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A Novel Method for Sequencing Non-Radioactive Nucleic Acids

Hiroyuki SUGISAKI*

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A simple and precise method for obtaining fingerprints from non-labeled nucleic acids has been developed. In this method, the 5′-hydroxyl groups of oligonucleotides produced by specific digestion of nucleic acids are phosphorylated with ^{32}P in the polynucleotide kinase reaction and then fractionated by two-dimensional chromatography on polyethyleneimine (PEI)-cellulose thin layer plates. Under the condition used for fingerprinting, ($\gamma^{32}P$)ATP as phosphate donor stays near the origin, so that the reaction mixture for phosphorylation can be directly applied onto PEI-cellulose plates without removing ATP. This makes the fingerprinting procedure more simple and reproducible. It is also possible to deduce the sequences of non-labeled oligonucleotides precisely. This method will be very useful in studying the primary structure of nucleic acids.

INTRODUCTION

Since the fingerprinting technique for radioactive oligonucleotides has been developed by Sanger *et al.*,[1] this method has widely been applied for the sequence determination of various RNA species. For sequencing RNA by this method, it is necessary to obtain RNA uniformly labeled with ^{32}P at high specific activity. Not all RNA can, however, be obtained in a highly labeled form. It is especially difficult to prepare uniformly labeled RNA from eukaryotic cells. If fingerprints from non-labeled nucleotides can be obtained, it would be very useful not only for the sequence analysis but also for the characterization of nucleic acid species. In order to obtain fingerprints from non-labeled nucleic acids, Székely and Sanger first attempted to use the polynucleotide kinase method[2], which has been applied for analysis of the 5′-terminal nucleotide sequences.[3,4] The procedure developed by Székely and Sanger was further modified by Simsek *et al.*[5,6] and applied for the characterization of cytoplasmic initiator tRNA of eukaryotic cells. In this method, oligonucleotides containing 5′-hydroxyl groups are phosphorylated with ^{32}P in the polynucleotide kinase reaction, and then fractionated by two-dimensional electrophoresis on cellulose acetate strip-DEAE-cellulose paper. For a maximum phosphorylation of 5′-hydroxyl groups, however, an excess amount of ($\gamma^{32}P$)ATP as phosphate donor is required, and after the reaction, unreacted ATP should be removed from the reaction mixture before fractionation, because ATP streaks on the paper and obscures some of the labeled oligonucleotides. Thus, Székely and Sanger destroyed ATP with myosin ATPase[2] and Simsek *et al.* with hexokinase.[5,6]

In developing this polynucleotide kinase method further, I found that ATP

* 杉崎弘幸 : Laboratory of Molecular Biology, Institute for Chemical Research, Kyoto University, Uji, Kyoto.

stays near the origin on polyethyleneimine(PEI)-cellulose thin layer plates under the conditions for fingerprinting oligonucleotides. By using this two-dimensional system, therefore, the reaction mixture used for the polynucleotide kinase reaction can be directly applied onto the thin layer plates without removing ATP: this makes the fingerprinting procedure more simple and reproducible. This procedure is also applicable for analysis of the base composition and sequence of an oligonucleotide. By applying these procedures, I have constructed the total sequence of a tRNA species and also determined the sequence of pyrimidine tracts of DNA. This paper describes the detailed procedures I have developed for the sequence determination of non-labeled nucleic acids.

MATERIALS AND METHODS

1. Preparation of Oligonucleotides for Fingerprinting

a) *RNase-digests of RNA*: About 25 μg of RNA with adequate size (*e.g.* tRNA, ribosomal 5s RNA or transcripts of DNA fragments) was mixed with 0.15 unit of *E. coli* alkaline phosphatase and either 3 units of T1 RNase or 3 μg of pancreatic RNase in 45 μl of 0.01 M tris-HCl (pH 7.5). The mixture was incubated for 3 hrs at 37°C. Five μl of 0.05 M EDTA (pH 8.0) was added, and incubation was continued for an additional 20 min. The solution was heated in a boiling water bath for 3 min to inactivate phosphatase, chilled and used for the subsequent polynucleotide kinase reaction.

b) *Pyrimidine tracts of DNA*: Pyrimidine tracts of DNA were prepared by the method of Burton.[7] About 25 μg of DNA in 10 μl of water was mixed with 20 μl of 3% diphenylamine in 98% formic acid. The mixture was incubated for 16 hrs at 37°C, treated with ethylether and dried in vacuo. The pyrimidine tracts obtained were mixed with 0.15 unit of alkaline phosphatase in 45 μl of 0.01 M tris-HCl (pH 7.5). After incubation for 3 hrs at 37°C, 5 μl of 0.05 M EDTA (pH 8.0) was added and incubation was continued for an additional 20 min. The solution was heated in a boiling water bath for 3 min, chilled and used for the polynucleotide kinase reaction.

c) *Dinucleoside monophosphates*: Four species of dinucleoside monophosphates (ApU, CpG, GpC, and UpG), which were used for assay of polynucleotide kinase, were prepared from *E. coli* ribosomal RNA. Pancreatic and T1 RNase digest of the RNA were fractionated by chromatography on DEAE-Sephadex and Dowex I columns, and the corresponding dinucleotides were isolated.[8,9] The dinucleotides were then treated with alkaline phosphatase to obtain dinucleoside monophosphates.

d) *Desalting nucleotides*: Since the resolution of oligonucleotides on PEI-cellulose thin layer chromatography is markedly influenced by the presence of salts in samples, samples were usually desalted by the following procedure. Samples were diluted with water to a salt concentration less than 0.01 M, and charged onto DEAE-cellulose columns (Whatman DE23, 0.5 cm $\times$ 1 cm). After washing the columns with 5 ml of 0.01 M triethylamine bicarbonate (pH 8), nucleotides were eluted with 2 M triethylamine bicarbonate (pH 8), and dried in vacuo.

2. (γ^{32}P)ATP

(γ^{32}P)ATP of about 2 Ci/mmole was prepared by the procedure of Glynn and Chappell[10]. The reaction mixture usually contained 1.5 μmole ATP, 5 mCi ^{32}P-orthophosphate (carrier free), 1 μmole 3-phosphoglycerate(tricyclohexylammmonium salt), 0.15 mg glyceraldehyde-3-phosphate dehydrogenase, and 15 μg 3-phosphoglycerate kinase in 0.5 ml of 0.06 M tris-HCl (pH 7.8)-6 mM $MgCl_2$-0.01 M 2-mercaptoethanol. The mixture was incubated at 26°C for 1 hr and applied onto a Dowex I column (0.5 cm × 1 cm). After washing the column with 10 ml of 0.05 M NaCl-0.01 N HCl to remove ^{32}Pi, (γ^{32}P)ATP was eluted with 1 ml of 0.3 M NaCl-0.01 N HCl. The eluate was neutralized with tris base, diluted with water, and desalted as in the previous section. (γ^{32}P)ATP thus prepared was dissolved in water to a concentration of 2 μmoles/ml and stored in a freezer (−20°C).

3. Enzymes

Polynucleotide kinase was prepared from phage T4 amN82 infected *E. coli* A19 cells by the procedure of Richardson[11], except for the chromatographic conditions: the enzyme was eluted from the DEAE-cellulose column (Whatman DE52) with a linear gradient of 0.002 M to 0.1 M potassium phosphate buffer (pH 7.5) containing 0.01 M 2-mercaptoethanol, and from the phosphocellulose column (Whatman P11) with a linear gradient of KCl (0 to 0.5 M) in 0.01 M potassium phosphate buffer (pH 7.5)-0.01 M 2-mercaptoethanol, respectively. The purified enzyme was dialyzed against 0.01 M potassium phosphate buffer (pH 7.5)-0.01 M 2-mercaptoethanol-40% glycerol and stored in a freezer (−20°C) at a concentration of about 1,000 units/ml (1 unit: activity transferring 1 nmole P within 30 min at 37°C). Alkaline phosphatase was prepared from *E. coli* A19 as described by Garen and Levinthal,[12] dissolved in 0.01 M tris-HCl (pH 7.5)-40% glycerol to a concentration of about 100 units/ml (1 unit: activity releasing 1 μmole P within 1 hr at 37°C) and stored in a freezer (−20°C). T1 and T2 RNases were purchased from Sankyo Co. (Tokyo) and pancreatic RNase and snake venom phosphodiesterase from Worthington Biochem. Co. (USA). These enzymes were stored in a freezer (−20°C) under the following conditions; T1 RNase: 1000 units/ml in water, T2 RNase: 5 units/ml in water, pancreatic RNase: 1 mg/ml in water, venom phosphodesterase: 1 μg/ml in 0.1 M triethylamine bicarbonate (pH 8). Muscle glyceraldehyde 3-phosphate dehydrogenase and yeast 3-phosphoglycerate kinase were purchased from Boehringer Mannheim Co. (West Germany).

4. Phosphorylation of Nucleotides with Polynucleotide Kinase

Unless otherwise noted, the 5′-hydroxyl group of nucleotides was phosphorylated under the following condition (standard condition). About 50 pmoles to 1 nmole of substrates (mono- and oligonucleotides containing 5′-hydroxyl groups) were mixed with (γ^{32}P)ATP (about 4 nmoles/1 nmole substrate) and polynucleotide kinase (about 2 units/1 nmole substrate) in 20 μl of 0.01 M $MgCl_2$-0.01 M 2-mercaptoethanol-0.01 M tris-HCl (pH 8.0). The substrates were usually desalted before phosphorylation. When samples have been prepared as in the section 1 (a), (b), however, 10 μl each was used without desalting. Incubation was made for 45 min at 37°C, and

terminated by heating in a boiling water bath for 2 min. The reaction mixture was directly applied onto PEI-cellulose thin layer plates for chromatography.

5. Two-Dimensional Chromatography on PEI-Cellulose Thin Layer Plates

Chromatography on PEI-cellulose thin layer plates (20 cm×20 cm, products of Macherey-Nagel Co., West Germany) was carried out by the procedure essentially identical to that described by Mirzabekov and Griffin.[13] The plates were washed through the following cycle of solvents;[14] 10 min soaking in 10% (w/v) NaCl, followed by drying at room temperature: 10 min soaking in water, followed by drying at room temperature: 10 min soaking in 2 N formic acid adjusted to pH 2.2 with pyridine, followed by transfer to water. After washing with water two times, the plates were dried at room temperature and stored at 4°C and dark.

Development in the first dimension for T1 RNase-digest was usually made with 1.4 M lithium formate-7 M urea, (pH 3.5) up to 10 cm above the origin, and then with 2.3 M lithium formate-7 M urea, (pH 3.5) up to the end of the plate. For pancreatic RNase-digest, the lithium formate concentrations were reduced to 1.3 M for the first step and 1.8 M for the second step, respectively. The plate was washed with methanol for three times, dried at room temperature and then developed in the second dimension with 0.6 M lithium chloride (for pancreatic RNase-digest) or 0.8 M lithium chloride (for T1 RNase-digest) in 7 M urea-0.02 M tris, (pH 8.0). For separation of pyrimidine tracts of DNA, development in the first dimension was made with 1.3 M lithium formate-7 M urea, (pH 3.5) up to the end of the plate and in the second dimension with 0.8 M lithium chloride-7 M urea-0.02 M tris, (pH 8.0). To obtain better separation of shorter oligonucleotides, the concentration of lithium salts in the both dimension was slightly reduced.

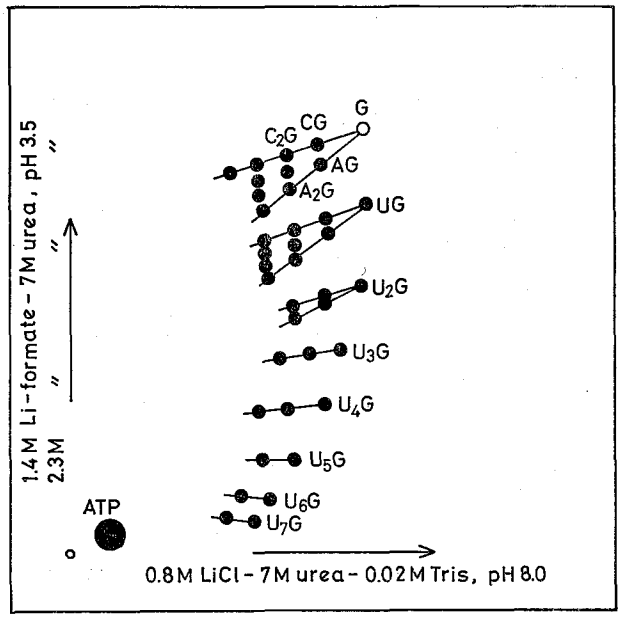

Fig. 1a.

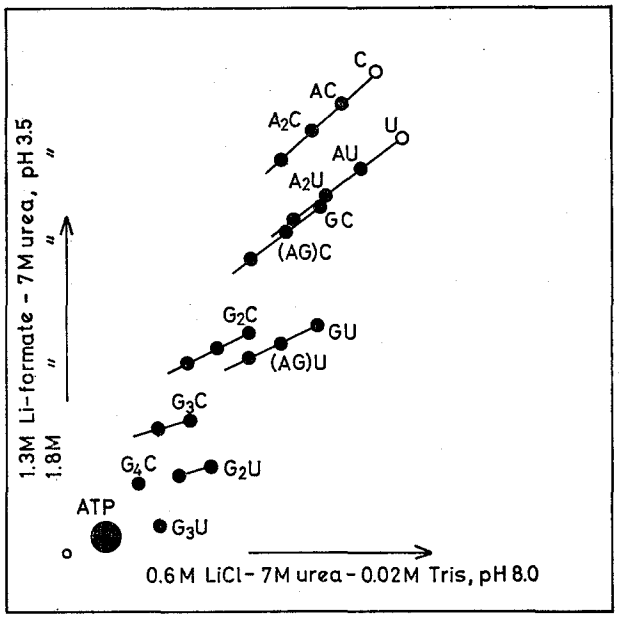

Fig. 1 b.

Fig. 1. Diagram illustrating the relationship between the composition of a nucleotide obtained in the polynucleotide kinase reaction and its map position on two-dimensional PEI-cellulose thin layer chromatography.

(a): nucleotides from T1 RNase digest. Development in the first dimension was made with 1.4 M lithium formate-7 M urea, (pH 3.5) and then with 2.3 M lithium formate-7 M urea, (pH 3.5) and in the second dimension with 0.8 M lithium chloride-7 M urea-0.02 M tris, (pH 8.0). (b): nucleotides from pancreatic RNase digest. Development in the first dimension was made with 1.3 M lithium formate-7 M urea, (pH 3.5) and then with 1.8 M lithium formate-7 M urea, (pH 3.5) and in the second dimension with 0.6 M lithium chloride-7 M urea-0.02 M tris, (pH 8.0). The solutions used for development were made up in the following way. For the lithium formate solution, a solution of formic acid at a given molarity was made up in 7 M urea, and pH was adjusted to 3.5 by dissolving solid lithium hydroxide. For the lithium chloride solution, a solution of lithium chloride at a given molarity was made up in 7 M urea-0.02 M tris base, and pH was adjusted to 8.0 by adding concentrated HCl.

Under the conditions used for chromatography, the position of an oligonucleotide on the fingerprint largely depends on its nucleotide composition. The relationship between the composition of an oligonucleotide and its map position is given in Fig. 1. Such a relationship is very similar to that obtained for oligonucleotides containing P at the 3-hydroxyl groups.[13] The nucleotide which has the greatest effect on the mobility in the first dimension is U. The fingerprint of T1 RNase-digest therefore yields a regular arrangement of oligonucleotides with increasing number of U (Fig. 1a). The pancreatic RNase-digest results in more simple fingerprints, since oligonucleotides containing C and U are markedly separated each other (Fig. 1b). Deoxyribonucleotides also yield a very similar fingerprint, since T behaves like U (see Fig. 6).

6. Nucleotide Composition Analysis of Oligonucleotides

For analysis of the nucleotide composition of oligonucleotides fractionated by two-dimensional chromatography on PEI-cellulose thin layer plates, spot regions were collected into small cone-shaped tubes with cotton stoppers. After washing with 5 mM triethylamine bicarbonate (pH 8), nucleotides were eluted with 2 M triethylamine bicarbonate (pH 8). To an oligonucleotides thus prepared (about 50 pmoles) was added 0.05 unit of T2 RNase dissolved in 10 μl of distilled water. After digestion for 3 hrs at 37°C, 10 μl of 0.02 M $MgCl_2$-0.02 M 2-mercaptoethanol-0.02 M tris-HCl (pH 8) containing about 0.3 nmole (γ^{32}P)ATP and 0.15 unit polynucleotide kinase were added. The mixture was incubated for 45 min at 37°C and directly applied onto a PEI-cellulose thin layer plate (3 cm×20 cm). Development was carried out with 2 M sodium formate (pH 3.5) up to the end of the plate. The chromatographic positions of four nucleoside(3′, 5′)diphosphates are shown in Fig. 4b.

7. Partial Digestion of Oligonucleotides by Venom Phosphodiesterase

To a (5′^{32}P)oligonucleotide prepared as in the above section (about 50 to 100 pmoles) was added 0.01 μg of snake venom phosphodiesterase dissolved in 50 μl of 0.02 M triethylamine bicarbonate (pH 8). The mixture was incubated at 20°C. At 5 min intervals, 10 μl each was withdrawn into a small tube containing a drop of phenol saturated with water. The combined solution was treated with ethylether and dried in vacuo. The digest was dissolved in 10 μl of water and used for the sequence analysis.

RESULTS AND DISCUSSION

1. Determination of the Conditions for Maximum Phosphorylation

For the application of the polynucleotide kinase method to sequence analysis, all the 5-hydroxyl groups of nucleotides should be phosphorylated quantitatively. The optimal condition for phosphorylation was determined by using four dinucleoside monophosphates each containing different species of nucleoside at the 5′-position (ApU, CpG, GpC and UpG). ApU was first incubated at different ratios of (γ^{32}P) ATP and polynucleotide kinase, and the extent of phosphorylation was determined. The result of analysis is shown in Fig. 2. In the presence of an excess enzyme, the addition of four times as much ATP as the substrate by molar ratio results in the complete phosphorylation within 45 min at 37°C. Under the condition, three other dinucleoside monophosphates were also completely phosphorylated, indicating that the acceptability of P at the 5′-hydroxyl group was not influenced by the species of bases. According to this result, the standard condition for phosphorylation was decided to carry out by incubation for 45 min at 37°C in the presence of about 4 nmoles (γ^{32}P) ATP and 2 units enzyme per 1 nmole substrate. The extent of phosphorylation at different substrate concentrations is shown in Fig. 3. The result indicates that, under the standard condition, the complete phosphorylation takes place at a substrate concentration higher than 50 pmoles/10 μl. Although the extent of phosphorylation considerably decreased at the concentration lower than this (*e.g.* 70% at 1 pmole/

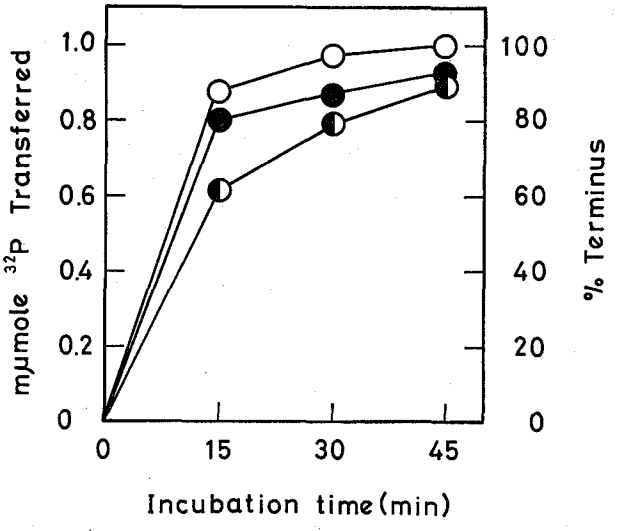

Fig. 2. The extent of phosphorylation at different ratios of ATP and enzyme to substrate.
Incubation mixture contained 1 nmole ApU as substrate and following amounts of (γ^{32}P)ATP and polynucleotide kinase in 20 μl of 0.01 M $MgCl_2$-0.01 M mercaptoethanol-0.01 M tris (pH 8.0). (a): 4 nmoles (γ^{32}P)ATP and 1 unit polynucleotide kinase. (-◐—◐-) (b): 2 nmoles (γ^{32}P)ATP and 2 units polynucleotide kinase. (-●—●-) (c): 4 nmoles (γ^{32}P)ATP and 2 units polynucleotide kinase. (-○—○-) Following incubation at 37°C, aliquots were withdrawn, heated in a boiling water bath and applied onto a PEI-cellulose thin layer plate. After development with 1.3 M lithium formate-7 M urea, (pH 3.5), ^{32}P transferred to ApU was measured.

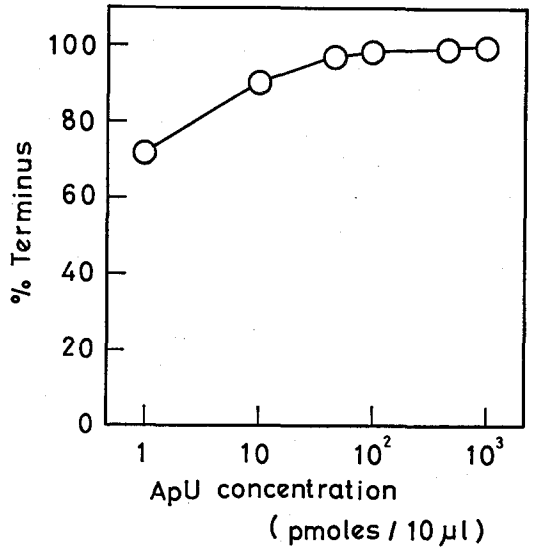

Fig. 3. The extent of phosphorylation at different substrate concentrations.
At constant ratios of (γ^{32}P) ATP and polynucleotide kinase to substrate (4 nmoles ATP and 2 units enzyme per 1 nmole substrate), the concentration of ApU as substrate was varied from 1 pmole to 1 nmole in 10 μl of 0.01 M $MgCl_2$-0.01 M mercaptoethanol-0.01 M tris (pH8.0). Incubation was made for 45 min at 37°C and ^{32}P transferred to ApU was measured.

10 μl), phosphorylation was completed by increasing the ATP concentration by two-fold. As described in the following section, it was confirmed that, under the standard condition, oligonucleotides produced from tRNA by pancreatic and T1 RNase digestion were completely phosphorylated.

2. Determination of Nucleotide Composition

Four species of nucleoside diphosphates(pNp) are clearly separated by one-dimensional chromatography on PEI-cellulose thin layer plates under the condition that ATP stays near the origin. After the complete digestion of DNA and RNA into nucleoside 3′-monophosphates, therefore, it is possible to determine the nucleotide composition by phosphorylating their 5′-hydroxyl groups with ^{32}P in the polynucleotide kinase reaction. This principle is also applicable for analysis of the nucleotide composition of oligonucleotides fractionated on the two-dimensional thin layer system. For example, p*CpUpCpG (*: ^{32}P) obtained by phosphorylation of CpUpCpG yields the following nucleotides and guanosine by digestion with T2 RNase:

$$\text{p*CpUpCpG} \xrightarrow[\text{T2 RNase}]{} \text{p*Cp+Up+Cp+G} \xrightarrow[\text{kinase+}(\gamma^{32}\text{P})\text{ATP}]{\text{Polynucleotide}} \text{p*Cp+p*Up+p*Cp+G}$$

By phosphorylating this digest with ($\gamma^{32}P$)ATP, nucleoside 3′-monophosphates produced from the inside of the original oligonucleotide are phosphorylated into (5′^{32}P)

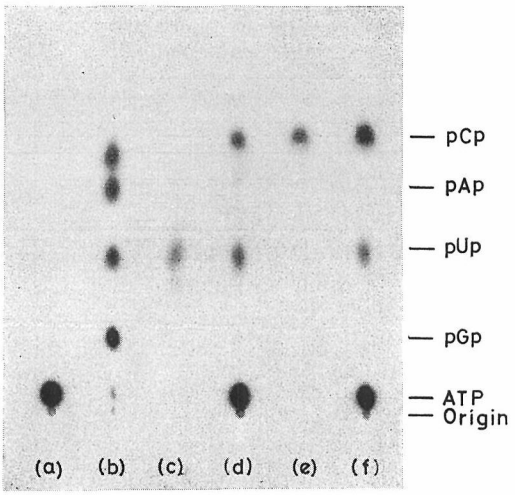

Fig. 4. Nucleotide composition analysis of oligonucleotides.

Fifty pmoles each of p*CpUpG and p*CpUpCpG obtained from a fingerprint was mixed with 0.1 unit of T2 RNase in 20 μl of distilled water. After incubation at 37°C for 3 hrs, 10 μl each was withdrawn and immediately applied onto a PEI-cellulose thin layer plate (c, e). To remainder, 300 pmoles ($\gamma^{32}P$)ATP and 0.15 unit polynucleotide kinase, dissolved in 10 μl of 0.02 M MgCl-0.02 M mercaptoethanol-0.02 M tris (pH 8.0),were added and the mixture was incubated for 45 min at 37°C, and applied onto a PEI-cellulose thin layer plate (d, f). The specific activity of ($\gamma^{32}P$)ATP was identical with that used for phosphorylation of original oligonucleotides. Development was made with 2 M sodium formate (pH 3.5) up to the end of the plate. (a): ATP alone, (b): four pNp as maker.

nucleoside diphosphates. Therefore, the species of the 5′-terminal nucleotide and nucleotide composition are determined by resolving (^{32}P)nucleotides on PEI-cellulose thin layer chromatography, before and after the phosphorylation (Fig. 4).

3. Sequencing of Oligonucleotides by Partial Digestion

As shown in Fig. 1, the position of an oligonucleotide on the fingerprint depends on its nucleotide composition. It is, therefore, possible to deduce the sequence of an oligonucleotide by analyzing its sequential degradation products. For example,

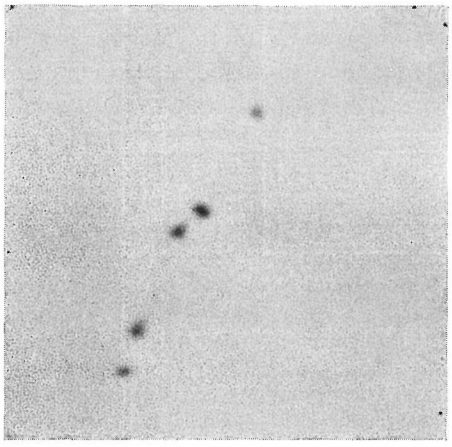

Fig. 5a.

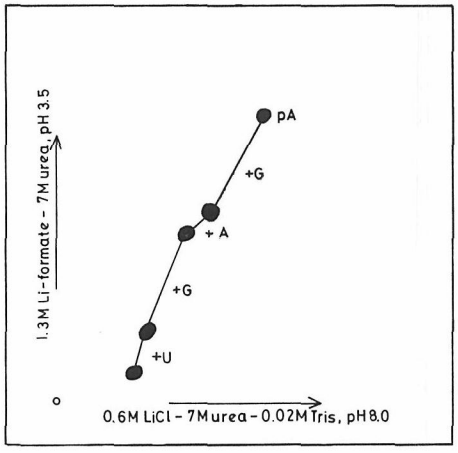

Fig. 5b.

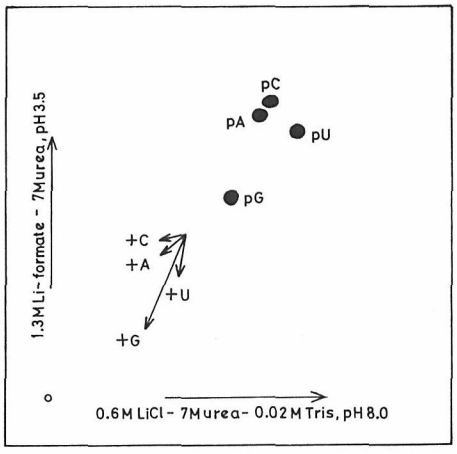

Fig. 5c.

Fig. 5. Sequence analysis of oligonucleotides.

To 50 pmoles of p*ApGpApGpU obtained from a fingerprint was added 0.01 μg of snake venom phosphodiesterase dissolved in 50 μl of 0.02 M triethylamine bicarbonate (pH 8). The mixture was incubated at 20°C. At 5 min intervals, 10 μl each was with drawn, treated as in the method section and chromatographed as in Fig. 1b. (a): radioautograph, (b): line diagram showing the sequence, (c): map position of four pN and direction and relative distance of the shift caused by addition of one nucleotide.

p*ApGpApGpU yields a series of oligonucleotides containing ^{32}P at the 5′-position by partial digestion with snake venom phosphodiesterase.

p*ApGpApGpU	⟶	p*ApGpApG+pU
	Venom	p*ApGpA+pG+pU
	phosphodiesterase	p*ApG+pA+pG+pU
	partial digestion	p*A+pA+2pG+pU

By resolving the digest on the two-dimensional system, the sequence of the original oligonucleotide can be deduced from the mobility shift of the sequential degradation products (Fig. 5a, b). The direction and relative distance of the mobility shift caused by the addition of one nucleotide to an oligonucleotide strikingly depends on the species of the one added (Fig. 5c). By using the PEI-cellulose thin layer system, the sequence of oligonucleotides up to octamer can be determined. For analysis of longer oligonucleotides, however, it is necessary to apply other two-dimensional systems such as the cellulose acetate electrophoresis-homochromatography system, developed by Brownlee and Sanger[15].

4. Application of the Polynucleotide Kinase Method

4–1. Analysis of Pyrimidine Tracts of DNA

The pyrimidine tracts ($p(Py)_np_n$) were prepared from calf thymus DNA, treated with alkaline phosphatase, and rephosphorylated with ^{32}P in the polynucleotide kinase reaction. The labeled nucleotides thus obtained ($p^*(Py)_np_{n-1}$) were resolved on the PEI-cellulose thin layer system. A typical pattern is shown in Fig. 6. The pyrimidine tracts are resolved in the first dimension mostly depending on their T content and in the second dimension in their C content. Therefore, the composition of each spot can be predicted from the map position, as indicated in Fig. 6b. Although the fingerprint shown in Fig. 6a was taken from the total calf thymus DNA, much more simple patterns are obtained from shorter DNA fragments such as the one produced by digestion with restriction endonucleases, and the sequence of each spot can be determined by the partial digestion method described in the section 3.

4–2. Sequence Analysis of E. coli $tRNA^{fmet}$

E. coli $tRNA^{fmet}$ of which the sequence has already been determined by Dube *et al.*[16] was digested with either pancreatic RNase or T1 RNase and dephosphorylated with alkaline phosphatase. The digest was then phosphorylated with ^{32}P under the standard condition and chromatographed on the two-dimensional PEI-cellulose thin layer system. The extent of phosphorylation of each oligonucleotide was also determined. A typical fingerprint obtained from the T1 RNase-digest is shown in Fig. 7. The approximate molar yields of each spot estimated from the ^{32}P activity are shown in Table I. The nucleotide composition of most spots could be predicted from their map positions (see Fig. 1). However, some spots appeared at unexpected positions, due to the presence of minor nucleotides. To determine their sequence precisely, each spot was eluted, digested with T2 RNase and the nucleotide composition was determined as in the section 2. The chromatographic position of minor nucleotides were estimated by comparing with those of authentic markers.

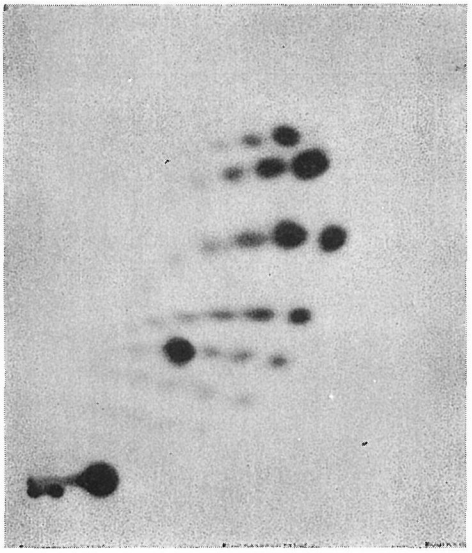

Fig. 6a.

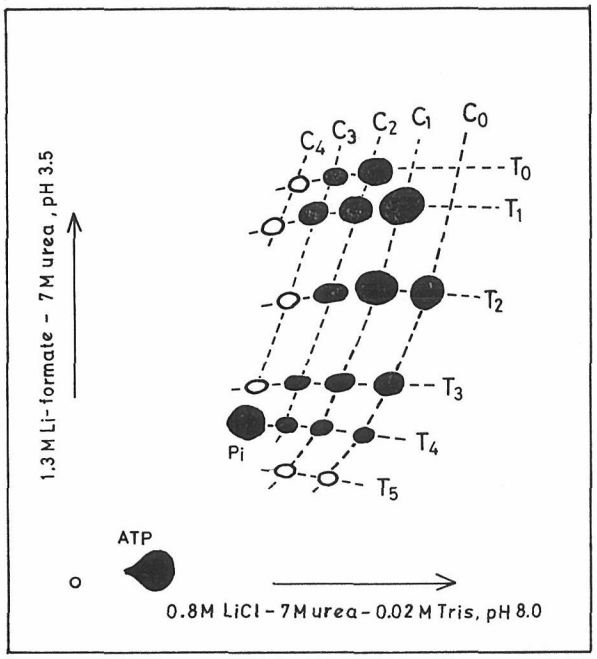

Fig. 6b.

Fig. 6. Fingerprint obtained from pyrimidine tracts of calf thymus DNA by the polynucleotide kinase method.

The pyrimidine tracts were prepared from calf thymus DNA as in the method section, phosphorylated under the standard condition, and chromatographed. (a): radioautograph, (b): line diagram illustrating the relationship between the nucleotide composition and map position.

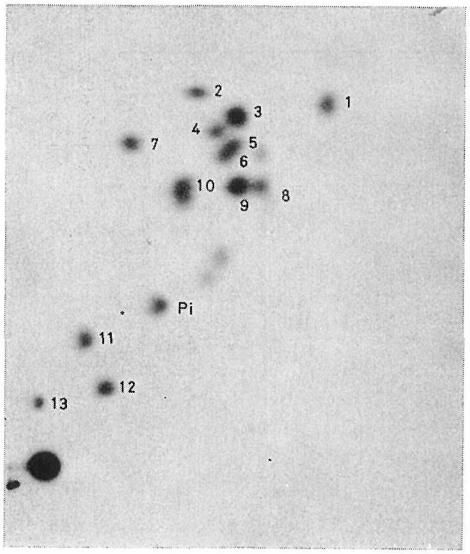

Fig. 7a.

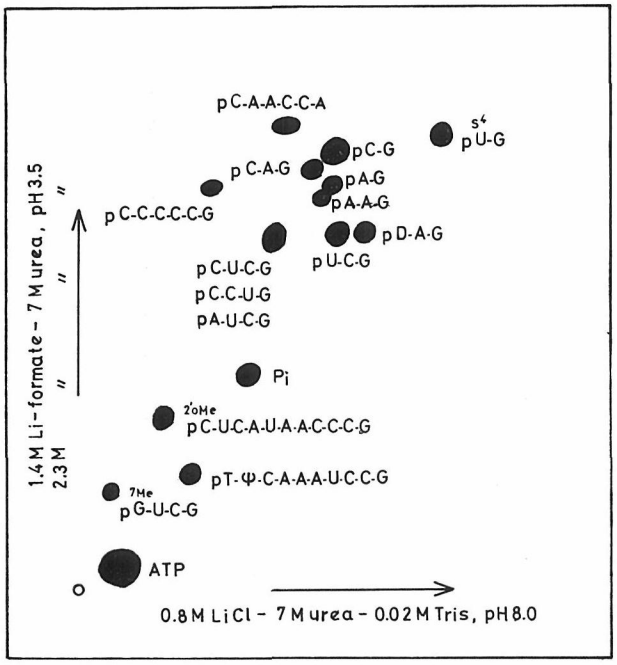

Fig. 7b.

Fig. 7. Fingerprint obtained from T1 RNase digest of *E. coli* t-RNA^{fmet} by the polynucleotide kinase method.

The T1 RNase digest of *E. coli* t-RNA^{fmet} was prepared as in the method section, phosphorylated under the standard condition, and fingerprinted as in the legend to Fig. 1a. *E. coli* t-RNA^{fmet} was a gift from Dr. S. Nishimura of the National Cancer Center Research Institute (Tokyo). (a): radioautograph, (b): line diagram showing the sequence of each spot.

Table I. The extent of phosphorylation of combined T1 RNase and alkaline phosphatase digest of *E. coli* $tRNA^{fmet}$ in the polynucleotide kinase reaction

Spot No.	Sequence	Molar ratio (mole/mole tRNA)	
		Obtained*	Expected**
1	$p^{S4}UpG$	0.91	1
2	pCpApApCpCpA	0.95	1
3	pCpG	2.2	2
4	pCpApG	1.2	1
5	pApG	0.86	1
6	pApApG	1.2	1
7	pCpCpCpCpCpG	1	1
8	pDpApG	0.93	1
9	pUpCpG	2.0	2
10 a	pCpUpCpG	2.2	1
b	pCpCpUpG		1
c	pApUpCpG		0.25
11	$p^{2'oMe}$CpUpCpApUpApApCpCpCpG	0.86	1
12	pTpψpCpApApApUpCpCpG	0.96	1
13	p^{7Me}GpUpCpG	0.68	0.75

* Values were indicated by ratios to Spot No. 7.

** Values were calculated from the sequence determined by Dube *et al.*[16]. Gp produced by T1 RNase digestion are not detected by the polynucleotide kinase method due to the characteristics of this enzyme which requires 3′-phosphate.[11]

The sequences of shorter oligonucleotides were simply determined from the nucleotide composition analysis. For the determination of the sequences of longer nucleotides, each spot was partially digested with venom phosphodiesterase and chromatographed as in the section.[3] The sequences thus obtained for each nucleotide are shown in Table I, in comparison with those expected from the sequence of this tRNA species. All the oligonucleotides which were expected to be produced from $tRNA^{fmet}$ was identified. It was also confirmed that these oligonucleotides were quantitatively phosphorylated under the standard conditions.

I have further constructed the total sequence of this tRNA species from the analysis of partial T1 RNase-digest. In this experiment, $tRNA^{fmet}$ was partially digested with T1 RNase by using the conditions of Brownlee,[17] dephosphorylated with alkaline phosphatase and rephosphorylated with ^{32}P in the polynucleotide kinase reaction. The labeled products were then resolved on 15% acrylamide gel electrophoresis, as in a previous paper.[18] The band regions detected by radioautography were eluted and then completely digested with T1 RNase. The products were treated with alkaline phosphatase, rephosphorylated with ^{32}P in the polynucleotide kinase reaction, and chromatographed on the two-dimensional PEI-cellulose thin layer system. Oligonucleotides produced from each band by digestion with pancreatic RNase were also analyzed by the same procedure to obtain information in the G-clusters regions, since Gp producted by T1 RNase digestion are not detected by the procedure used in the present study. On the basis of the information obtained by these analyses, the total sequence

of $tRNA^{f met}$ was successfully constructed by the overlapping method.

Finally, the procedure described above is generally applicable for the sequence determination of RNA with appropriate size. In addition, short DNA fragments are available these days because of the discovery of a number of restriction endonucleases with different specificities.[19] Such a fragment can be further cleaved into smaller pieces with T4 endonuclease IV, which is known to cleave T–C linkages preferentially.[20,21] Therefore, it would be possible to apply the procedure described above for the construction of DNA sequences.

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