved by the micro method of Randerath (15) which involves successive elimination of the 3'-terminal base by periodate oxidation and subsequent base elimination. Therefore it appears feasible that a combination of the three methods will allow sequence determination of nonradioactive tRNA in microgram amounts.

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4. Abbreviations used: T₁ RNase, T₁ ribonuclease; PNPase, polynucleotide phosphorylase; nucleotides are abbreviated according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (1970) *Biochemistry* 9, 4022; GDP, ppG, guanosine 5'-diphosphate; Gp, guanosine 3'-phosphate; UDP, ppU, uridine 5'-diphosphate; Up, uridine 3'-phosphate.
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