Volume 4 Number 12 December 1977 Nucleic Acids Research

The use of nuclease P_1 in sequence analysis of end group labeled RNA

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Received 2 September 1977

ABSTRACT

A method is described for the direct sequence analysis of 20-25 nucleotides from the termini of 5'- or 3'-end-group [^{32}P] labeled RNA. The method involves partial endonucleolytic digestion of the labeled RNA with nuclease P_1 (from Penicillium citrinum) followed by separation of the partial digestion products by two-dimensional homochromatography, the nucleotide sequence being determined by mobility shift analysis. This procedure has been applied to the sequence analysis of the terminal regions of tRNAs and of high molecular weight RNA, such as messenger RNA or viral RNA. A further application involves its use in conjunction with snake venom phosphodiesterase to determine the sequences of 5'-end group labeled oligonucleotides, containing modified bases, derived from T_1 or pancreatic RNase digestion of tRNA.

INTRODUCTION

We have recently described the use of *in vitro* labeling in the nucleotide sequence analysis of several eukaryotic cytoplasmic and organellar tRNA species, most of which can neither be conveniently labeled *in vivo* with [^{32}P] to high specific activity nor obtained in large quantity[1-6]. A first step in this work was the development of methods for *in vitro* labeling, at the 5'-end of oligonucleotides present in T_1- or pancreatic RNase digests, of non-radioactive tRNAs[1,4,7]. This *in vitro* labeling reaction, which involves the use of T4 polynucleotide kinase and γ-[^{32}P]ATP, can be carried out on enzymatic digests obtained from as little as 0.5 µg of tRNA. The resulting mixtures of 5'-[^{32}P] labeled oligonucleotides are separated by two-dimensional electrophoresis ("fingerprinted")[8]. The sequence of the 5'-[^{32}P] labeled oligonucleotides is then established by partial digestion with snake venom phosphodiesterase, followed by mobility shift analysis using one-dimensional paper

electrophoresis[1,3,9], or two-dimensional homochromatography[4,7,10] of the resulting homologous series of partial digestion products.

To complete a tRNA sequence, the oligonucleotides present in T_1-RNase or pancreatic RNase digests must be ordered into a unique total sequence. Towards this end, we have previously described a method for specific cleavage of tRNA adjacent to the m^7G, which is present in many tRNAs, and isolation by polyacrylamide gel electrophoresis of the two fragments thus produced[5,11]. By fingerprinting these two fragments, the oligonucleotides originally identified in complete T_1-RNase or pancreatic RNase digests of the whole tRNA may be localized to the 5'- or 3'- "half" of the tRNA[2,5]. In this paper, we describe a method which allows the direct derivation of the sequence of 20-25 nucleotides at both the 5'- and the 3'-terminus of a tRNA and thereby reduces significantly the amount of additional overlap information necessary for establishing the final sequence of the tRNA. This procedure, which is general and can, in principle, be applied to any RNA, involves the use of nuclease P_1 from Penicillium citrinum[12] as an endonuclease for obtaining partial digests of 5'- or 3'-[^{32}P] labeled tRNAs. The partial digestion products are separated by two-dimensional homochromatography; from the resulting autoradiographic patterns, terminal sequences of over 20 nucleotides may generally be read directly by mobility shift analysis. As with fingerprint analysis, end group labeling for nuclease P_1 digestion may be performed on as little as 0.5 µg of tRNA.

Partial digestion with nuclease P_1 can also be used for establishing the sequence of 5'-end group labeled oligonucleotides, such as those obtained from fingerprints, and has been found especially valuable in complementing partial digestion with snake venom phosphodiesterase for the sequence analysis of oligonucleotides containing modified bases. A further use of nuclease P_1 has been in the determination of the 5'-terminal nucleotide sequences of 5'-[^{32}P] labeled rabbit α and β globin mRNA[13] and Alfalfa Mosaic Virus RNA 4[14] and of the 3'-terminal nucleotide sequence of 3'[^{32}P] labeled Turnip Yellow

Mosaic Virus RNA[7].

MATERIALS AND METHODS

General. Sources of materials were as follows. Escherichia coli $tRNA_{II}^{Tyr}$ was purified as described previously[15]. Neurospora crassa cytoplasmic $tRNA_i^{Met}$ was kindly provided by L.I. Hecker and W.E. Barnett[24]. T4 polynucleotide kinase was prepared according to Panet et al.[16]. Highly purified tRNA nucleotidyl transferase was a gift from A. Prochiantz and D. Carre. Penicillium citrinum nuclease P_1 was from Yamasa Shoyu Co. (Japan); the lyophilized powder was dissolved at 1 mg/ml in 50 mM Tris-maleate, pH 6.0, and stored at -20°C. Ribonucleases T_1 and T_2 were from Sankyo Co. (Japan), bacterial and calf intestinal alkaline phosphatases were from Boehringer Mannheim Corp., and yeast hexokinase was from P.L. Biochemicals. Snake venom phosphodiesterase was from Worthington Biochemicals. Bovine serum albumin (A grade) was from Calbiochem. γ-[^{32}P]ATP was prepared by a modification of the procedure of Glynn and Chappell[17] at 500-1000 Ci/mmole, and was purified by DEAE Sephadex column chromatography in triethylammonium bicarbonate buffer. α-[^{32}P]ATP was purchased from New England Nuclear Corp. at 100-300 Ci/mmole.

Methods for T_1 or pancreatic RNase digestion of tRNA, 5'-end group labeling with [^{32}P], and fingerprinting were as described previously[4,7]. Location of radioactive oligonucleotides by autoradiography and their elution, recovery and 5'-end group analysis by complete digestion with T_2-RNase followed by thin layer chromatography were as published[4,7]. In addition, nuclease P_1 was used for 5'-end group analysis of 5'-[^{32}P] labeled RNA. The reaction mixture (10 µl) contained 1 µg P_1 per 1-10 µg carrier yeast tRNA in 50 mM ammonium acetate buffer, pH 5.3. Incubation was at 37°C for 5 hr, and analysis was by thin layer chromatography with appropriate UV marker compounds, as for 5'-end-group analysis using T_2-RNase. Partial digestion of 5'-[^{32}P] labeled oligonucleotides with snake venom phosphodiesterase was as described[7].

5'-End Group Labeling of tRNA with [^{32}P]. The tRNA was first treated with phosphatase and then labeled with [^{32}P] at

it's 5'-end using polynucleotide kinase. The incubation mixture (10 µl) for the first step contained 0.5-2.5 µg of RNA, bacterial or calf intestinal alkaline phosphatase (0.005 unit) and 50 mM Tris-HCl, pH 8.0. Incubation was at 55°C for 30 min. The phosphatase activity was destroyed by making the solution 5 mM in nitrilotriacetic acid (NTA) and incubating for 20-30 min at room temperature and then 2 min (bacterial alakline phosphatase) or 4 min (calf intestinal alkaline phosphatase) at 100°C. The phosphatase reaction may also be terminated by extraction with phenol: chloroform = 1:1 (for details of extraction procedure, see below under "3'-Labeling"). The incubation mixture (10 µl) for the second step contained 0.1-0.5 µg of the phosphatase-treated RNA, T4 polynucleotide kinase (2 units), 25-100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ (where necessary, additional $MgCl_2$ was added to balance the NTA concentration), 15 mM β-mercaptoethanol and 50-100 µM γ-[^{32}P]ATP. Incubation was at 37°C for 30 min. Recently, we have found that the phosphorylation reaction is stimulated somewhat by substituting the following buffered salt solution in the incubation mixture (other components being identical); 25-50 mM Tris-HCl, pH 8,0, 10 mM $MgCl_2$, 10 mM dithiothreitol (Calbiochem), 10 µg/ml bovine serum albumin and 10% glycerol. At 30 min, the reaction mixture was lyophilized and then subjected to polyacrylamide gel electrophoresis.

3'-End-Group Labeling of tRNA with [^{32}P]. The tRNA was first treated with snake venom phosphodiesterase under mild conditions to remove part of the 3'-terminal C-C-A, and then labeled with [^{32}P] at the 3'-end by using tRNA nucleotidyl transferase in the presence of α-[^{32}P]ATP and non-radioactive CTP. The incubation mixture (10 µl) for the first step contained 5 µg of tRNA and 0.25 µg of snake venom phosphodiesterase in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$; incubation was at room temperature for 10-15 min. The mixture was then extracted twice with equal volumes of phenol/chloroform (1:1). After two back extractions of the pooled organic phases with water, the pooled aqueous phases were extracted six times with ether; the solution was left in an open tube for 15 min at 37°C to drive off residual ether, and was then lyophilized and redis-

solved in a small volume. For the second step, 0.5-2.5 µg of snake venom phosphodiesterase-treated tRNA was incubated (10 µl) with 1 µg of tRNA nucleotidyl transferase and 25-30 µM each of α-[^{32}P]ATP and non-radioactive CTP in 25-50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 8 mM dithiothreitol. Incubation was at 37°C for 45 min. The reaction mixture was lyophilized and then subjected to polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis and Recovery of Radioactive Material. Electrophoresis was in 12% or 15% w/v polyacrylamide gel slabs prepared with recrystallized acrylamide and N,N'-methylene-bis-acrylamide in a ratio of 20:1. The gel buffer system was 90 mM Tris, 90 mM borate, 4 mM EDTA, pH 8.3[18], 7 M urea (ultrapure grade, Schwarz-Mann); no urea was used in the running buffer. Lyophilized samples of RNA were generally dissolved in 98% formamide (deionized with Fisher Rexyn I-300 mixed bed ion exchange resin) containing 20 mM sodium phosphate, pH 7.6, 20 mM EDTA and 0.1% each bromophenol blue and xylene cyanole blue, heated for 5 min at 55°C, and then used for electrophoresis. Electrophoresis was at room temperature with a constant voltage gradient of 10-20 V/cm. Several layers of Whatman DE-81 paper were placed under the bottom of the gel slab in the anode electrolyte chamber to trap the fast-moving [^{32}P]ATP as it left the bottom of the gel.

Radioactive bands were located by autoradiography, excised from the gel, and counted (Cerenkov) in a liquid scintillation counter. The material in the excised bands was recovered by electrophoretic elution as described by Knecht and Busch[19]. Electrophoresis was in Pasteur pipettes with shortened tips that were plugged with 5% polyacrylamide in either gel buffer (without urea) or in 20-25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA (the latter buffer proved easier to remove by dialysis in a subsequent step). Dialysis sacs containing approximately 0.5 ml of buffer were then attached to the pasteur pipettes, the excised bands were inserted into the pipettes and 10-50 µg of carrier yeast tRNA (free of ribonuclease) in 25% sucrose was layered onto the surface of the plug. The carrier tRNA was found to improve the recovery of the [^{32}P] RNA. Electrophoresis was in fresh running buffer at room temperature for

6-14 hr at 1-2 mA/tube. The [^{32}P] RNA was generally freed of salt by dialysis and stored at -80°.

Partial Digestion of End-Group Labeled RNA with Nuclease P_1. The incubation mixture contained 5'- or 3'-[^{32}P] labeled RNA (or oligonucleotide), carrier yeast tRNA and nuclease P_1 at a ratio of 7.5 ng enzyme per 50 µg of carrier RNA. Incubation was in 50 mM ammonium acetate buffer, pH 5.3, at 20°C. Generally, 5-10 x 10^4 cpm of material was incubated in a total volume of 20-50 µl with 10-25 µg of carrier RNA. Aliquots (5 µl) were removed at various times, boiled 4 min in 5 mM EDTA to inactivate the enzyme, and 0.5-1 µl of each aliquot was analyzed for the extent of digestion by one-dimensional homochromatography. Appropriate aliquots were then pooled and analyzed by two-dimensional homochromatography[4,7]. For tRNA, removal of aliquots from the digestion reaction at 2 min and 5 min was found to be sufficient to give a good distribution of partial degradation products, so the one-dimensional homochromatography analysis could generally be dispensed with; for oligonucleotides, removal of aliquots at 5 min, 10 min and 20 min was generally found most useful. At least 10,000 cpm was used for two-dimensional homochromatography.

Homochromatography. RNA digests ("homomixes") for DEAE-cellulose thin layer chromatography were prepared[7] by adding KOH to 3.2-3.5% yeast RNA in 7.8-8.2 M urea to bring the pH to 7.0. Additional KOH was then added to bring the KOH concentration to 10, 25, 50 or 75 mM over that required to neutralize the RNA solution. The RNA was then incubated at 65°C for 20-24 hr. After cooling to room temperature, the pH of the solution was reduced to 4.5-4.7 with acetic acid, and the volume then adjusted to give a final RNA concentration of 3% in 7 M urea. The less KOH in the digestion step, the greater the average chain length of the RNA in the respective solution, and the stronger its elution power. Long (20 x 40 cm) plates were run in 10 mM KOH strength homomix; the best results were obtained when this homomix was titrated down to pH 4.5 to provide additional buffering capacity for prolonged chromatographic runs.

DEAE-cellulose thin layer plates were either homemade or

from Analtech, Inc., and were of two types. 20 x 20 cm plates were a 250 μ layer of cellulose MN 300 HR/MN 300 DEAE = 15/2; 20 x 40 cm plates were a 250 μ layer of cellulose MN 300 HR/ Avicel/MN 300 DEAE = 10/5/2.

Chromatography in homomix was at 65°. 20 x 20 cm plates were run in glass chromatography tanks and 20 x 40 cm plates were run in custom-designed plexiglass tanks built for us by Wilbur Scientific (Boston, Mass., USA). Two-dimensional homochromatography was as described by Sanger and coworkers[10], using electrophoresis on cellulose acetate or cellogel strips at pH 3.5 in the first dimension and DEAE-cellulose thin layer chromatography in homomix in the second dimension. Samples were lyophilized and resuspended in 2-3 μl for loading onto the cellulose acetate strip; where larger quantitites of RNA (>8 μg) were to be loaded on a strip, cellogel strips (Kalex, Inc., N.Y.) were used instead of the usual cellulose acetate. When the xylene cyanole dye spotted alongside the sample had migrated the predetermined distance (usually 8.5-10 cm), the strips were removed and the sample was transferred to a DEAE-cellulose thin layer plate by the procedure of Southern[20]. After the transferred region had dried, the plate was topped with a Whatman 3 MM paper wick, prerun at 65°C to 2/3 its height in distilled water, and then transferred to a tank of the appropriate homomix. In later work with 40 cm long plates, it was found useful to also cover the bottom of the plate with a 3 MM paper wick during the prerun and homochromatography; this prevented the DEAE-cellulose from flaking off the bottom of the plate during prolonged chromatography at 65°C.

RESULTS

5'- and 3'-End-Group Labeling of tRNA. As described above, tRNA can be labeled with [^{32}P] at the 5'-end by sequential treatment with alkaline phosphatase and polynucleotide kinase (in the presence of γ-[^{32}P]ATP), and can be labeled with [^{32}P] at the 3'-end by sequential treatment with snake venom phosphodiesterase and tRNA nucleotidyl transferase (in the presence of α-[^{32}P]ATP and non-radioactive CTP). Using these methods, the extent of tRNA labeling with [^{32}P] at the 5'-end

generally varies between 25% and 75%, while the extent of labeling at the 3'-end is generally 75-95% of the theoretical maximum; in each case, the extent of [^{32}P] incorporation varies with the species of tRNA. In general, because of its greater heat stability, calf intestinal alkaline phosphatase gives more complete dephosphorylation of tRNA at 55°C (this elevated temperature is necessary for effective removal of the 5'-terminal phosphate group). In addition, we have recently found that the extent of phosphorylation by polynucleotide kinase may be stimulated by use of a somewhat modified reaction buffer, as described above (Materials and Methods). One occasional problem with both the 5'- and the 3'-end group labeling procedures is that commercial enzyme preparations (i.e., alkaline phosphatase and snake venom phosphodiesterase) may be contaminated with significant ribonuclease activity. It is, therefore, sometimes necessary to test several batches of enzyme to find a satisfactory one. An alternative would be to purify these enzymes further.

The purity of 5'-[^{32}P] labeled tRNA recovered after gel electrophoresis may be checked and the 5'-end-group identified by 5'-end group analysis, as described above (Materials and Methods). The [^{32}P]-labeled end is either converted to a nucleoside 3',5'-diphosphate (*pNp) by complete digestion with T_2-RNase or to a nucleoside 5'-monophosphate (*pN) by complete digestion with nuclease P_1, and is then identified by thin layer chromatography[4,7]. The purity of 3'-end-group labeled RNA is generally at least as good as that of the starting material, since tRNA nucleotidyl transferase will transfer a [^{32}P]AMP residue only onto the 3'-terminus of a tRNA. In our experience, end group purity of at least 80% is essential for unambiguous sequence analysis by partial digestion with nuclease P_1.

Sequence Analysis by Partial Digestion with Nuclease P_1. The procedure we have used for this is illustrated schematically in Fig. 1. The partial P_1 digests are used directly for analysis by two-dimensional homochromatography. A typical analysis of 5'-[^{32}P] labeled tRNA, that of Neurospora crassa $tRNA_i^{Met}$, is illustrated in Fig. 2, and a typical analysis of

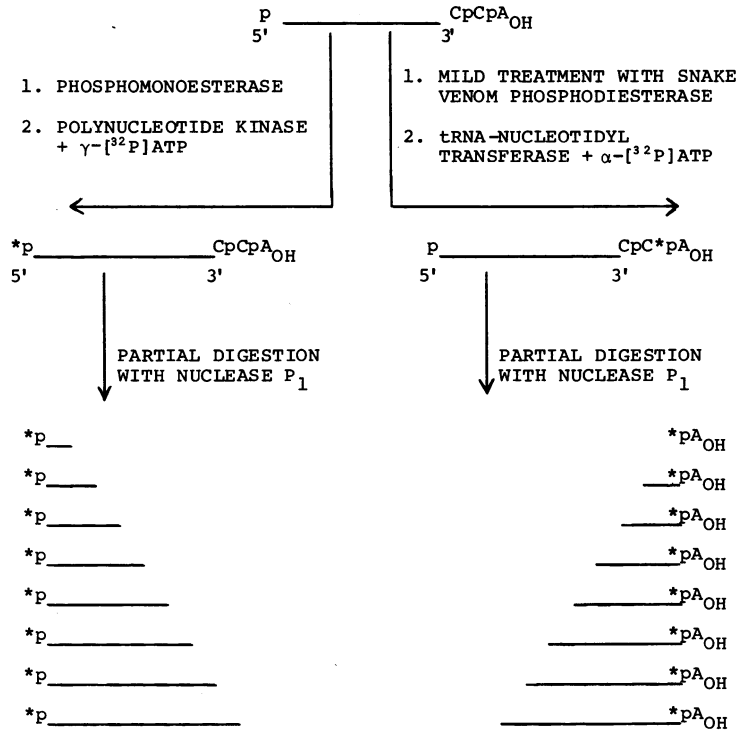

Fig. 1. Scheme for the use of nuclease P_1 in the 5'- and 3'-terminal sequence analysis of end-group labeled tRNA.

3'-[^{32}P] labeled tRNA, that of E. coli $tRNA_{II}^{Tyr}$, is illustrated in Fig. 3. Since only the [^{32}P]-labeled products of the partial digest are visualized by autoradiography, the patterns of Figs. 2 and 3 represent homologous series of successively longer oligonucleotides with common [^{32}P]-labeled end groups (Fig. 1). The nucleotide sequences represented by the patterns can be determined by analysis of the two-dimensional mobility shifts between successive oligonucleotide spots[4,7,10,21-32], as illustrated in the schematic drawings (Figs. 2 and 3). Sequences of over 20 nucleotides can frequently be determined from a single pattern, the actual number being limited mainly by the resolution of the two-dimensional separation.

As nuclease P_1 is not entirely random in its selection of cleavage sites[12], certain internucleotide bonds may be cut less frequently than others; this results in a final autoradi-

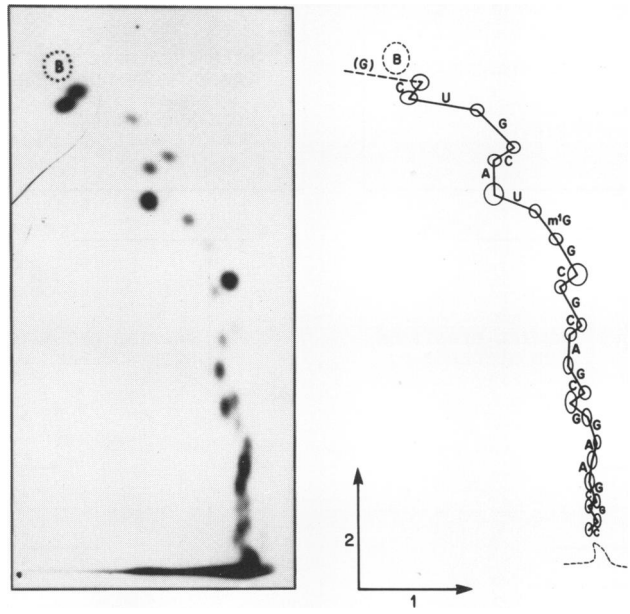

Fig. 2. Autoradiogram of a partial nuclease P_1 digest of 5'-[^{32}P] labeled Neurospora crassa $tRNA_i^{Met}$. First dimension, electrophoresis on cellulose acetate, pH 3.5; second dimension, homochromatography in 10 mM KOH-strength "homomix". The 5'-end group, pA, was not transferred to the plate in this case. B, circled, is xylene cyanole dye.

ogram containing lighter and denser spots. Polypyrimidine clusters, especially oligo C stretches, in single-stranded conformations show up as a series of lighter spots (Fig. 5B); base-paired oligo C stretches are, however, cleaved more readily by nuclease P_1 (Fig. 2). Similarly, the phosphodiester bond on the 3'-side of certain modified nucleosides is resistant to nuclease P_1, and under the partial digestion conditions used, the corresponding oligonucleotide spot may be entirely missing from the autoradiogram (Fig. 5B and below). Also, modified nucleosides, even when they are not strongly inhibitory toward nuclease P_1, may give rise to unusual mobility shifts between successive oligonucleotide spots in a homologous partial digestion series (the nature of these unusual mobility shifts, and their identification will be discussed elsewhere -- Silberklang, Gillum, Simsek and RajBhandary, in

Fig. 3. Autoradiogram of a partial nuclease P1 digest of 3'-[32p] labeled E. coli tRNA^{Tyr}; note that the 3'-end group, pA, is diffuse and has moved rapidly in the second dimension. This is due to the presence of urea on the plate (from the cellulose acetate strip) during the water prerun; pA, being only slightly negatively charged at pH 3.5 (the pH upon transfer), is not adsorbed strongly on to the DEAE-cellulose. The first mobility shift, pA⟶pCpA, is, therefore, artifactually larger in the second dimension.

preparation). The "gaps" and unusual mobility shifts encountered in two-dimensional homochromatographic patterns are rare, but, when they occur, are readily apparent. The sequence determination of such regions in a tRNA molecule must rely on prior knowledge of the modified nucleosides present and of the sequences of the oligonucleotides in complete T_1- and pancreatic RNase digests of the tRNA and recognition of these sequences within the two-dimensional homochromatographic pattern of the end-group labeled tRNA.

Use of Nuclease P_1 in the Sequence Analysis of Oligonucleotides Containing Modified Bases. When polynucleotide kinase is used to prepare T_1-RNase or pancreatic RNase "fingerprints" of a tRNA, the oligonucleotides recovered are labeled with [^{32}P] only at their 5'-ends. The sequence of most of these oligonucleotides can be determined by partial enzymatic degradation with snake venom phosphodiesterase, followed by analysis by one-dimensional DEAE-paper electrophoresis or two-dimensional homochromatography[1,3,4,7]. A special problem arises, however, with oligonucleotide sequences that contain 3'- or internal modified nucleoside residues that block the stepwise 3'$\longrightarrow$5' exonucleolytic progress of snake venom phosphodiesterase. In such cases, the terminally [^{32}P]-labeled partial degradation products resulting from cleavage at phosphodiester bonds on the 5'-side of the modified nucleoside can be obtained in better yield by partial endonucleolytic digestion with nuclease P_1. Since both snake venom phosphodiesterase and nuclease P_1 cleave phosphodiester bonds to leave 3'-hydroxyl and 5'-phosphate ends, aliquots of partial digests produced by each of these enzymes can subsequently be combined to give an optimal representation of all the terminally [^{32}P]-labeled partial degradation products of an oligonucleotide. This is illustrated schematically in Fig. 4. The combined partial digests may then be analyzed by two-dimensional homochromatography.

The use of this method for the sequence analysis of an oligonucleotide (*pC-U-C-A-U-t^6A-A-C-C-C-G) present in T_1-RNase digests of Neurospora crassa $tRNA_i^{Met}$ is illustrated in Fig. 5[24]. The snake venom phosphodiesterase digestion (Fig.

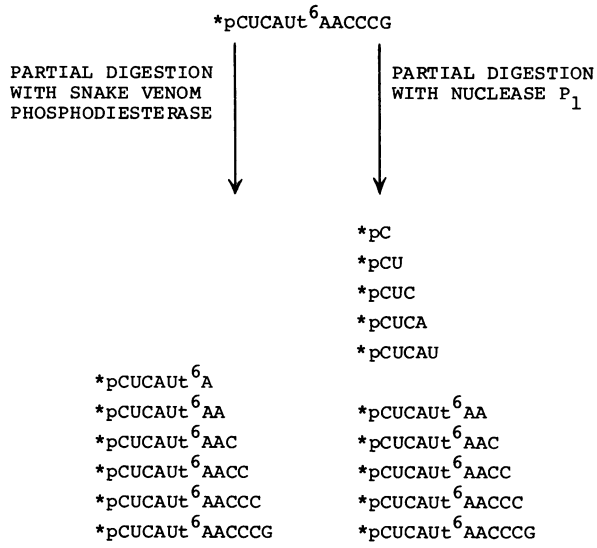

Fig. 4. Scheme for the use of nuclease P_1 in conjunction with snake venom phosphodiesterase in the sequence analysis of 5'-[^{32}P] labeled oligonucleotides containing modified bases which block the stepwise exonucleolytic progress of snake venom phosphodiesterase.

5A) is blocked at the t^6A residue. The nuclease P_1 digest, on the other hand, being a nearly random endonucleolytic digest, accumulates the other intermediates except the one resulting from cleavage at the t^6A-A phosphodiester bond (Fig. 5B). The two digests are, therefore, complementary, and when pooled and analyzed together yield the complete sequence of the oligonucleotide (Fig. 5C). The nature of the unusual mobility shift displayed by t^6A was known from previous work on similar oligonucleotides present in T_1-RNase digests of several eukaryotic initiator methionine tRNAs[1,2,4,5] (A. Gillum, unpublished). (The spot marked X which is seen in Figs. 5A and C is probably *pCUCp caused by contamination of the snake venom phosphodiesterase used by a specific endonuclease which cleaves within the sequence C-A (Dr. J. Heckman, personal communication).

We have found that, although the same modified nucleoside residue may inhibit both exonucleolytic digestion by snake venom phosphodiesterase and endonucleolytic attack by nuclease P_1 (as was illustrated in Fig. 5 for t^6A), in all cases so far

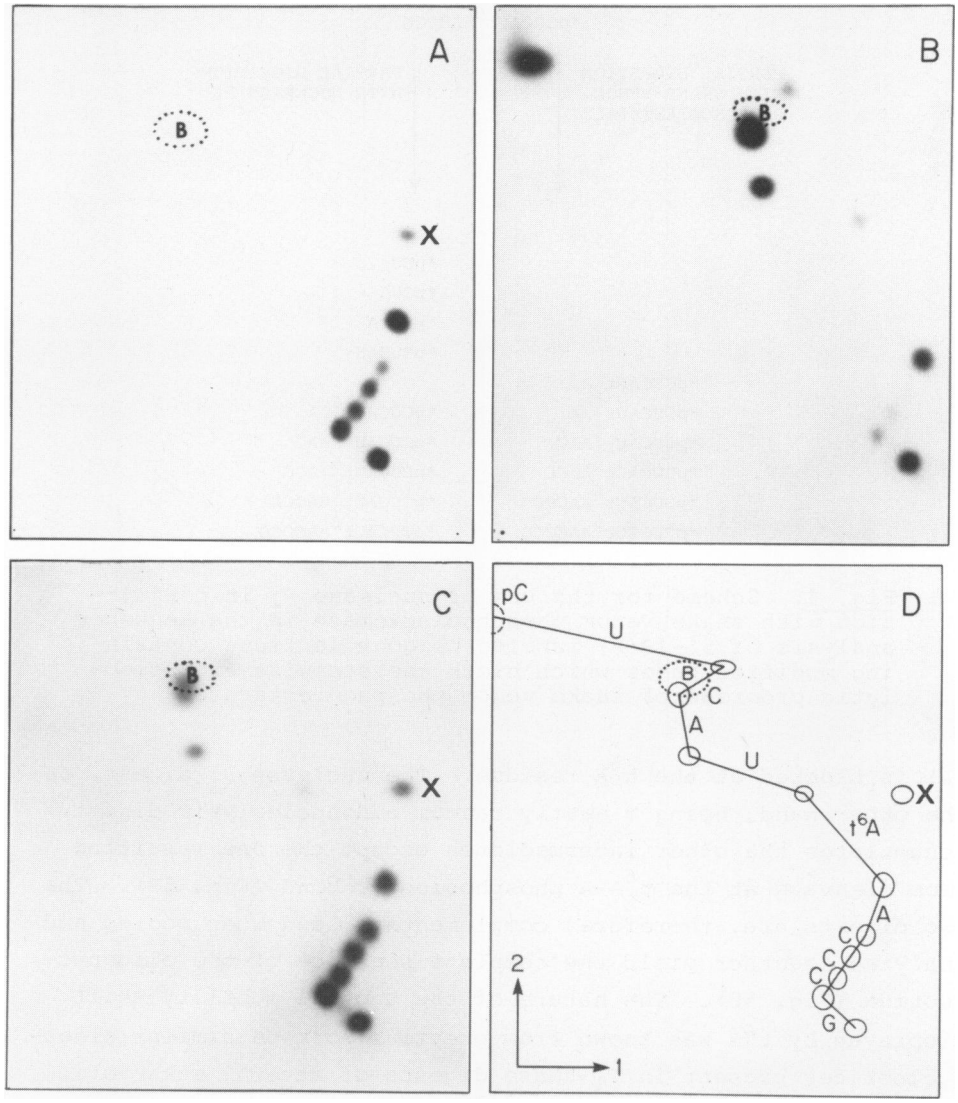

Fig. 5. Autoradiogram of partial digests of [^{32}P]C-U-C-A-U-t^{6}A-A-C-C-C-G. First dimension, electrophoresis on cellulose acetate, pH 3.5; and second dimension, homochromatography in 50 mM KOH-strength "homomix". A. Partial digestion with snake venom phosphodiesterase; B. partial digestion with nuclease P_1; C. combined aliquots of digests indicated in A and B. D is a replica of C indicating identification of mobility shifts. B, circled, is xylene cyanole dye.

examined analysis of combined partial digests affords a useful method toward the unambiguous sequence analysis of 5'-[^{32}P] labeled oligonucleotides containing such modified residues. Modified nucleosides in this category include t^6A, i^6A, Y, 2'-O-methylated nucleosides and, depending on the surrounding sequence, Ψ and D. The identification of the particular modified nucleoside in each case must be confirmed by other means[25]; this will be the subject of a separate communication (Silberklang, Gillum, Simsek and RajBhandary, in preparation).

DISCUSSION

Besides the results reported in this paper, the procedure which we describe herein has found application in the sequence analysis of several tRNAs, including a chloroplast[6] and a mitochondrial tRNA[25], and terminal sequence analysis of mRNAs from rabbit globin[13], Alfalfa Mosaic Virus[14] and Turnip Yellow Mosaic Virus[7]. The method is, therefore, quite general and allows one to determine the sequence of 20 or more nucleotides at the 5'- or 3'-terminus of any RNA which can be specifically labeled with [^{32}P] at its ends. While T4 polynucleotide kinase can be used to label the 5'-ends of tRNAs[3], high molecular weight rRNAs[26] and eukaryotic mRNAs (subsequent to the removal of "cap" structures[27] present in these RNAs[13,14]), selective labeling of RNAs with [^{32}P] at the 3'-end has so far been possible only in the case of tRNAs[15] and of plant viral RNAs which have a "tRNA-like" structure at their 3'-end[7]. An application of this technique for sequencing the 3'-ends of rRNAs or of mRNAs which do not contain "tRNA-like" sequences at their 3'-end will require the development of a method for labeling the 3'-ends of these RNAs with [^{32}P].

Since the derivation of nucleotide sequences, as described herein, relies on mobility shift analysis, interpretation of any mobility shifts which are found to be unusual must be done carefully and with an understanding of the factors which govern them[10,21]. Many of the phenomena discussed by Tu *et al*[21] for DNA sequence determination by mobility shift analysis also apply to RNA sequence determination. While the conditions used for two-dimensional homochromatography in our work are somewhat different from theirs, the nature of the mobility

shifts observed are qualitatively similar to those observed by them for DNA.

In the homochromatography dimension, for example, the distance between two oligonucleotides in a homologous partial digestion series which differ by a purine nucleotide is always larger than that between those which differ by a pyrimidine nucleotide[21], and can, therefore, be used to distinguish between purine and pyrimidine nucleotides. Oligonucleotides in a homologous series which differ by a modified nucleotide containing a positively or negatively charged base (e.g. t^6A, Fig. 5), however, may be separated by an unusual distance; this phenomenon will be discussed in detail elsewhere (Silberklang, Gillum, Simsek and RajBhandary, in preparation).

In the pH 3.5 electrophoresis dimension, the relative mobilities of two oligonucleotides in a homologous series which differ by a single nucleotide will depend not only on the pKa of the ribonucleotide by which they differ but also on the size and base composition of the remainder of sequence common to them[21]. Thus, addition of pC or pA to an oligonucleotide pX will result in a faster, slower or unchanged mobility of the longer oligonucleotide with respect to pX, depending upon the base composition of pX. Consequently, in the two-dimensional homochromatography patterns of partial digests, the direction of mobility shifts due to the addition of pC or pA to an oligonucleotide can vary. This is illustrated in Fig. 3, where addition of pA to pCpCpA (where the sequence consists exclusively of A and C residues) results in an increased electrophoretic mobility of pApCpCpA with respect to pCpCpA in the first dimension; whereas addition of pA to a longer oligonucleotide, pUpC.....pCpCpA (where the sequence contains some U residues) results in little change in the mobility in the first dimension and is, therefore, seen as a vertical mobility shift in the two-dimensional pattern.

Finally, our use of partial endonuclease digestion with nuclease P_1 of end group labeled RNA (Fig. 1) for sequence analysis by two-dimensional homochromatography is, in principle, also applicable to end group labeled DNA; it is known that nuclease P_1 does act as an endonuclease towards DNA[28].

Because of its relatively uniform accumulation of partial digestion intermediates (Figs. 2 and 3), from the mononucleotide through long oligonucleotides, the use of nuclease P_1 also presents advantages over the use of pancreatic DNase for this purpose[23]. Recently, partial digestion with nuclease P_1 has, in fact, been successfully applied to the terminal sequence analysis of 5'-[^{32}P] labeled (A.M. Gillum, unpublished) and 3'-[^{32}P] labeled (Dr. J. Petruska, personal communication) DNA molecules.

ACKNOWLEDGEMENTS

This work has been supported by grant GM17151 from the National Institute of General Medical Sciences and grant NP-114A from the American Cancer Society. M.S. and A.M.G. were supported by USPHS predoctoral training grants.

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