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Rapid print-readout technique for sequencing of RNA's containing modified nucleotides

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ABSTRACT

A rapid, simple, and highly sensitive method for sequence analysis of RNA was developed, which consists of the following steps: (i) controlled hydrolysis of the RNA by brief heating in water; (ii) (^{32}P)-labeling of 5'-hydroxyl groups of the fragments produced in (i); (iii) resolution of labeled fragments by size on polyacrylamide gels giving the familiar "ladder"; (iv) contact transfer ("print") of the ladder from the gel to a PEI-cellulose thin layer; (v) in situ treatment of the ladder with RNase T_2 resulting in the release of 5'-(^{32}P)-labeled nucleoside-3',5' diphosphates; (vi) contact transfer and thin-layer separation of (^{32}P)-labeled nucleotides on PEI-cellulose in ammonium sulfate and ammonium formate solvents; (vii) autoradiography. The chromatographic behavior of the 4 major and 18 modified nucleotides was determined. The positions of major and modified nucleotides in the sequence can be read directly from the separation patterns displayed on X-ray film. As this is the only sequencing method presently available that allows one to display and identify directly the positions in the RNA chain of major and modified nucleotides, no additional procedures are required to analyze the latter.

INTRODUCTION

During the past 1-2 years sensitive readout techniques for the sequence analysis of RNA have been reported[1-5]; these are related to the chemical DNA sequencing technique[6], differing from the latter mainly by the use of enzymes rather than chemicals for the controlled digestion of a terminally labeled polynucleotide. While representing useful new tools for the sequence analysis of RNA, the application of these techniques is restricted mainly due to 2 problems: (i) highly base-paired regions of RNA are resistant to enzymatic cleavage even under denaturing conditions and (ii) the enzymes used to cleave the RNA do not sufficiently discriminate the modified nucleotides from each other and from the major nucleotides. Because of the first point it is difficult or impossible to read accurately the sequence in some regions of the RNA; because of the second point additional procedures are needed to establish the identity and the location of modified nucleotides.

We have developed a new RNA sequencing method which circumvents the difficulties resulting from the resistance of highly hydrogen-bonded areas to enzymatic digestion and also allows one to identify directly the positions of modified nucleotides in the RNA chain. The method is in part based on the observation recently reported by Stanley and Vassilenko[7] that under sufficiently mild conditions of chemical degradation of RNA the cleavage products arise mostly from random single "hits", leading to the formation of 2 sets of fragments, the first set consisting of chains extending from the phosphorylated 5'-terminus of the RNA (e.g., tRNA) to internal residues carrying 3'-terminal phosphate groups, the second set containing chains extending from internal positions carrying a free 5'-hydroxyl group to the 3'-terminus of the RNA. In the newly developed method, we partially hydrolyze the RNA by brief heating in water to generate the 2 sets of fragments, label the free 5'-hydroxyl groups of the second set of fragments with ^{32}P by the polynucleotide kinase reaction, separate the fragments on a denaturing polyacrylamide gel into a series of 5'-terminally (^{32}P)-labeled fragments, each differing from its neighbor by the addition or the removal of a single 5'-terminal nucleotide, contact-transfer the radioactive fragments to a PEI-cellulose anion-exchange thin-layer sheet ("print" step), digest the fragments *in situ* with RNase T_2 [8,9], and resolve the 5'-(^{32}P)-labeled nucleoside-3',5' diphosphates released in the previous step by thin-layer chromatography. After autoradiography the nucleotide sequence can be read directly from the spot pattern displayed on the X-ray film. This is the only method presently known that allows one to display and identify directly the positions in the nucleic acid chain of modified nucleotides along with those of the major nucleotides.

MATERIALS

Polynucleotide kinase (P-L Biochemicals), RNase T_2 (Sankyo), nuclease P_1 (Yamasa Shoyu Co., Tokyo), and potato apyrase (Sigma, grade I) were used without further purification. $tRNA_1^{Val}$ of *E. coli* B[10] (Medical Research Laboratories, Irvine, CA.) and $tRNA^{Tyr}$ (a mixture of $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ [11]) of *E. coli* MRE600 (Boehringer Mannheim) were purified by electrophoresis on a 12% acrylamide, 7 M urea gel and isolated as described[12]. The nucleoside-3',5' diphosphates, pAp, pCp, pUp, and pGp were from P-L Biochemicals.

The modified nucleoside-3',5' diphosphates, pm^1Ap, pac^4Cp, pm^5Cp, pm^1Gp, $pm_2^2G>p$, and pm_2^2Gp were prepared in (^{32}P)-labeled form by RNase T_2 digestion of oligonucleotides in RNase A and RNase T_1 digests of yeast $tRNA_{CUA}^{Leu}$ [13], yeast $tRNA_{UUG}^{Leu}$ [14], and human placenta $tRNA^{Phe}$ [15], followed by (^{32}P)-labeling of the

resulting nucleoside-3' monophosphates with (γ-^{32}P)ATP (ICN) and polynucleotide kinase. pDp, pVp, pm^{6}Ap, and pm^{7}Gp were obtained from E. coli tRNA$_1^{Val}$, pm^{2}Gp, pUm-Up, and pΨm-Cp from human placenta tRNA$_{CCC}^{Gly}$ (R.C. Gupta, B.A. Roe, and K. Randerath, manuscript in preparation), and pQp, pms^{2}i^{6}Ap, ps^{4}Up, pTp, pΨp, and pGm-Gp from E. coli tRNA^{Tyr} by subjecting the tRNAs to the print-readout sequencing technique as described in this paper.

Ultrapure urea was obtained from Schwarz/Mann, and acrylamide and methylene bisacrylamide from Bio-Rad.

Macherey and Nagel PEI-cellulose sheets (40 cm x 20 cm and 20 cm x 20 cm) without indicator were purchased from Brinkmann Instruments. Prior to use, the smaller sheets were pre-developed in water[16]. After the front had migrated to the top of the sheets, the cover of the chromatographic tank was slightly moved back so as to allow water to evaporate from the upper portion of the sheets. Development was for 8-15 hrs. The sheets were allowed to dry at room temperature for several hrs before use. If not used the same day they were stored at -20°. The 40 cm x 20 cm sheets were used for the print step (see below) without pre-development. X-Omat R films were from Eastman-Kodak. Cronex 4 films and calcium tungstate Lightning Plus intensifying screens were from DuPont. (^{99}Tc)ink was prepared by diluting (^{99}Tc)NH_4TcO_4 (New England Nuclear) with regular ink.

Button-type Alnico magnets (#372A, 3/8" high x 1/2" dia., General Hardware Manufacturing Co., New York, N.Y. 10013) were purchased from a local stationary store.

BASIC PROCEDURE AND EXAMPLE

In this section E. coli tRNA^{Tyr} will be used as an example to illustrate the method.

Controlled hydrolysis of RNA. A sealed tube containing 2-10 µg of RNA in 5-10 µl of double-distilled water is placed in a water bath at 80° for 6 min. The solution is evaporated in a current of cool air.

(^{32}P)-labeling of 5'-ends. The residue is dissolved in 5-10 µl of 40 mM Tris-HCl, pH 8.7, 13 mM $MgCl_2$, 10 mM dithiothreitol, 25 µM (γ-^{32}P)ATP (200-800 Ci/mmole). 0.5 µl (3-5 units) of polynucleotide kinase is added and the solution kept at 38° for 25 min. One µl of apyrase solution (4 milliunits/µl) is added per 5 µl of incubation mixture and the solution kept at 38° for 20 min. The solution is evaporated in a current of cool air.

Gel electrophoresis. The residue is dissolved in 20-40 µl of 90 mM Tris

base, 90 mM boric acid, 1 mM EDTA, 7 M urea containing about 10 μg each of bromophenol blue (BP) and xylene cyanol FF (XC). 5-μl aliquots of the sample are fractionated on a slab of polyacrylamide (50 cm x 30 cm x 0.06 cm; 14 wells of dimensions 1 cm x 1 cm x 0.06 cm) prepared from 12% acrylamide, 0.4% methylene bisacrylamide, 7 M urea, 90 mM Tris base, 90 mM boric acid, 1 mM EDTA, 0.03% ammonium persulfate, 0.03% N,N,N',N'-tetramethylethylenediamine. Prior to electrophoresis, the gel is allowed to age at least 8 hrs and then pre-electrophoresed 8-15 hrs at 900 volts, using 90 mM Tris base, 90 mM boric acid, 1 mM EDTA as the electrophoresis buffer. The sample is loaded in alternate wells at 0, 4, 8, and 11 hrs and electrophoresed at 1200 volts (constant), about 20 milliamps for a total of 14 hrs.

Print step. A PEI-cellulose sheet (40 cm x 20 cm) is soaked in 500 ml of water for 10 min. One glass plate is removed from the gel by gently prying with a spatula. The gel surface is wetted thoroughly with water. The drained PEI-cellulose layer is placed on top of the gel. Trapped air is extruded by gently stroking the plastic backing of the thin layer. The gel/PEI-cellulose assembly is wrapped in thin plastic foil such as "Saran" wrap. With the layer on top of the gel, the assembly is covered with a piece of aluminum foil and then a plain glass plate, and 3 or 4 glass thin-layer chromatography tanks (or similar weight) are placed on top. After 15-24 hrs at room temperature, the PEI-cellulose sheet is removed, dried in a current of warm air, and soaked in 300-500 ml of methanol for 5-10 min. Before autoradiography, the layer is marked with (^{99}Tc)ink dots for alignment after exposure. A sheet of Cronex 4 film is sandwiched between the layer and the sensitive side of an intensifying screen. Exposure is at -20° or -70° for 20 min to a few hrs depending on the amount of radioactivity to be detected. Sensitivity is 5-10 times greater at -70° than at -20°.

Fig. 1 depicts polyacrylamide gel ladders (a-d) and a PEI-cellulose print ladder (e) obtained by applying this procedure to E. coli $tRNA^{Tyr}$. Chain lengths of up to 25-30 nucleotides are being transferred almost quantitatively by the printing technique. Transfer of larger fragments becomes progressively less efficient, but enough is being transferred for terminal analysis of chain lengths of up to 120. (Larger fragments have not been investigated thus far.) It should be noted that only the prints have to be autoradiographed; the gel autoradiogram is presented mainly to show that the procedure affords a reasonably regular spectrum of terminally labeled products. After contact transfer, ladders a, b, c, and d are suitable for the determination of positions 51-84, 32-62, 10-47, and 2-35, respectively, encompassing almost the entire sequence

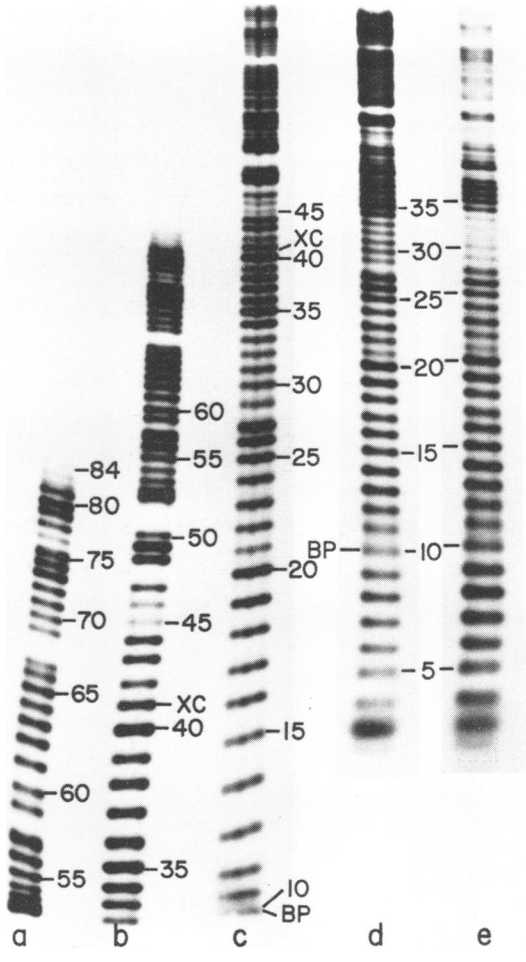

Fig. 1.

Gel ladders (a-d) and a print ladder (e) obtained by heating 5 μg of *E. coli* $tRNA^{Tyr}$ in 5 μl of water at 80° for 6 min, followed by 5'-terminal (^{32}P)-labeling. Specific activity of (γ-^{32}P)ATP was 300 Ci/mmole. Four identical aliquots were loaded on a 12% polyacrylamide slab gel (50 cm x 30 cm x 0.06 cm) at 0 (a), 4 (b), 8 (c), and 11 (d) hrs and electrophoresed at 1200 volts for 14 hrs. (e) depicts the PEI-cellulose print ladder obtained from (d). Ladders (d) and (e) were lowered to facilitate reproduction. Autoradiography with intensifying screen for 30 min at -20°. BP and XC, positions of tracking dyes, bromophenol blue and xylene cyanol FF, respectively. Positions are numbered in 3' to 5' direction.

of the RNA (Fig. 2).

Note the large gaps between bands 47 and 48, 50 and 51, and 67 and 69. Fragments 48 and 51 are retarded on the gel because of the presence at their 5'-ends of the hypermodified nucleosides ms^2i^6A and Q, respectively. Band 68 is missing due to the resistance to hydrolysis of the phosphodiester bond linking Gm69 and G68. In contrast, bands 27 and 28, 57 and 58, and 75 and 76 are incompletely resolved. This appears to be a secondary structure effect which occurs when pG or pC is added at the 5'-end of a stem region, resulting in the formation of a G = C base pair, cf. Fig. 2.

5'-terminal analysis and reading of the sequence. This involves the following manipulations: (i) excision of the ladder from the print; (ii) treatment of the ladder with RNase T_2; (iii) preparation of the treated ladder for contact transfer; (iv) chromatography of RNase T_2 digestion products after contact transfer to a fresh PEI-cellulose acceptor sheet; and (v) autoradiographic detection of products and reading of the sequence.

(i) Film and print are aligned and the positions of the ladders are marked by punching holes through the film into the layer. The print sheet is

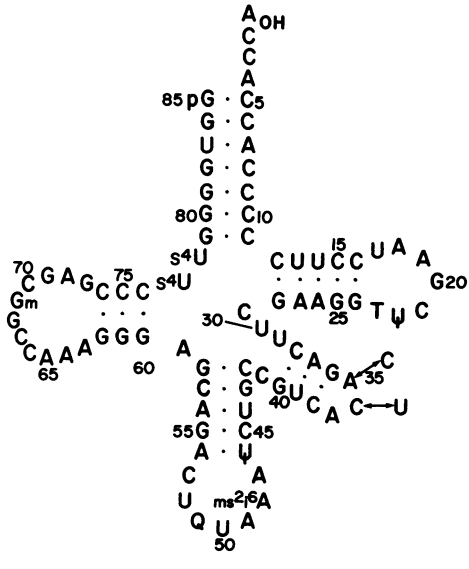

Fig. 2.

The nucleotide sequence of E. coli $tRNA^{Tyr}$ 11 arranged in the clover-leaf configuration. Positions in the sequence are numbered from 3' to 5' so that the numbers correspond to the chain lengths of the fragments indicated in Fig. 1.

cut midway between individual ladders of interest, leaving 6-10 mm wide non-radioactive margins parallel to the ladder (Fig. 3a and e). Strips are marked appropriately with pencil.

(ii) Up to 5 ladder strips are soaked in 200 ml of 0.15 M sodium acetate, pH 4.5, for 10 min with agitation. Without drying, the strips are soaked in 200 ml of methanol for 10 min and dried. A solution of RNase T_2 is prepared containing 0.2 unit/µl in water. (This stock solution can be kept at -20° for several months.) 20-µl portions of this enzyme solution are applied rapidly and evenly by streaking with a disposable micropipet to a strip of the ladder containing about one third to one quarter of each gel band (Fig. 3b) adjacent to the non-radioactive part of the layer (Fig. 3a). One 20-µl portion is sufficient for 5-6 cm. Leaving the center of the ladder (Fig. 3c) untreated, one proceeds with the other part (Fig. 3d) until the entire length of the ladder to be analyzed has been moistened with enzyme solution. The treated strip is covered with Teflon tape and wrapped tightly in Parafilm to retard evaporation. The strips are sandwiched between glass plates and kept at 38° for 2-3 hrs or overnight.

(iii) The strips are cut lengthwise into 2 narrower strips, each comprising the RNase T_2-treated zone, as well as an about 6 mm wide strip from the adjacent non-radioactive area (total width, about 10 mm; length, up to 18 cm), see Fig. 3.

(iv) Following contact transfer, one of the 2 strips is analyzed by chromatography on PEI-cellulose in an ammonium sulfate system[17], the other (from the same part of the ladder) in a pH 3.5 ammonium formate system. For contact transfer and chromatography of digestion products in ammonium sulfate,

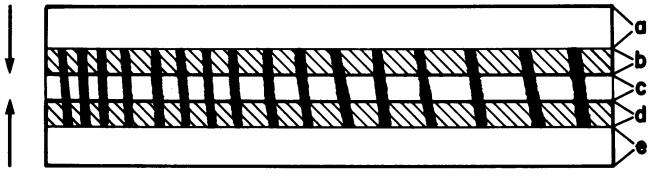

Fig. 3.

Preparation of the 2 donor strips from the print ladder (schematic). a and e denote non-radioactive regions of the layer; b and d, regions of the ladder treated with RNase T_2; and c, the untreated region of the ladder. After RNase T_2 treatment the center portion of the ladder is excised so that 2 separate RNase T_2-treated strips are obtained for subsequent contact transfer of the released (^{32}P)-labeled digestion products to a PEI-cellulose sheet (see text). The arrows indicate the direction of solvent migration through the donor strip during contact transfer/chromatography.

a Whatman 1 wick is attached at the top of a PEI-cellulose sheet (20 cm x 20 cm) by stapling and the layer of the donor strip is then placed on the acceptor sheet in such a way that its non-radioactive portion is below the origin line (about 2.5 cm from the bottom). The donor strip and the acceptor sheet are sandwiched between 2 strong magnetic bars. The preparation of such bars from individual magnets is described in ref. 1[18]. After the 3' → 5' direction of the donor ladder has been indicated on the acceptor layer with pencil the chromatogram is developed at 4° with water to the origin line, then with 0.55 M ammonium sulfate to 3-4 cm on the wick. After removal of the magnetic bar and the donor strip, the layer is dried in a current of warm air and the wick is cut off.

For the analysis of the released 5'-terminal nucleotides in the ammonium formate system, donor strip and acceptor sheet are pre-treated with pH 3.5 buffer prior to the development in ammonium formate. Several (up to 5) ladder strips are soaked in 100 ml of 0.1 M ammonium formate, pH 3.5, for 7 min and dried with warm air. In this case, contact transfer and chromatographic separation are being performed as 2 separate operations. For transfer, the layer of the donor strip is placed on the PEI-cellulose acceptor sheet so that its non-radioactive portion is below the origin line (1.5 cm from bottom) and held in place with magnets as described above. The chromatogram is developed at room temperature with water to the origin line, then with 4 M lithium formate, 7 M urea, pH 3.5, to 5 cm from the bottom of the acceptor sheet. The nucleotides will migrate onto the acceptor sheet in this solvent. After removal of the magnets and the donor strip, the wet sheet is soaked in 200 ml of methanol for 7 min, dried, and then soaked in 200 ml of 0.1 M ammonium formate, pH 3.5, for 7 min. After drying (5 min in warm air), a Whatman 1 wick is attached to the top of the sheet, which is finally developed in 1.75 M ammonium formate, pH 3.5, to 4 cm on the wick. After drying in a current of warm air, the wick is cut off.

(v) For autoradiography, X-Omat R film is sandwiched between the layer and the sensitive side of an intensifying screen. Exposure is at -20° or -70° for up to several hrs or overnight as required. If the chromatogram has weakly and strongly labeled nucleotides exposure should be repeated for a different length of time.

Fig. 4 illustrates the resolution in the ammonium sulfate system of 5'-terminally (^{32}P)-labeled nucleotides released by *in situ* RNase T_2 treatment of print ladders from *E. coli* $tRNA^{Tyr}$. The sequence shown extends from the 5'-side of the D-stem of the RNA to the apex of the large variable loop. Fig.

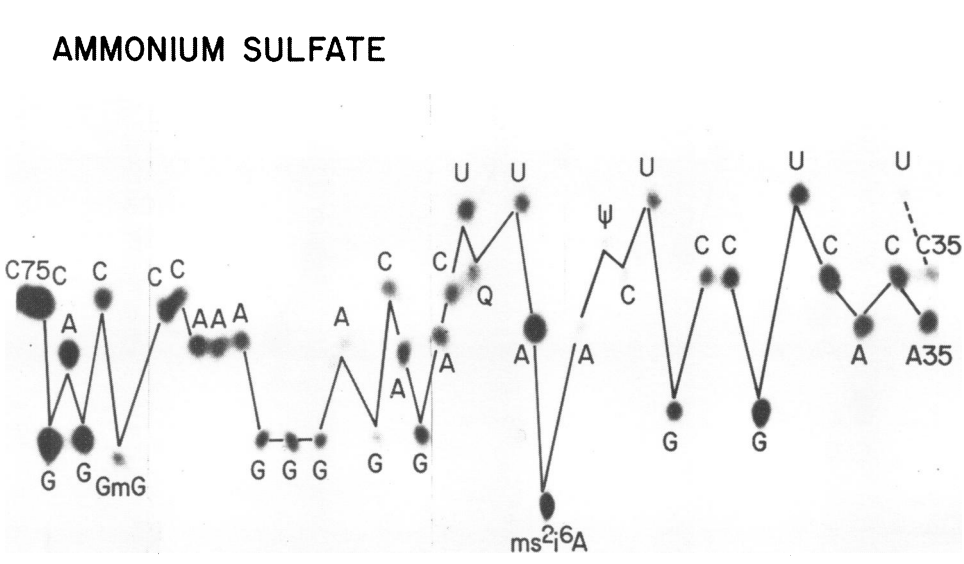

Fig. 4.

5'-terminal analysis of fragments 35-75 from the prints of E. coli $tRNA^{Tyr}$ ladders a and b (Fig. 1). After RNase T_2 treatment in situ, the released 5'-terminal nucleotides were contact-transferred to a PEI-cellulose sheet for further chromatographic analysis in 0.55 M ammonium sulfate. Autoradiography, as shown, enables to read the sequence from position 35 (from 3'-end) to position 75. Two termini each are found for positions 35 and 36, indicating that the tRNA used for the analysis was a mixture of $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ [11].

5 depicts a print-readout in ammonium sulfate extending from the T-loop to the third nucleotide from the 3'-end of the RNA. Note that pψp and pGm-Gp as well as the hypermodified nucleotides pQp and pms^2i^6Ap are well resolved from each other and the major nucleoside-3',5' diphosphates.

Figs. 6 and 7 exemplify print-readouts in the ammonium formate system extending from the 5'-side of the D-stem of $tRNA^{Tyr}$ to the anticodon loop and from the 5'-side of the T-stem to the acceptor stem, respectively. The modified nucleotides are seen again to be quite well separated from each other and the major nucleotides. pAp was found to travel in a second front, sometimes trailing slightly. Note the fast migration of pQp in this acidic solvent, explained by the secondary amino group of the side chain of the Q base[19]. pms^2i^6Ap forms a double-spot in this solvent, the faster of which may be the cyclic nucleotide, $pms^2i^6A>p$.

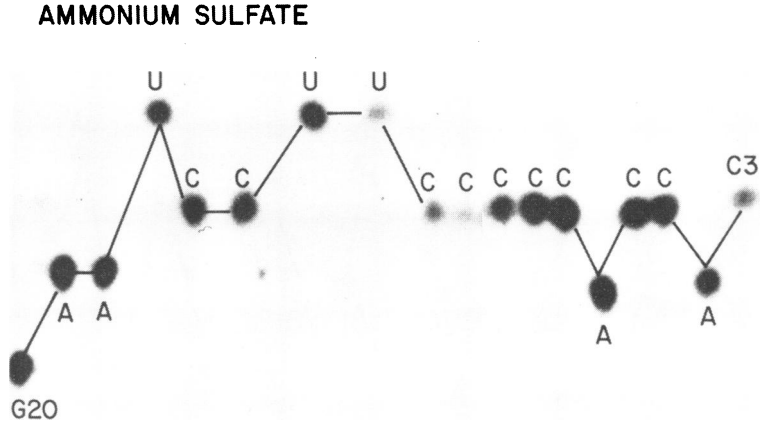

Fig. 5.

5'-terminal analysis of fragments 3-20 from the prints of ladders c and d (Fig. 1). For further details, consult text and legend of Fig. 4.

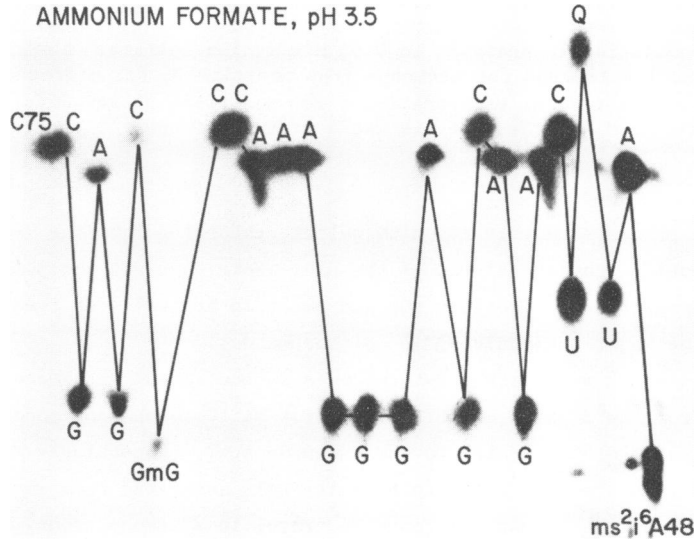

Fig. 6.

5'-terminal analysis of fragments 48-75 from the prints of ladders a and b (Fig. 1). Chromatography in 1.75 M ammonium formate, pH 3.5. C66 and C67 are not clearly resolved; a shorter exposure (not shown) indicated 2 spots which also separate in the ammonium sulfate system (Fig. 4). In addition, the ladder (Fig. 1a) shows 2 bands corresponding to these positions.

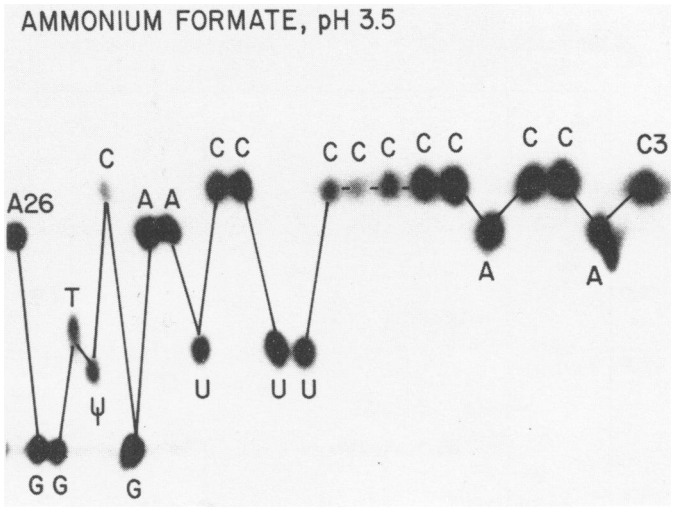

Fig. 7.

5'-terminal analysis of fragments 3-26 from a print of ladder d (Fig. 1e). Chromatography in 1.75 M ammonium formate, pH 3.5. For further details, consult text and legend of Fig. 4.

ANALYSIS OF NUCLEOSIDE-3',5' DIPHOSPHATES

The chromatographic behavior of the 3',5' diphosphates of the 4 major and the most common modified nucleosides on PEI-cellulose was investigated. Among several systems, unbuffered ammonium sulfate and ammonium formate, pH 3.5, were found to give the most satisfactory results. Solubility effects have been shown to play a major role in the chromatography of nucleotides on PEI-cellulose in ammonium sulfate[17]. In accord with this observation, 0.55 M ammonium sulfate was found to be a particularly powerful solvent for the separation of methylated and other modified nucleoside-3',5' diphosphates from the parent nucleotides. Results obtained in the 2 systems complement each other so that most modified tRNA constituents can be identified on the basis of their relative R_F values in ammonium sulfate and ammonium formate, pH 3.5 (Fig. 8). The ammonium sulfate system may be used at room temperature, but resolution of nucleotides is somewhat better at 4°.

For identifying (^{32}P)-labeled nucleoside-3',5' diphosphates that do not separate well in both systems (such as pac^{4}Cp/pTp and pm^{6}Ap/pCp), one converts these compounds to the corresponding (^{32}P)-labeled nucleoside-5' monophosphates by treatment of the spots with nuclease P_1, followed by contact transfer and

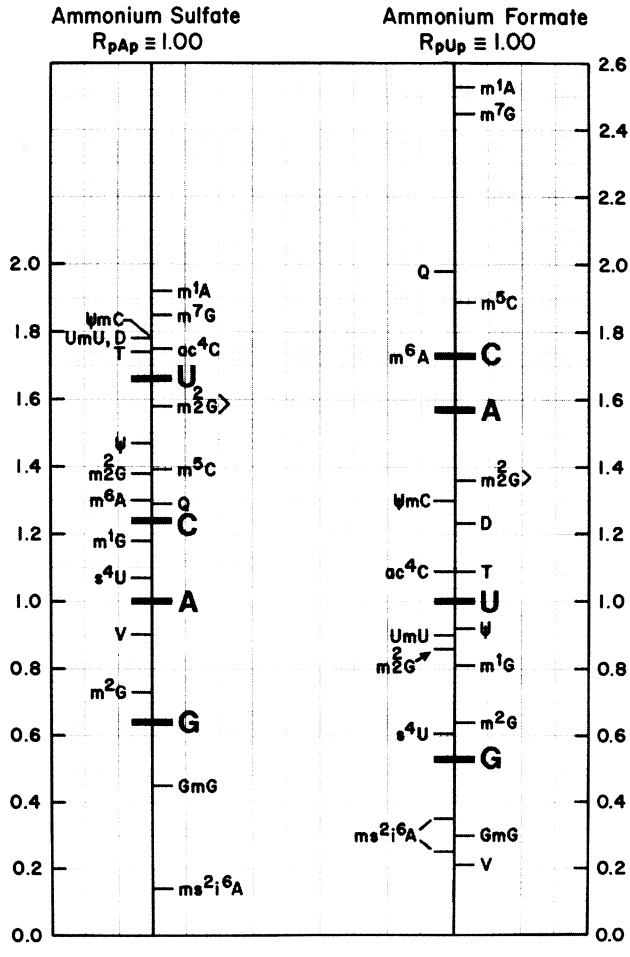

Fig. 8.

Diagram of relative R_F values of nucleoside-3',5' diphosphates and ribose-methylated dinucleoside triphosphates in ammonium sulfate at 4° ($R_{pAp} \equiv 1.00$) and in ammonium formate, pH 3.5 at 23° ($R_{pUp} \equiv 1.00$). V, Q, and ms^2i^6A denote nucleoside-3',5' diphosphates of uridine-5-oxyacetic acid[20], 7-(4,5-cis-dihydroxy-1-cyclopenten-3-yl-aminomethyl)-7-deazaguanosine[19], and 2-methylthio-N^6-isopentenyladenosine[21], respectively.

chromatography. Individual nucleoside-3',5' diphosphate spots to be analyzed are cut from the ammonium formate chromatogram. The cut-out(s) are soaked in 20-50 ml of methanol for 5 min and dried. 4-5 µl of a solution containing 0.2 µg/µl of nuclease P_1 in 50 mM Tris-HCl, pH 7.3, are applied to the center

of the cut-out. After the cut-outs have been covered with Teflon tape and Parafilm they are sandwiched between two glass plates and kept at 38° overnight. The treated cut-outs are soaked in methanol and dried. One µl of a mixture of non-radioactive major nucleoside-5' monophosphates (about 5 nmoles each) is applied to the center of the cut-out. The compounds are contact-transferred with the aid of button magnets[8] to a PEI-cellulose layer (20 cm x 20 cm) by development in either acetic acid/formic acid or Tris-HCl, pH 8.0, as described previously for the separation of nucleoside-5' monophosphates[22]. After removing the magnets and cut-outs, the chromatogram is dried in a current of warm air and non-radioactive nucleotides are located in ultraviolet light and radioactive nucleotides by autoradiography.

COMMENTS AND DISCUSSION

The starting material should be at least 90% pure and undegraded; for example, RNAs or RNA fragments freshly isolated from denaturing polyacrylamide gels were found to meet the requirements of the procedure. A control experiment is recommended in which a sample of the RNA is subjected to 5'-(^{32}P)-labeling and gel electrophoresis without prior heating in order to check for breaks. The parent molecule should have a phosphorylated or otherwise blocked 5'-terminal hydroxyl group to prevent labeling of fragments encompassing the 5'-end of the RNA, and chemical degradation of the RNA should be performed in such a way as to avoid overdigestion resulting in double "hits". These requirements hold regardless of whether cleavage is achieved by heating in formamide[7] or water. An acceptable level of digestion was obtained by heating the intact tRNA in water at 80° for 6-10 min. Digests obtained by heating at 90° for 2-3 min or at 100° for 0.5-1 min appeared to be still suitable for sequence analysis. Simple hydrolysis in water offers advantages over formamide digestion, because the sample is ready for the labeling reaction without additional steps, whereas formamide interferes with the polynucleotide kinase reaction and thus needs to be removed by ethanol precipitation of the RNA or by freeze-drying[7].

In spite of non-uniform labeling of 5'-ends in the polynucleotide kinase reaction, the extent of labeling of most modified nucleotides appeared sufficient for their subsequent detection on the readout chromatograms. However, prolonged exposure times may be required to detect weakly labeled modified compounds such as m_2^2G and ribose-methylated nucleotides. The apyrase digestion step serves to remove unreacted (γ-^{32}P)ATP and other unidentified (^{32}P)-labeled contaminants which interfere with the detection of some shorter

fragments (chain length < 10) of the ladder. At the dilution used, the commercial apyrase preparation was found not to contain phosphatase and nuclease activities interfering with subsequent analysis.

Fifty cm long thin[23] (0.06 cm) 12% or 15% gels appeared best suited for resolving the labeled constituents in tRNA hydrolysates; longer gels and/or higher acrylamide/bisacrylamide content may be required for larger RNAs. Compared with 20% gels, the lower percentage gels offer additional advantages in terms of saving of material and time; in addition, transfer of larger RNA chains was more rapid from these gels than from 20% gels (data not shown). The lack of diffusion on the print (Fig. 1e) is probably due to binding of the transferred polynucleotides to polyethyleneimine. As a rule of thumb, the distance between 2 adjacent gel bands should be about 3 mm or larger to enable their subsequent analysis. As mentioned earlier (see also Fig. 1), 2 adjacent bands may be incompletely resolved in rare instances under the experimental conditions described. Because this appears to be due to the secondary structure, the effect may be overcome by running the denaturing gels at higher voltages so that the helical regions are denatured at the resultant higher temperature (about 50°)[7,23].

In situ digestion on PEI-cellulose thin layers of 3'-terminally labeled polyribonucleotides with RNase T_2 was previously shown to be a convenient means of releasing labeled 3'-terminal groups for subsequent chromatographic identification[8,9]. In the case of 5'-(^{32}P)-labeled polyribonucleotides, this treatment results in the extensive release of the end groups as 5'-(^{32}P)-labeled nucleoside-3',5' diphosphates, except that ribose-methylated nucleotides are obtained as dinucleotide derivatives and m_2^2G partially forms the corresponding 2',3'-cyclic nucleotide derivative. Hypermodified compounds such as ms^2i^6A may also give rise to partial formation of cyclic nucleotides, as mentioned earlier.

The pairs pac^4Cp/pTp and pm^6Ap/pCp are not sufficiently resolved in the 2 chromatographic systems described. Since the modified nucleosides in the RNA to be sequenced have usually been identified independently by base analysis of the intact RNA and ac^4C, rT, and m^6A, respectively, occur usually in specific positions in the RNA chain, the chance of misidentifying these nucleotides is small. The simple expedient of digesting the nucleoside-3',5' diphosphate to a nucleoside-5' monophosphate by in situ treatment of the spot with nuclease P_1, followed by contact transfer, allows one to resolve these compounds as (^{32}P)-labeled nucleoside-5' monophosphates. Separation of pac^4C from pT and pm^6A from pC on PEI-cellulose thin layers has been described previously[22].

Yields of nucleoside-5' monophosphates after *in situ* nuclease P_1 treatment of nucleoside-3',5' diphosphate spots at pH 7.3 were found to depend on the base, amounting to 20-50% for pyrimidine derivatives and 60-80% for purine derivatives. The influence of pH and nucleotide base on the dephosphorylation of nucleoside-3' monophosphates by nuclease P_1 has been investigated[24].

Since the procedure described does not allow one to identify the 5'-terminus and the 3'-terminus of the RNA, these have to be analyzed separately, for example by (^{32}P)-labeling of the 5'-terminal nucleotide after dephosphorylation[2,5] and by (^{3}H)-labeling of the 3'-end[1], followed by RNase T_2 digestion of the RNA and chromatographic identification (this paper and ref.[8]).

The procedure described is accurate, technically simple, and highly sensitive. A total of 1-2 µg of tRNA is sufficient to generate 4-6 ladders. At a specific activity of (γ-^{32}P)ATP of 400 Ci/mmole, 10^3-10^4 dpm are obtained in individual bands of the print ladder. This is ample radioactivity for subsequent digestion and analysis. In the case of RNAs containing modified nucleotides, the print-readout technique provides sequence information more rapidly than any other presently known procedure.

Part of this work has been reported in preliminary form[25].

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ABBREVIATIONS

Abbreviations used are as recommended (1979) by the IUPAC-IUB Commission on Biochemical Nomenclature (see Biochim. Biophys. Acta 247, 1 (1971)). Additional abbreviation: PEI-cellulose, anion-exchange cellulose material obtained by treating cellulose with polyethyleneimine[16].

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