Fingerprinting nonradioactive ribonucleic acid with the aid of polynucleotide phosphorylase

Kwok S. Szeto and Dieter Söll

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520

Received 19 November 1973

ABSTRACT

We describe a method for obtaining radioactive fingerprints from non-radioactive ribonucleic acid. Fragments derived by T1 ribonuclease digestion of RNA are dephosphorylated with bacterial alkaline phosphatase. When these fragments are used as primers for the reaction of primer dependent polynucleotide phosphorylase with [α - 32 P]GDP in the presence of T1 ribonuclease the 3'-hydroxyl group of each fragment becomes phosphorylated. The degree of phosphorylation is reasonably uniform. The method has been applied to T1 ribonuclease digests of *Escherichia coli* tRNA^{Met}; the oligonucleotides were further analyzed by spleen phosphodiesterase F digestion. In a similar manner fingerprints of pancreatic ribonuclease digests of RNA can be obtained, when [α - 32 P]UDP, polynucleotide phosphorylase and pancreatic ribonuclease are used.

INTRODUCTION

The electrophoretic and chromatographic techniques developed by Sanger and his colleagues for the fractionation of radioactive oligonucleotides^{1,2} have been used extensively for determination of nucleotide sequences of RNAs. However, many RNA species cannot be labeled sufficiently *in vivo* or are unavailable for labeling by *in vitro* transcription³. Therefore methods for the fingerprinting of non-radioactive RNAs are needed. To date one such method exists^{4,5}: the enzymatic phosphorylation of the 5'-hydroxyl group in ribooligonucleotides with polynucleotide kinase.

In this paper we describe an alternate approach which allows phosphorylation of the 3'-hydroxyl group of ribooligonucleotides with [32 P]phosphate. It is based on the action of primer dependent polynucleotide phosphorylase⁶, an enzyme which has been successfully used in the synthesis of ribooligonucleotides⁷⁻⁹. The principle of our method is outlined on Chart I. tRNA

is first digested with T₁ RNase¹⁰ to oligonucleotides ending in -Gp. Their terminal phosphomonoester group is removed by alkaline phosphatase to yield a free 3'-hydroxyl group. The resulting oligonucleotides serve as primers for primer dependent PNPase in a reaction with [α -³²P]GDP. The simultaneous action of T₁ RNase cleaves the added oligoguanylate sequence after the 3'-terminal G of the original oligonucleotide, which is now terminated with a radioactive 3'-phosphomonoester group. The oligoguanylate is degraded to Gp, resulting in an overall conversion of GDP to Gp.

Chart I

MATERIALS AND METHODS

General. [α - 32 P]GDP with a specific activity of 14.6 Ci/mmol was obtained from New England Nuclear Corporation. Purified *E. coli* K12 tRNA_f^{Met} with a specific methionine acceptor activity of 1.5 nmoles/A₂₆₀ unit was obtained from Dr. A.D. Kelmers of the Oak Ridge National Laboratory. T1 RNase (Sankyo) was purchased from CalBiochem, bacterial alkaline phosphatase and pancreatic RNase were obtained from Worthington Biochemical Corp., and spleen phosphodiesterase was a product of Boehringer-Mannheim Corp. U2 RNase was a kind gift of Dr. H. Okazaki, Sankyo Chemical Co., Tokyo. Primer dependent *M. luteus* PNPase⁶ was a generous gift of Dr. M.F. Singer.

3'-End Group Labeling of Oligonucleotides Derived from tRNA by Action of T1 RNase. The procedure (outlined in Chart I) 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.

The incubation mixture (100 μ l) contained 0.5 A₂₆₀ units pure tRNA_f^{Met} 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 labeling experiments.

For the labeling of the 3'-hydroxyl groups 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), 0.7 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, taken up in 5 μ l of water and then fingerprinted by standard two-dimensional electrophoretic procedures^{1,2}. Under these conditions all GDP was converted to Gp. When a more quantitative labeling of CG containing oligonucleotides (e.g., CpG) was desired, 2 nmoles of [α - 32 P]GDP and 1 unit of T1 RNase was used in the reaction. In this case not all the GDP was converted to Gp and oversplitting (by T1 RNase) of larger oligonucleotides was observed.

RESULTS

The preparation of the dephosphorylated tRNA fragments for labeling was performed essentially as described by Simsek, *et al.*⁵: The tRNA was digested with T1 RNase, the resulting oligonucleotides dephosphorylated by alkaline phosphatase, and the latter enzyme inactivated by incubation with nitrilotriacetic acid.

The addition of guanylate residues to the resulting oligonucleotides by primer dependent PNPase was carefully investigated. In order to give uniform labeling, excess [α - 32 P]GDP (over the 3'-hydroxyl termini of the primers) and a high concentration of PNPase is desired. This in turn requires a high concentration of T1 RNase to cleave the oligoguanylate chains added to the primer. However, high concentrations of the latter enzyme cause overdigestion^{1,4} of the larger oligonucleotide primers, leading to lower molar yields of those oligonucleotides and giving rise to the appearance of new ones. The experimental conditions given above represent the compromise by which both uniform labeling and least RNA breakdown were best realized. Moreover the excess of [α - 32 P]GDP was converted to radioactive Gp, the product of final degradation.

In order to test our method, purified *E. coli* tRNA_F^{Met} was chosen, since its nucleotide sequence is known¹¹ and two-dimensional fingerprints of

uniformly labeled tRNA have been published¹². The autoradiogram of the 3'-[³²P]phosphate-labeled oligonucleotides in a T1 RNase digest is shown in Fig.1. The fingerprint resembles that of the uniformly labeled tRNA¹². As can be seen the labeling of the various oligonucleotides is not quite uniform and some oversplits have occurred. Table I gives a summary of the molar yields

Figure 1.

Two-dimensional fractionation of a T1 RNase digest of *E. coli* tRNA_f^{Met}. The structure of the nucleotides is given in Table I.

TABLE I
MOLAR YIELDS AND CHARACTERISTICS OF OLIGONUCLEOTIDES

Spot Number	Structure of Oligonucleotide ^a	Molar Yield		Theoretical (from sequence ¹¹)	Digestion Products Found	
		Standard GDP concentration ^c	High GDP concentration ^c		Pancreatic RNase	U2 RNase
t1	CG	0.2	0.8	2	G	
t2	AG	0.6	1.0	1	AG	
t3	CAG	0.6	0.5	1	AG	
t4	AAG	1.1	0.8	1	AAG	
t5	CCCCCG	0.6	0.6	1	G	
t6	UG	---	---	1		
t7	DAG	1.0	1.0	1	AG	
t8	UCG	2.0	1.7	2	G	
t9	CUUG/CCUG ^b	2.4	2.5	2	G	
t10	m ⁷ GUUG ^b	0.7	0.6	1	G	
t11	CAACCAG	0.8	0.9	2	AG	
t12	CmUCAUAACCG ^b	0.5	0.3	1	G	CCCG
t13	T ₄ CAAAUCCG ^b	0.6	0.4	1	G	(U,C ₂)G
X		0.9	1.1		AG+G	

^aSequences identical to those determined by Dube, *et al.*¹¹.

^bPartial sequences determined were consistent with reported structure¹¹

^cSee Materials and Methods.

of each oligonucleotide. As expected from previous work⁷ CpG is not a good primer. This may be due to aggregation of this compound in solution. Heating of the fragment mixture before labeling with PNPase⁷ may increase the yield of enzymatic phosphorylation of CG rich oligonucleotides.

The lower molar ratios of fragments t5 and t11 may have a similar explanation. The lower yield of the large fragments t12 and t13 may be due in part to loss during transfer as is often observed, but also to oversplitting by T1 RNase as suggested by the data in Table I. Such split products may account for the high yield of fragment t9. However, it must be kept in mind that the starting unlabeled tRNA, although at least 90% pure, may be contaminated with other oligonucleotides or tRNAs which would also be labeled

by PNPase. The most striking omission in this map is the virtual absence of fragment t6, UG.

This is not due to the inability of PNPase to use UG as a primer. In a fingerprint (not shown) obtained by the same method of non-radioactive *E. coli* tRNA₁^{Leu} UG was formed in good yield. The oligonucleotide UG in tRNA_f^{Met} should contain 4-thiouridine (nucleotide #8 from the 5'-end). This modified nucleotide is known to cross-link with the cytidine residue (nucleotide #13)¹³. From other experiments¹⁴, we know that about 50% of our tRNA_f^{Met} preparation was cross-linked. On T1 RNase digestion such a cross-linked tRNA would yield UG covalently linked to CAG, fragment t3. This might be the reason for the lower yield of fragment t3 and for the appearance of spot X. This spot, found also by Dube, *et al.*¹² and by Szekeley and Sanger,⁴ contains AG and G (see Table I and ref. 12). On the whole, the extent of labeling obtained by our procedure is satisfactory and compares with that given for the polynucleotide kinase method⁵.

A first characterization of the oligonucleotides was obtained by digestion with pancreatic and U2 RNase (Table I). The sequence of nucleotides was established by partial digestion with spleen phosphodiesterase. The digests were applied to electrophoresis at pH 1.9 on DEAE paper and the products identified on the basis of M values¹ and cochromatography with some markers (Fig. 2). This procedure gives clear results for short oligonucleotides, but only partial sequences are obtained with some larger oligonucleotides. Confirming evidence comes from the pancreatic and U2 RNase digests. As can be seen from Fig. 1 and from the characterization of the radioactive oligonucleotides our data are in agreement with those of Dube, *et al.*¹² obtained by sequencing uniformly labeled tRNA_f^{Met}. Two differences on the autoradiogram obtained by the two methods are pertinent to mention. The oligonucleotide pCpGp located at the 5'-end of the tRNA is missing in our fingerprints, since after phosphatase treatment and labeling it will be converted to CpGp.

Figure 2. Diagram of partial spleen phosphodiesterase digests of some T1 RNase fragments of *E. coli* tRNA^{Met} fractionated on DEAE paper at pH 1.9. Thick lines indicate position of undigested controls.

Likewise, the 3'-terminal oligonucleotide CpApApCpCpA_{OH} is converted after PNPase treatment to CpApApCpCpApGp (t11) and will be in a different position on the fingerprint. In addition, it contains two radioactive phosphate groups.

DISCUSSION

The work presented in this paper shows that primer dependent PNPase can be used to label digests of very small amounts of non-radioactive RNA. Approximately one μ g of tRNA is needed for a fingerprint. Using [α -³²P]GDP, PNPase and T1 RNase, the ratios of the expected oligonucleotides found in a fingerprint of tRNA_f^{Met} were satisfactory. However, some additional spots were observed. Because the concentration of inorganic phosphate cannot be higher than that of GDP and is very low, these probably do not arise from phosphorylytic cleavage of the oligonucleotides by PNPase. Apart from contamination of the original RNA preparation, a reason for side products is the ability of T1 RNase to produce oversplits^{1,4}.

The same technique can also be applied to obtain fingerprints of pancreatic RNase fragments of RNA. In this case [α -³²P]UDP and PNPase in conjunction with pancreatic RNase must be used. Since of the four nucleoside diphosphates tested, UDP gave the highest yield in reactions with nucleoside diphosphates as primers⁷, much more uniform labeling would be expected in such fingerprints.

Since this method labels each oligonucleotide in the 3'-phosphate, further analysis of these oligonucleotides can be obtained by degradation with pancreatic RNase, by U2 RNase or by partial degradation with spleen phosphodiesterase. The last method yields clear information, since the action of contaminating endonucleases will give products which lack the 3'-terminal nucleotide and are therefore unlabeled. The nucleotide sequence of shorter oligonucleotides can be determined this way. However, the complete sequence analysis of longer oligonucleotides is difficult by these methods. But in

analogy to the standard RNA sequencing methods, RNA fragments from primary T1 RNase digestion labeled in the 3'-phosphate could be subjected to secondary analysis by degradation with pancreatic RNase. The resulting oligonucleotides, after dephosphorylation, could be phosphorylated by PNPase with [α - 32 P]UDP in the presence of T1 and pancreatic RNase.

The most useful application of labeling the 3'-hydroxyl ends of oligonucleotides may be in conjunction with the enzymatic phosphorylation of the 5'-hydroxyl groups by polynucleotide kinase: RNA is digested with T1 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 similar⁴. The only exception would be the oligonucleotide from the 3'-end of the original RNA if the terminal base is not G. A comparison of the partial venom phosphodiesterase digest of a 5'-labeled oligonucleotide with that of the partial spleen phosphodiesterase digest of the corresponding 3'-labeled oligonucleotides should allow complete sequence determination even of large oligonucleotides.

ACKNOWLEDGEMENTS

We are indebted to Drs. H.U. Blank and M.F. Singer for many stimulating discussions in the initial stages of this project. We thank Drs. J. Steitz and S. Weissman for advice on the fingerprinting of radioactive RNA, and we are grateful to Drs. M.F. Singer, A.D. Kelmers, and H. Gordon for their generous gifts of materials. This work was supported by grants from the National Institutes of Health (GM-15401) and from the National Science Foundation (GB-36007X) and (GB-39868).

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10. Abbreviations used: T1 RNase, T1 ribonuclease; PNPase, polynucleotide phosphorylase; nucleotides are abbreviated according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (1971), *J. Mol. Biol.* 55, 299-305; GDP, guanosine 5'-diphosphate; Gp, guanosine 3'-phosphate.
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