Volume 5 Number 1 January 1978 Nucleic Acids Research

Sequence analysis of 5'[^{32}P] labeled mRNA and tRNA using polyacrylamide gel electrophoresis

Raymond E. Lockard*, Birgit Alzner-Deweerd*, Joyce E. Heckman*, Joseph MacGee+, Marvin Wilson Tabor+, and Uttam L. RajBhandary*

*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, and +V. A. Hospital, and Departments of Biological Chemistry and Medicine, College of Medicine, University of Cincinnati, Cincinnati, OH 45267, USA

Received 2 November 1977

ABSTRACT

Sequence analysis of 5'-[^{32}P]labeled tRNA and eukaryotic mRNA using an adaptation of a method recently described by Donis-Keller, Maxam and Gilbert for mapping guanines, adenines and pyrimidines from the 5'-end of an RNA is described. In addition, a technique utilizing two-dimensional polyacrylamide gel electrophoresis for identification of pyrimidines within a sequence is described.

5'-[^{32}P] Labeled rabbit β-globin mRNA and N. crassa mitochondrial initiator tRNA were partially digested with T_1-RNase for cleavage at G residues, with U_2-RNase for cleavage at A residues, with an extracellular RNase from B. cereus for cleavage at pyrimidine residues and with T_2-RNase or with alkali for cleavage at all four residues. The 5'-[^{32}P] labeled partial digestion products were separated according to their size, by electrophoresis in adjacent lanes of a polyacrylamide slab gel and the location of G's, A's and of pyrimidines extending 60-80 nucleotides from the 5'-end of the RNA determined.

Two-dimensional polyacrylamide gel electrophoresis was used to separate the 5'-[^{32}P] labeled fragments present in partial alkali digests of a 5'-[^{32}P] labeled mRNA. The mobility shifts corresponding to the difference of a C residue were distinct from those corresponding to a U residue and this formed the basis of a method for distinguishing between the pyrimidines.

INTRODUCTION

We have recently described[1-4] a variety of methods utilizing in vitro labeling with [^{32}P] for the sequence analysis of non-radioactive tRNAs. One of these is the use of nuclease P_1 and two-dimensional homochromatography for the sequencing of 20-30 nucleotides from the 5'- or 3'-ends of any RNA which can be selectively labeled at these termini.[5] Along with a general method that we developed for removing the "cap" structure from the 5'-ends of eukaryotic mRNAs and the subsequent labeling of

these mRNAs with [^{32}P] at their 5'-ends, the above procedure has led to the sequencing of the 5'-termini of rabbit α- and β-globin mRNAs[6], alfalfa mosaic virus RNA[7], and the 3'-end of turnip yellow mosaic virus RNA[8].

More recently, Donis-Keller, et. al.[9] have described a procedure for sequence analysis of 5'-[^{32}P] labeled RNA by partial chemical and enzymatic digestion of the 5'-[^{32}P] RNA followed by separation of the partial digestion products by polyacrylamide gel electrophoresis. In such studies, 5'-[^{32}P] yeast 5.8S RNA was partially digested in urea at elevated temperature with T_1-RNase to generate partial cleavage after G residues, with U_2-RNase for partial cleavage after A residues and with alkali for partial cleavage after all four residues. The 5'-[^{32}P] labeled fragments thus generated were separated according to their chain length by polyacrylamide gel electrophoresis under denaturing conditions and the relative locations of G, A and by difference that of pyrimidine residues from the 5'-end of a labeled RNA could be determined. The procedure as described cannot be used to distinguish among the pyrimidine residues and a search for enzymes which can help discriminate between C and U residues is currently under way (H. Donis-Keller, personal communication).

In the present paper we describe our adaptation and extension of the above scheme for sequencing 5'-[^{32}P] labeled tRNA and eukaryotic mRNA. We have expanded the variety of RNase digestions employed, and have used T_2-RNase and a pyrimidine specific extracellular RNase from a strain of *B. cereus*[10]. T_2-RNase hydrolyzes almost all of the phosphodiester bonds in RNA and is most useful on tRNAs which contain modified nucleosides such as D, m^7G, etc., where the use of partial alkaline digests for obtaining cleavage at all the phosphodiester bonds may lead to partial destruction or alteration of certain modified nucleosides. The pyrimidine specific RNase from *B. cereus*, unlike pancreatic RNase which is more sequence specific[9], cleaves after almost all of the pyrimidines in an RNA and thereby provides a more direct evidence for the location of pyrimidine residues from the 5'-end of an RNA. In addition, we describe the use of two-dimensional polyacrylamide gel

electrophoresis for analyzing partial alkaline digests of 5'-[^{32}P] mRNA and its use in distinguishing between the pyrimidines.

MATERIALS

N,N,N',N'-tetramethylglycinamide (TMGA) and cyclohexylamine were obtained from Eastman Kodak Company. Ultra-pure urea was obtained from Schwarz-Mann, and acrylamide and bis-acrylamide from Bio-Rad. The sources of bacterial alkaline phosphatase, T_1-, T_2-, U_2- and pancreatic RNases were as described elsewhere.[5] T_4-polynucleotide kinase was either purified according to Panet, et. al.[11] or purchased from New England Biolabs. High specific activity γ-[^{32}P]ATP (2000-2500 Ci/mmole) was prepared by a modification of the procedure of Glynn and Chappell.[12] Polyacrylamide gel electrophoresis was carried out using a Model 100 Electrophoresis Power Supply (Wilbur Scientific Co., Boston, MA). Dupont Cronex Lightning plus intensifying screens for autoradiography were obtained from a local supplier of X-ray films.

METHODS

Preparation of Reagents Used for β-elimination. N,N,N',N'-tetramethylglycinamide (TMGA)-HCl was prepared as follows. To 27 ml. of TMGA, 20 ml. of H_2O was added, and the solution was cooled to 4°C in ice. Concentrated HCl was added with vigorous stirring until the pH was 5.0, not allowing the temperature to rise above 10°C. Two volumes of benzene were added, and H_2O was removed by rotary evaporation in vacuo overnight. The resultant TMGA-HCl was recrystallized from n-butanol and the crystals then dried overnight in vacuo. Cyclohexylamine-HCl was prepared similarly, except that the H_2O was removed by lyophilization and the cyclohexylamine-HCl was recrystallized from H_2O and dried overnight in vacuo.

Preparation of TMGA-HCl Buffer. To 4.0 ml. of H_2O .415 g TMGA-HCl and .011 g Na_2-EDTA was added. The solution was warmed to 45°C, the pH adjusted to 8.0 with concentrated NaOH and then brought to 5.0 ml. with H_2O to a final concentration of .5 M TMGA-HCl and 6 mM EDTA.

Preparation of TMGA-Buffered Cyclohexylamine. To 4.0 ml. of H_2O 1.36 g cyclohexylamine-HCl and .415 g TMGA-HCl was added.

The solution was warmed to 45°C, the pH adjusted to 8.0 with concentrated NaOH and then brought to 5.0 ml. with H_2O to a final concentration of 2.0 M cyclohexylamine-HCl and .5 M TMGA-HCl.

5'-End Group Labeling of Rabbit Globin mRNA

Isolation of rabbit reticulocyte polysomes and large scale purification of globin mRNA was as described elsewhere.[6] The 5'-end group labeling[6] of this mRNA required the removal of "cap" structure present at the 5'-end[6,13] by periodate oxidation followed by β-elimination and the subsequent removal of the 5'-terminal ppp-triphosphate end by incubation with E. coli alkaline phosphomonoesterase. The mRNA carrying the 5'-hydroxyl group was then labeled with [^{32}P] using γ-[^{32}P]ATP and T_4-polynucleotide kinase.

Globin mRNA, 5 A_{260} units in 400 µl. of H_2O and 0.5% SDS was reacted in the dark at 25°C with a 800-fold molar excess (approximately 500 nmoles) of freshly dissolved sodium periodate for one hour at a final RNA concentration of 10 A_{260} units/ml. The reaction was terminated by addition of 0.3 vol. of 20% sodium acetate (pH 5.5) and 2.5 vol. of ethanol. After leaving overnight at -20°C, the oxidized mRNA was recovered by centrifugation, washed once with ethanol-0.3 M LiCl, (2:1 V/V), once with 95% ethanol, and was dissolved and then dialyzed overnight against 2 liters of 0.1 mM EDTA, pH 7.2, to remove any residual periodate.

The oxidized mRNA was dissolved in H_2O to a concentration of 10 A_{260} ml., to this solution was added one-sixth the volume of TMGA-HCl buffer and one-fifth the volume of the TMGA-cyclohexylamine buffer. The reaction mixture, now approximately 0.2 M TMGA-HCl (pH 8.0), 0.4 M cyclohexylamine-HCl, and 1 mM EDTA, was incubated for 45 minutes at 45°C. SDS used earlier[6] was found to slow the β-elimination reaction appreciably, hence was eliminated in this revised procedure. The reaction was terminated by addition of LiCl to 0.4 N followed by 2.5 vol. of ethanol, and left overnight at -20°C. The mRNA was again pelleted by centrifugation, and washed and dialyzed to remove residual cyclohexylamine by the same procedure used previously to remove residual periodate.

The dialyzed chemically "decapped" mRNA was lyophilized to dryness, dissolved in 250 µl. of 40 mM Tris-HCl (pH 8.3), and incubated for 40 minutes at 45°C at a concentration of 0.3 units/ml. of alkaline phosphatase. The mRNA was then phenol extracted to remove phosphomonoesterase, ethanol precipitated, and washed as described elsewhere for globin mRNA preparation.[6] Overall recovery of mRNA at this stage was between 50 to 75%.

Incubation mixture for the polynucleotide kinase reaction (120 µl.) contained the "decapped" and dephosphorylated mRNA, 3 nmoles of γ-[^{32}P]ATP and 25 units of polynucleotide kinase in 25 mM Tris-HCl, pH 8.4, 10 mM $MgCl_2$, and 10 mM DTT. After incubation for 30 minutes at 37°C, the mRNA was precipitated by addition of 0.3 vol. of 25% sodium acetate (pH 5.5) and 2.5 vol. of ethanol and left at -90°C for two hours. The mRNA was recovered by centrifugation, washed once with 95 % ethanol, and dissolved in 10 µl. of H_2O for further purification.

Purification of 5'-[^{32}P] Labeled Rabbit α- and β-Globin mRNA. The 5'-[^{32}P] labeled rabbit globin mRNA (10 µl.), was mixed with 100 µl. of gel loading solution (99% deionized formamide with 0.1% w/v xylene cyanole and 0.1% w/v bromophenol blue dyes), and was applied on four separate slots of a 7% polyacrylamide slab gel. Conditions for electrophoresis were as described before[6]. The 5'-[^{32}P] RNA bands were recovered by electrophoretic elution into dialysis bags using 40 mM Tris-acetate (pH 7.8) in 2 mM EDTA as the elution buffer[6]. Recovery of mRNA from the excised gel bands was at least 90%. The electro-eluted mRNA was then made 0.2 M with respect to KCl, 0.2 A_{260} of unlabeled globin mRNA was added as carrier, and the mixture applied to a 1-2 ml. oligo(dT)-cellulose column at 4°C, previously equilibrated with 0.10 M KCl and 25 mM Tris-HCl (pH 7.5). The column was washed with 10-15 ml. of the same buffer at 4°C and the bound [^{32}P]-end labeled mRNA was then eluted with H_2O at 37°C, filtered free of cellulose by millipore filtration (HAWP 0.45; Millipore, Bedford, MA) and lyophilized. The residue was dissolved in 200 µl. of H_2O, dialyzed against 0.1 mM EDTA, pH 7.2, and the [^{32}P]-end labeled mRNA was made 10% in ethanol and stored at -90°C in aliquots until further use.

Preparation of 5'-[^{32}P] labeled Alfalfa Mosaic Virus RNA 4 and N. Crassa Mitochondrial Initiator tRNA

These were carried out as described elsewhere.[3,7]

Isolation of Pyrimidine Specific Ribonuclease from B. cereus

The organism Bacillus cereus was cultivated from soil (ground of V.A. Hospital, Cincinnati, Ohio) in a basal enrichment medium of 0.01% yeast extract and 0.05% NH_4Cl in tap H_2O, enriched with 0.1% polyuridylic acid as the source of carbon and phosphorous.[14] After overnight incubation of the enrichment tubes, the cultures were streaked onto agar plates with the following composition per liter: ribose 1.0 g; agar 15.0 g; yeast extract 1.0 g; malt extract 1.0 g; 20 ml. of solution A; 100 ml. of solution B; and tap H_2O. Solution A contained in 100 ml.: NH_4Cl, 0.5 g; $MgSO_4$, 2.5 g; $FeNH_4(SO_4)_2 \cdot 12H_2O$, 0.5 g; $CaCl_2$, 0.25 g; KCl, 0.25 g; $MnCl_2 \cdot 4H_2O$, 0.25 g; and distilled H_2O to 100 ml. Solution B was a mixture of 1 M K_2HPO_4, 1 M NaH_2PO_4, and distilled H_2O in a ratio of 1:1:4 by volume. Standard microbiological procedures were used to isolate pure cultures from these plates.

After isolation, the organisms were maintained on slants composed of the above medium. Two 5 ml. slants were inoculated and incubated 16-24 hours at 37°C. The entire growth of the slants was transfered to 100 ml. of liquid medium containing per liter: lactose, 5 g; neopeptone, 10 g; NaCl, 2 g; yeast extract, 2 g; beef extract, 5 g. After incubation of the standing growth flask at 37°C for 24 hours the contents were mixed by swirling and a small portion was diluted 1 to 20 with H_2O. The absorbance of the diluted sample was read at 550 nm against H_2O. The amount of standing culture to be used as an inoculum for the growth flask was calculated as follows:

$$\text{ml. inoculum per 300 ml. medium} = \frac{66.6}{A_{550} \times 20}$$

One 4 liter flask containing 1 liter of liquid medium was inoculated and incubated at 35°C for 7 hours with vigorous shaking in a reciprocating shaker-water bath. Growth was terminated by cooling the medium to 4°C in ice, 2-4 drops of n-octanol was added and concentrated H_2SO_4 (10 ml.) was added slowly while the medium was stirred with a magnetic stirrer.

The flask was covered and stored overnight at 4°C before further processing, but could be stored for several days without loss of enzymatic activity.

The contents of the flask were centrifuged at 10,000 xg for 30 minutes to pellet cells. The supernatant was adjusted to pH 7.5 by dropwise addition of 10 M NaOH. The neutral fluid was then concentrated to 40 to 100 ml. using an Amicon Model 402 Concentrator-Dialyzer fitted with a PM10 membrane.[14] The sample was continuously dialyzed in the concentrator-dialyzer with 0.02 M sodium acetate (pH 4.8) until the pH of the effluent was 4.8. (Alternatively, the enzyme could also be dialyzed against 0.1 M potassium borate, pH 8.0.) The enzyme was stored at 4°C in either buffer without appreciable loss of activity for two to four months. For some of the experiments the enzyme was partially purified by chromatography on a column of CM-cellulose.

Partial Digestion of 5'-[^{32}P] Labeled RNA for Analysis by Polyacrylamide Gel Electrophoresis

Partial digestion of 5'- [^{32}P]-end labeled mRNA with both T_1 and U_2-RNase was carried out using the same reaction cocktail and incubation condition as by Donis-Keller, et. al.[9], except that the carrier RNA concentration was 1 µg/µl rather than 0.25 µg/µl. For 5'-[^{32}P] end labeled tRNA, however, to insure complete denaturation, the RNA was first heated in the reaction cocktail containing 7M urea at 100°C for 90 seconds and quickly chilled in ice prior to addition of enzymes. Incubations were at 50°C. Appropriate nuclease/RNA ratios (units of RNase/µg. RNA) for partial digestion had to be determined for each individual RNA preparation. These conditions were established by serial dilution of enzyme into reaction mixtures containing buffers, 7M urea and 5'-[^{32}P] RNA, as described previously.[9] One to two µl. of RNase T_1 (Sankyo), 0.1 unit/µl., was added to the first of three tubes of reaction mixture (volume of incubation mixture, 20 µl.) followed by two successive ten-fold serial dilutions. RNase U_2 (Sankyo), 1 unit/µl., was similarly diluted. These enzyme dilutions gave a range of digestion products from which two were usually chosen for electrophoresis on polyacrylamide gel. In general, levels of T_1-RNase used varied from 10^{-2} to 5 x 10^{-4} units per

µg. carrier RNA and those of U_2-RNase varied from 10^{-1} to 5 x 10^{-3} units per µg. carrier tRNA.

Partial digestions with pancreatic RNase were carried out essentially as for T_1- and U_2-RNases. Incubations usually contained 10^{-3} and 10^{-4} units of pancreatic RNase per µg. of carrier tRNA.

Partial digestions with T_2-RNase usually contained 10^{-2} and 10^{-3} units of the enzyme per µg. of carrier tRNA.

Partial alkaline hydrolysis of 5'-[^{32}P] labeled RNA contained in a volume of 10 µl., lyophilized 5'-[^{32}P] labeled RNA, including 10 µg. of carrier tRNA, 0.1 M NaOH and .001 M EDTA. Incubation was at 37°C. At 2, 4 and 8 minutes, 3 µl. aliquots were removed and added immediately to a solution containing 400 µl. ethanol and 75 µl. of 0.3 M sodium acetate (pH 5.5). The mixture was stored for 30 minutes at -90°C after which the precipitated oligonucleotides were collected by centrifugation and washed once with 95% ethanol. The precipitate was dissolved in 20 µl. of a loading solution consisting of 10 M urea, 0.1% each of xylene cyanole and bromophenol blue and used directly for polyacrylamide gel electrophoresis. These alkaline hydrolysis conditions routinely gave a wide range of 5'-[^{32}P] labeled digestion products from two to one hundred or more nucleotides.

Partial digestion of 5'-[^{32}] end labeled RNA with the pyrimidine specific RNase from B. cereus was carried out using the same reaction cocktail used for T_1- and U_2- RNase, except that the 7 M urea was omitted. The B. cereus enzyme when assayed by digestion of H^3-PolyC had an activity comparable to 4 units/ml of pancreatic ribonuclease. To obtain the proper range of 5'-[^{32}P] labeled digestion products for sequence analysis, 0.5 µl. to 4.0 µl. of enzyme was added to various reaction tubes in arbitrary 0.5 µl. increments. The reaction mixture was incubated for 20 minutes at 50°C with one-half of the reaction removed after 10 minutes of incubation and terminated by addition of an equal volume of 10 M urea. After 20 minutes, the two portions of the incubation mixture were combined and used for gel electrophoresis.

The partial digests were fractionated in adjacent lanes

of a polyacrylamide slab gel (0.15 cm. x 20 cm. x 40 cm.).[9] The gel was pre-electrophoresed for 4 hours at 500 volts prior to loading samples, and then electrophoresed at 1000 volts, 10-12 milliamps. RNA fragments about 26 nucleotides long run with the xylene cyanole marker dye, while fragments about 7-8 nucleotides long run with the bromophenol blue. After electrophoresis, one glass plate was removed from the polyacrylamide slab, the gel covered with Saran Wrap, and then autoradiographed at -20°C with approximately 50 lb. of weight placed upon the film envelope during exposure. The additional weight was found to enhance the resolution of gel bands greater than 25 nucleotides.

Two-Dimensional Polyacrylamide Gel Electrophoresis of Partial Alkaline Digests on 5'-[^{32}P] labeled mRNA

Partial alkaline digests were carried out as above on about 150,000 cpm. of 5'-[^{32}P] labeled mRNA. The ethanol precipitate was dissolved in 20 µl. of loading solution which contained 7 M urea, 25% Sucrose, 0.1% bromophenol blue and 0.3% xylene cyanole and was used for two-dimensional electrophoresis. Electrophoresis in the first dimension was in a 10% polyacrylamide slab gel run at pH 3.5 and in the second dimension was in a 20% polyacrylamide slab gel run at pH 8.3. Gel solution for the first dimension contained 10% acrylamide, 0.5% N,N'-methylene bis acrylamide, 7 M urea and 25 mM citric acid and had a pH of 3.5. The gel solution was deaerated for 30 minutes at reduced pressure and then polymerized as described by De Wachter and Fiers.[15] The gel (0.2 x 20 x 40 cm.) was pre-electrophoresed for 2 hours at 200 volts, electrophoresis buffer was 25 mM citric acid and 4 mM EDTA adjusted to pH 3.5 with sodium hydroxide. The slots (1 cm. wide) were filled with the same buffer, except that they also contained 7 M urea.

Following pre-electrophoresis, the slots were cleaned and filled with electrophoresis buffer and samples were applied. Electrophoresis was carried out at 200 volts until the bromophenol blue tracking dye had migrated 23 cm. (for analysis of oligonucleotides up to 40 long) or 33 cm. (for analysis of oligonucleotides 40 and longer). After the electrophoresis, a strip of gel 1 cm. wide and 16 cm. long was cut from an appropriate region of the gel and placed lengthwise across a

glass plate (20 cm. wide and 40 cm. long) 1 cm. from the bottom of the plate and the gel strip was sealed between two glass plates.

The gel solution (20% acrylamide, 1% N,N'-methylene bis-acrylamide, 7 M urea, 90 mM Tris-borate, pH 8.3) for the second-dimension was poured in two stages. In the first stage enough gel solution was poured to cover the upper edge of the first-dimensional gel strip. To ensure rapid polymerization, this gel solution contained .25% ammonium persulfate. After polymerization of the gel block the remainder of the gel solution was poured, and allowed to polymerize. The direction of electrophoresis of the slab gel (0.2 x 20 x 40 cm.) was from bottom to top with 90 mM Tris-borate pH 8.3 and 4 mM EDTA as the running buffer. Electrophoresis was carried out at 300-400 volts at room temperature for varying periods, depending upon the region of the first dimensional gel being analyzed. For analysis of oligonucleotides 4 to 40 long, electrophoresis was carried out until the bromophenol blue tracking dye had migrated about 23 cm., for oligonucleotides 25 to 60 long, until the xylene cyanole dye had run about 27 cm. and for analysis of oligonucleotides 35 to 100 long, the xylene cyanole dye was run off the gel.

RESULTS

Preparation of 5'[^{32}P] Labeled β-Globin mRNA. Most eukaryotic mRNAs contain a m^7Gppp-"cap" structure at their 5'-end.[13] We have previously demonstrated[6] the existence of such a structure in rabbit globin mRNAs and outlined a method for the removal of the "cap" structure and subsequent labeling of the 5'-end of rabbit globin mRNAs with [^{32}P]. The 5'-[^{32}P] labeled rabbit α- and β- globin mRNAs can then be separated from each other and other contaminating RNAs by polyacrylamide gel electrophoresis (Figure 1). Using uniformly [^{32}P] labeled Vesicular stomatitis virus RNA, we have found that the removal of the "cap" structure goes essentially to completion (data not shown). The migration of the [^{32}P] labeled α- and β-globin mRNAs as discrete bands and the lack of background radioactivity in Figure 1 further indicate that the conditions used for the removal of the "cap" structure and subsequent labeling do not

FIGURE 1. Autoradiogram of 5'-[^{32}P] globin mRNA electrophoresed on a 7% polyacrylamide slab gel in 7 M urea. Direction of electrophoresis is from top to bottom.

cause any noticeable degradation of the mRNAs.

Partial Digestions on 5'-[^{32}P] Rabbit β-Globin mRNA and Analysis by Polyacrylamide Gel Electrophoresis

The 5'-[^{32}P] rabbit β-globin mRNA was recovered from the gel (Figure 1) and purified by chromatography on a column of oligo dT cellulose. The 5'-[^{32}P] mRNA was then partially digested with a pyrimidine specific RNase from B. cereus, T_1-RNase and U_2-RNase and with alkali and subjected to electrophoresis on a 20% polyacrylamide slab gel. Figure 2 shows the results obtained. In this experiment the first two nucleotides from the 5'-end have been electrophoresed off the gel. The incubation of 5'-[^{32}P] β-globin mRNA without enzymes results in very little non-specific fragmentation of the RNA, while

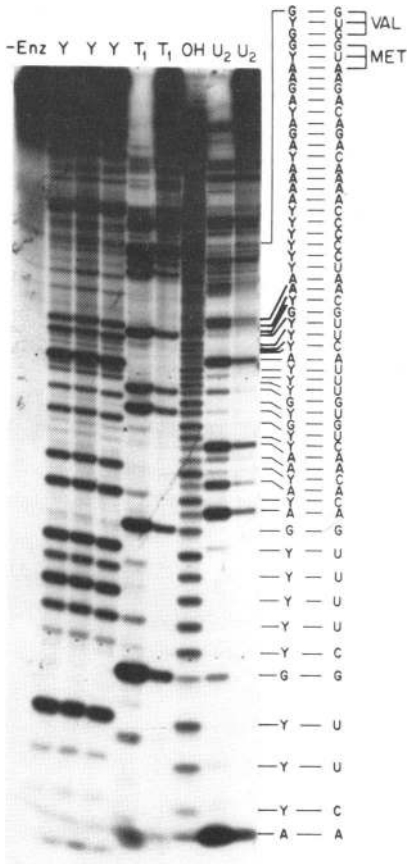

FIGURE 2. Autoradiogram of partial digests on 5'-[^{32}P] rabbit β-globin mRNA electrophoresed on a 20% polyacrylamide slab gel in 7 M urea. Approximately 100,000 cpm of [^{32}P] mRNA was used for each digestion except for the alkaline digests where 200,000 cpm was used. Electrophoresis was run for 9 hrs at 1,000 volts. Partial digests contained the following amounts of enzyme per μg carrier RNA and per μl. of incubation. From left to right:

-Enz, incubated with no addition of enzyme;
Y, 0.0001 unit *B. cereus* RNase;
Y, 0.0002 unit *B. cereus* RNase;
Y, 0.0003 unit *B. cereus* RNase;
T_1, 0.005 unit;
T_1, 0.0005 unit;
OH, partial alkaline digest;
U_2, 0.05 unit; and
U_2, 0.005 unit.

limited alkaline digestion generates an array of fragments from 3-100 nucleotides long. The strong bands present in partial T_1-RNase digests locate G residues and those present in similar digests of U_2-RNase locate A residues, from the 5'-labeled end. Since alkali cleaves every 3'→5' phosphodiester bond involving ribose residues, those bands which are present in the partial alkaline digests, but are not represented in either the T_1-RNase or U_2-RNase digests, must arise from cleavage of phosphodiester bonds on the 3'-side of pyrimidine residues.[9] More direct evidence for this is derived from partial digestion with a pyrimidine specific RNase (Y) from *B. cereus*. This enzyme cleaves after virtually every pyrimidine

residue (Figure 2; tracks 2-4 from the left), although it has some preference for Y-A and Y-G bonds. Unlike pancreatic RNase which is somewhat more specific[9] (also see below), uracil rich sequences, e.g., U_9-U_{12}, in the β-globin mRNA are easily cleaved by the B. cereus RNase, and cytosine rich sequences C_{37}-C_{41} are also cleaved, though less easily. Thus, the use of this enzyme for obtaining partial digests allows one to independently ascertain the location of most pyrimidines in an RNA and to resolve occasional ambiguities which may arise from the use of only RNase T_1- and U_2- and alkali for partial digestions. The radioactivity present in all tracks at the bottom of the gel is due to a salt front containing partial degradation products.

Based on these results, the sequence which can be deduced for the 5'-end of rabbit β-globin mRNA in which Y represents a pyrimidine residue is indicated on Figure 2. Also shown on Figure 2 is the known sequence of this region of rabbit β-globin mRNA from the work of Lockard and RajBhandary,[6] Baralle,[16] and Efstratiadis, et. al.[17] Except for the fact that the procedure used here does not distinguish C from U, the two sequences are in complete agreement.

Partial Digestion of 5'-[^{32}P] Labeled N. crassa Mitochondrial Initiator tRNA and Analysis by Polyacrylamide Gel Electrophoresis.

Figure 3 shows an example of the application of the above sequencing procedure to 5'-[^{32}P] labeled tRNA, including the use of pancreatic RNase, and of T_2-RNase instead of alkali, for obtaining partial digests. In this experiment, the 5'-terminal 14 nucleotides were run off the gel and the sequence which is shown begins with the fifteenth nucleotide from the 5'-end of the tRNA. The presence of additional bands in one of the partial T_1-RNase digests (Figure 3, track 2 from the left) besides those expected from cleavage at G residues is due to the fact that the 5'-[^{32}P] tRNA used had undergone some random cleavage during its isolation following polyacrylamide gel electrophoresis and storage. As in the case of rabbit β-globin mRNA (see above), the results of this experiment alone do not allow us to distinguish among the pyrimidines. However, as described elsewhere,[3] in this particular instance a knowledge of the se-

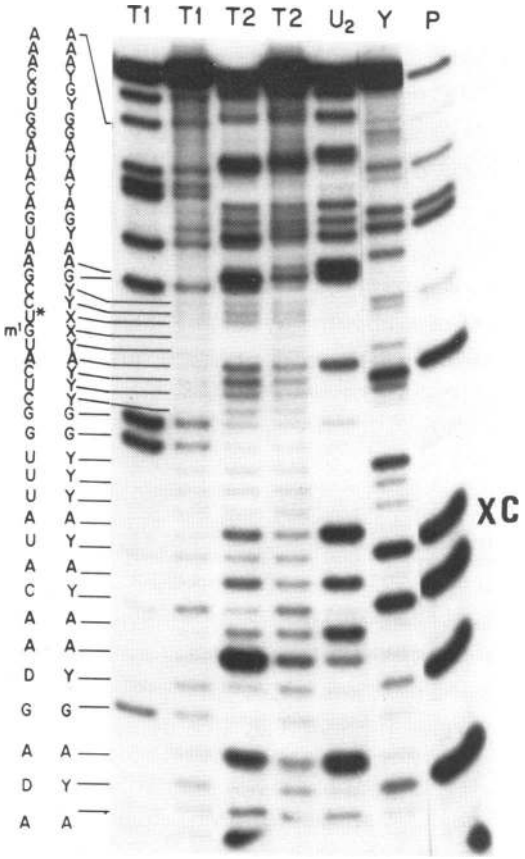

FIGURE 3. Autoradiogram of partial digests on 5'-[^{32}P] labeled N. crassa mitochondrial initiator tRNA. Approximately 40,000 cpm. of [^{32}P]RNA were used for each digestion. Partial digestions contained the following amounts of enzyme per µg. carrier RNA and per µl. of incubation. From left to right:

T_1, 0.01 unit;
T_1, 0.001 unit;
T_2, 0.01 unit;
T_2, 0.001 unit;
U_2, 0.1 unit;
Y, 0.001 unit; and
P, 0.0001 unit.
XC indicates location of xylene cyanole tracking dye.
Y, RNase from B. cereus.
P, pancreatic RNase.

quences of oligonucleotides present in complete T_1-RNase and pancreatic RNase digests of this RNA enabled us to align most of these oligonucleotides into a unique sequence for this tRNA.

The results shown in Figure 3 also demonstrate the striking difference in the partial digestion pattern obtained using the B. cereus enzyme from that using pancreatic RNase. Whereas the B. cereus RNase cleaves almost all the phosphodiester bonds on the 3'-side of pyrimidines, under the consitions used here, pancreatic RNase has a pronounced specificity and cleaves almost exclusively Y-A bonds.

Finally, the results in Figure 3 also exemplify the usefulness of T_2-RNase for obtaining partial digests at every phosphodiester bond in a tRNA. We have no evidence that the

relatively milder conditions used by Donis-Keller, et. al.[9] for partial alkaline digestions of 5'-[^{32}P] RNA compared to the one used here would destroy or alter modified nucleotides in tRNA to the extent that they would cause tRNA fragments to behave anomalously during gel electrophoresis. Should this be the case, however, our results suggest that partial digestion with T_2-RNase would be a good alternative to partial digestion with alkali.

Partial Digestion of 5'-[^{32}P] mRNA with Alkali: Use of Two-Dimensional Polyacrylamide Gel Electrophoresis to Distinguish C from U

Figures 4A and 4B show the results of experiments in which a 5'-[^{32}P] mRNA was partially digested with alkali and the digests were then subjected to two-dimensional gel electrophoresis. The mRNA chosen for this purpose was alfalfa mosaic virus RNA 4. Since electrophoresis in the first dimension was on a 40 cm. long gel and only a 16 cm. long strip from it was used for electrophoresis in the second dimension, the region from which the gel strip was cut and the time of electrophoresis in the first dimension will determine the size range of the partial fragments being analyzed. We have found it useful to apply the same partial digest in two parallel slots for the first dimension and to include an overlap of about 6 cm. or more among the two vertical strips which are subsequently used for electrophoresis in the second-dimension.

The basic principle behind the separation of the homologous oligonucleotides observed in Figures 4A and 4B is similar to that in two-dimensional electrophoresis--homochromatography[5,18,19] and the mobility shifts between two homologous oligonucleotides which differ by a C or A residue can be easily distinguished from those which differ by a U or G residue. In Figs. 4A and B, mobility shifts due to U or G are almost vertical whereas those due to C or A are at a sharp angle. We have some preliminary evidence that it might be possible to differentiate a purine nucleotide mobility shift from a pyrimidine nucleotide, as can be often done in homochromatography.[5,18,19] Thus, it may eventually be possible to additionally distinguish C from A and U from G using two-dimensional electrophoresis. In the current work, we have used the analyses as shown in

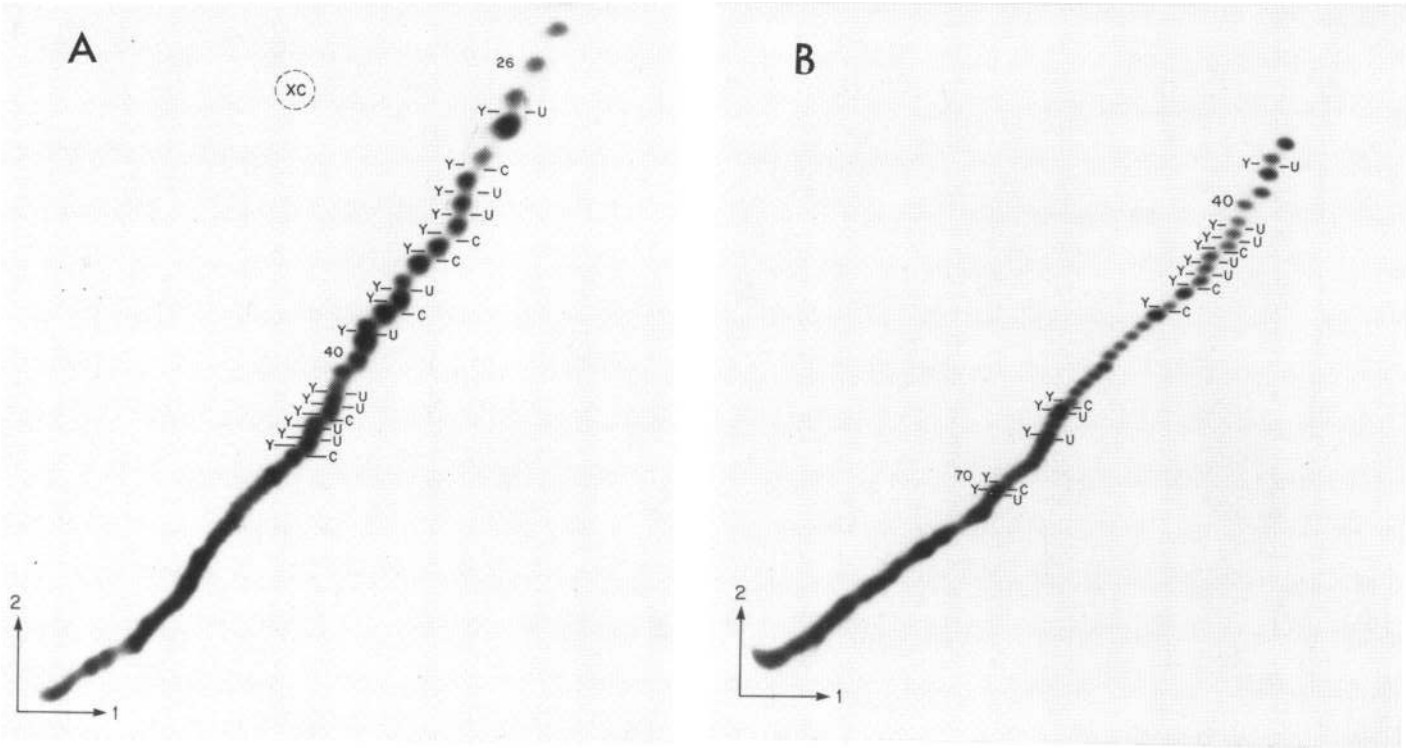

FIGURE 4. Autoradiograms of partial alkaline digests on 5'-[^{32}P] alfalfa mosaic virus RNA 4 as analyzed by two-dimensional polyacrylamide gel electrophoresis. Circled XC indicates location of xylene cyanole tracking dye. Numbers on the left indicate the size of the radioactive oligonucleotide. For other details, see text.

Figures 2 and 3 to locate the pyrimidine residues in an RNA and utilized the two-dimensional gel electrophoretic analysis to distinguish between the pyrimidines. Thus, in Figures 4A and 4B, Y on the left of a radioactive spot indicates that the mobility shift is due to a pyrimidine, and the letter C or U on the right identifies the pyrimidine residue as either cytosine or uracil.

DISCUSSION

Along with the work of Donis-Keller, et al.,[9] the work described in this paper provides a rapid and simple approach for the sequencing of up to 100 nucleotides from the 5'-end of an RNA. Although one of the main classes of RNAs of interest, eukaryotic mRNAs, contain a blocked "cap" structure at their 5'end,[13] we have outlined a method for their removal and subsequent labeling of the 5'-end of mRNAs with [^{32}P].[6] Using such a procedure and a combination of the methods described in this paper involving one-dimensional and two-dimensional polyacrylamide gel electrophoretic analysis of partial digests (Figures 2, 4A and 4B), we have recently determined the sequence of 74 nucleotides at the 5'-terminus of alfalfa-mosaic virus RNA 4 leading into the coat protein cistron[7]. Since the translational initiator codon in most eukaryotic mRNAs is likely to be 100 nucleotides or less from the 5'-end, sequence analysis of the 5'-untranslated regions of most eukaryotic mRNAs which are available in pure form or which can be purified subsequent to 5'-labeling will, therefore, be much simpler now.

The procedure originally described by Donis-Keller, et. al.[9] allows one to map the location of G's, A's and of pyrimidines from the end of a terminally labeled RNA using partial digestion with T_1-RNase for cleavage specifically at G residues, with U_2-RNase for cleavage at A residues, and with alkali for cleavage at all four residues. In our experience, non-specific cleavages by T_1-RNase and U_2-RNase are insignificant except at very high enzyme/substrate ratios. Any ambiguity that might arise in the interpretation of data due to such non-specific cleavages can be usually resolved by simultaneous electrophoresis in adjacent lanes of partial digests carried

out at several enzyme/substrate ratios.

A method for distinguishing between the pyrimidines has so far not been available. An ideal approach for this would be the use of an enzyme or a chemical reagent which under conditions of partial digestion cleaves all CpN bonds in an RNA without cleaving any of the UpN bonds or vice versa. Attempts along these lines are currently underway (H. Donis-Keller, personal communication; K. Randerath, personal communication). In the present work, we have used two-dimensional polyacrylamide gel electrophoresis of partial alkali digests to distinguish between C and U. While we were able to use this method for sequencing up to 74 nucleotides from the 5'-end of an RNA, it is quite possible that this can be extended to 100 or more through the use of lower percentage polyacrylamide gels in the second dimension to obtain better separation among the very long oligonucleotides. In addition, we are now using an apparatus for electrophoresis in the second dimension which can accommodate almost the entire length (30 cm.) of one slot from the first dimension and this will allow us to obtain the information necessary for distinguishing C from U on oligonucleotides up to 100 long using no more than two two-dimensional gel electrophoretic fractionations.

Finally, although the methods described in here (also ref. 9) are applicable to sequencing mRNAs and most other RNAs, an application of these methods alone may not be adequate for establishing the total sequence of tRNAs, since tRNAs contain many modified nucleotides--some of which are quite resistant to partial digestion with nucleases and others, such as 2'-O-methylated nucleotides, are totally resistant toward digestion by either nucleases or alkali. Consequently, it will still be necessary to have in hand a knowledge of oligonucleotide sequences present in complete RNase digests of the tRNA.[1-4,8,20] The usefulness of the methods discussed in here for sequencing tRNA lies more in the fact that these methods can provide most of the overlap information necessary for ordering these oligonucleotides into a unique sequence within a relatively short period and requires less than a microgram of the tRNA. Thus, using partly this method, we have recently completed the se-

quence analyses of initiator tRNAs from wheat germ (in collaboration with H.P. Ghosh and coworkers), mycoplasma[21], N. crassa mitochondria[3], and Drosophila melanogaster (in collaboration with D. Söll, G.M. Tener and their coworkers).

ACKNOWLEDGEMENTS

We thank Helen Donis-Keller, Allan Maxam and Walter Gilbert for their assistance and dissemination of their work prior to publication. We are grateful to Helen Donis-Keller also for providing us with the conditions for partial alkaline digestion used in here. This work was supported by grants from the National Institutes of Health (GM 17151) and the American Cancer Society (NP-114) to U.L.R. and in part by the Veteran's Administration Fund to J.M. R.E.L. was a postdoctoral fellow of the Medical Foundation, Boston, MA, supported by the Charles A. King Trust, and was earlier in the course of this work a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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