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A New Chemical Procedure for ^{32}P-Labeling of Ribonucleic Acids at Their 5′-Ends after Isolation

(5′-terminus/35S RNA of avian myeloblastosis virus/nonaqueous media)

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ABSTRACT A new technique, which utilizes the chemical reaction between [^{32}P]diimidazolidate of orthophosphate and the cetyltrimethylammonium salt of high-molecular-weight RNA in nonaqueous dimethyl formamide, has been developed for the ^{32}P-labeling of RNAs after isolation. The radioactive label of high specific activity is introduced onto a phosphorylated 5′-end of the RNA and renders it suitable for 5′-terminal group analysis. When the labeling reaction was applied to the 70S RNA of avian myeloblastosis virus, a labeled 35S RNA was isolated on sucrose–dimethyl sulfoxide gradients without apparent degradation.

One dominant technique is currently used for the ^{32}P-labeling of ribonucleic acids at the 5′-ends after isolation (1). It utilizes phosphatase-polynucleotide kinase treatment of RNA in the presence of [γ-^{32}P]ATP (1, 2). A few chemical procedures have also been reported for the purpose of radioactive labeling of the 5′-end (3, 4) but are seldom used in labeling of high-molecular-weight ribonucleic acids.

We now report a novel sensitive chemical procedure which introduces ^{32}P-label of high specific activity at phosphorylated 5′-ends of low- and high-molecular-weight RNAs. The method is based on the reaction of [^{32}P]diimidazolidate of orthophosphate, which reacts exclusively with primary phosphates (5–7) (Fig. 1). The internucleoside phosphate diesters are weak nucleophiles and remain unaltered. The ^{32}P-labeled ribonucleic acid of high specific activity can then be analyzed for 5′-end group or 5′-terminal oligonucleotide sequences.

The chemical procedure reported here enables the isolation of intact ^{32}P-labeled 35S RNA from the 70S RNA of avian myeloblastosis virus.

MATERIALS AND METHODS

Ribonucleic acids were precipitated from 0.2 M NaCl solutions with 2.5 volumes of ethanol at −20° for 12 hr. After centrifugation the precipitates were redissolved in a small amount of water and lyophilized. The dry residue (250 μg, about 5 A_{260} units, about 10^{-5} mmol of tRNAs or about 5×10^{-7} mmol of 16S or 18S RNAs) was dissolved in 250 μl of formamide. [^{32}P]Mono(tri-*n*-butylammonium) phosphate (prepared from 0.1 mg of orthophosphoric acid, 10^{-3} mmol, specific activity 5 Ci/mmol) was reacted under strict anhydrous conditions with carbonyldiimidazole (8.1 mg, 5×10^{-2} mmol) in anhydrous dimethyl formamide (250 μl) at room temperature and overnight to produce the [^{32}P]diimidazolidate of orthophosphate. Excess carbonyldiimidazole was destroyed by treatment with anhydrous methanol (50 μl) at room temperature for 30 min. The solution of the [^{32}P]diimidazolidate of orthophosphate (in 250 μl of dimethyl formamide) was then added to the formamide solution of the RNA, and reaction was allowed to proceed overnight at room temperature. The organic solution was dialyzed against two changes of 2 liters of water (treated with diethylpyrocarbonate) at 4°. The RNA was then precipitated from 1 M LiCl solutions with 2.5 volumes of ethanol and analyzed.

A modification of this procedure, suited for high-molecular-weight RNAs, was also used with similar results. The RNA was converted into its cetyltrimethylammonium salt (3, 8), which was washed with water and lyophilized. The dry RNA salt was then dissolved in a small volume of dimethyl formamide and reacted for 24 hr with [^{32}P]diimidazolidate of orthophosphate. At the end of the reaction, the solvent was removed under reduced pressure and the residue was washed with four portions of water (3 ml each) and lyophilized. The dry residue was dissolved in dimethyl sulfoxide (100 μl) followed by addition of 20 μl of aqueous buffer (10 mM Tris·HCl, 1 mM EDTA, pH 7.4). The combined solution was then applied on top of a sucrose–dimethyl sulfoxide gradient and analyzed (Fig. 2). The appropriate fractions were pooled, and RNA was precipitated by the addition of an equal volume of 2 M LiCl followed by 2.5 volumes of ethanol.

For 5′-end group analysis the RNA was hydrolyzed in 0.3 M KOH overnight at 37° and neutralized with $HClO_4$; the hydrolysate was absorbed, washed, and eluted from charcoal (9). Analysis of the alkaline hydrolysates is described in the legends to Figs. 3 and 4. Two unlabeled markers, ppGp and ppAp, were prepared for the identification of the 5′-terminal nucleotides resulting from alkaline hydrolysis. ppGp was prepared by alkaline hydrolysis (0.25 M KOH, 14 hr, room temperature) of ppGpp, which was a gift from Dr. M. Cashel. ppAp was prepared by reacting pAp with the diimidazolidate of orthophosphate and subjecting the crude product to alkaline hydrolysis. Analysis of T1 ribonuclease digests of labeled *Escherichia coli* 16S RNA is described in the legend to Fig. 5.

High-molecular-weight RNA (70S) from avian myeloblastosis virus was isolated by a published procedure (10).

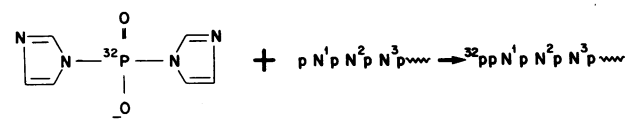

FIG. 1.

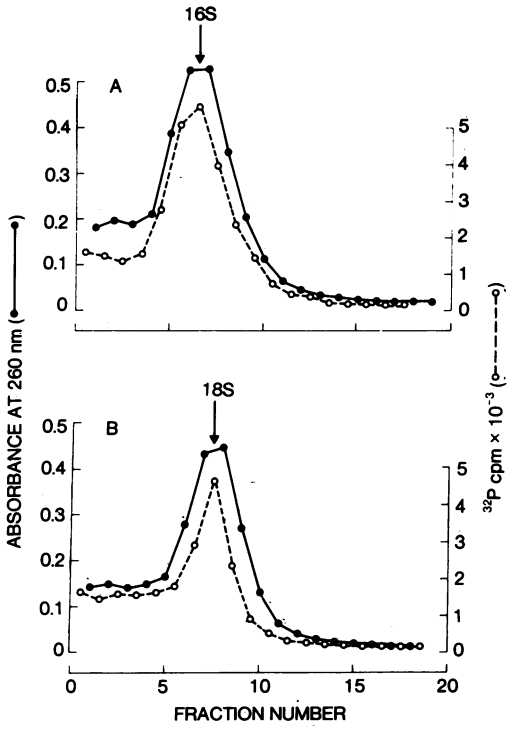

FIG. 2. (A) Sedimentation pattern of labeled *E. coli* 16S RNA through a 0–20% (w/w) sucrose gradient in 85% dimethyl sulfoxide–15% buffer [50 mM Tris·HCl, 5 mM EDTA, pH 7.4 (v/v)]. Centrifugation was performed in a SW56 rotor at 56,000 rpm for 18 hr at 23°. ^{32}P cpm represent acid-precipitable counts. (B) Sedimentation pattern of labeled 3T3 18S RNA under the same conditions.

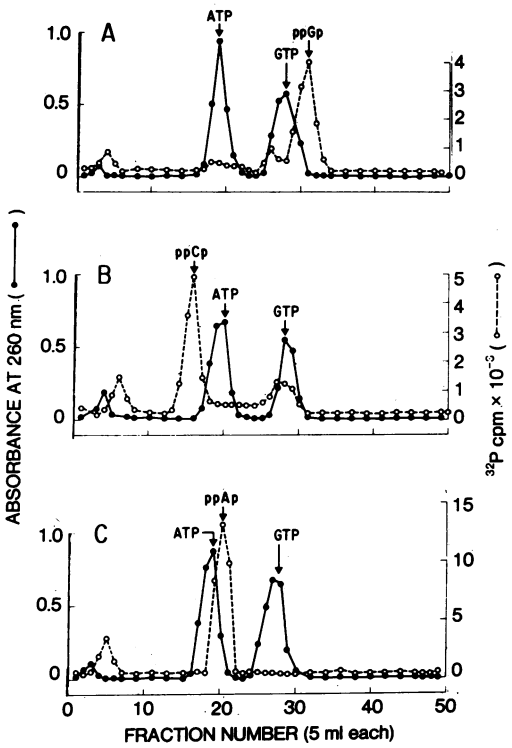

FIG. 3. (A) Chromatography of alkaline hydrolysate of labeled $tRNA^{Phe}_{yeast}$ on Dowex 1-X8 (200–400 mesh, formate form) column (0.5 × 4 cm). The material was eluted with 4.0 M formic acid (25 ml) followed by a linear gradient of 0.0–1.5 M ammonium formate in 4.0 M formic acid (250 ml). ATP and GTP were added as unlabeled markers. (B) Chromatography of alkaline hydrolysate of labeled $tRNA_f^{Met}$ (*E. coli*). (C) Chromatography of alkaline hydrolysate of labeled 16S RNA (*E. coli*).

The dry RNA pellet (5 A_{260} units) was dissolved in 1 ml of water (treated with diethylpyrocarbonate), and 1 ml of a 1% solution of cetyltrimethylammonium bromide was added. After 30 min at 4°, the RNA salt was collected by centrifugation, washed three times with water, and dried by lyophilization. The dry cetyltrimethylammonium salt of the 70S RNA was subjected to the chemical labeling procedure described above. After analysis on a sucrose dimethyl sulfoxide gradient (Fig. 6), the appropriate fractions were pooled, 0.5 volume of 4 M LiCl solution was added, and the labeled RNA was precipitated with 3 volumes of ethanol at −20° for 3 days.

RESULTS

Fig. 2 illustrates that *E. coli* 16S RNA and 3T3 cell line 18S RNA can be labeled by our techniques and remain virtually intact.

Figs. 3 and 4 describe the 5′-end group determination of the labeled RNAs. In the case of monophosphorylated 5′-ends, the groups identified are the various ^{32}ppNp. Since these nucleoside triphosphates contain one additional negative charge as compared with pppN, they elute behind the corresponding unlabeled nucleoside triphosphates, which are used as unlabeled markers, in both systems that are described here. The positions of the radioactive peaks, eluted by a linear gradient of ammonium formate in 4.0 M formic acid (11), was confirmed by the chromatographic properties of two authentic unlabeled markers, ppGp and ppAp. The labeled 5′-end groups were thus identified as ppCp, ppAp, and ppGp. The 5′-end group of $tRNA^{Phe}_{yeast}$ is known to be pG (12) and of $tRNA^{Met}$ of *E. coli* is pC (13). Our procedure led to the same assignments, although the $tRNA_f^{Met}$ preparation contained a small impurity (Figs. 3 and 4). The 5′-end group of *E. coli* 16S RNA was identified as pA (1, 14), and its 5′-terminal oligonucleotide obtained by T1 ribonuclease digestion elutes at a

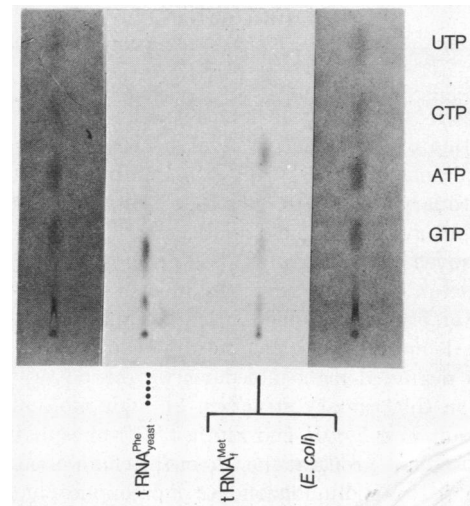

FIG. 4. Thin-layer chromatography of the alkaline hydrolysates of labeled $tRNA^{Phe}_{yeast}$ and $tRNA_f^{Met}$ (*E. coli*) on PEI-cellulose developed with 0.8 M ammonium sulfate, followed by autoradiography. The unlabeled UTP, CTP, ATP, and GTP were identified by their UV absorption.

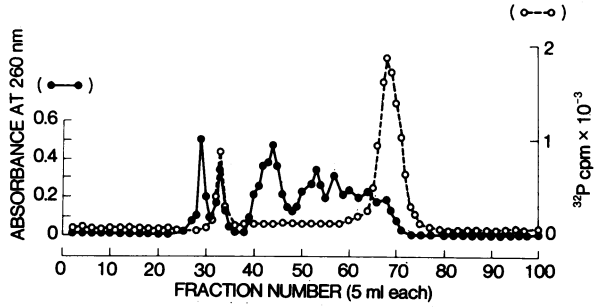

FIG. 5. Chromatography of T1 ribonuclease digest of labeled *E. coli* 16S RNA on a DEAE–Sephadex column. The digest was eluted with a linear gradient of 0.0–0.8 M sodium chloride in 50 mM Tris·HCl, 1 mM EDTA, 7 M urea, pH 7.4. Two A_{260} units of labeled 16S RNA and 2.5 mg of carrier high-molecular-weight yeast RNA were digested with 500 units of T1 ribonuclease at 37° for 1 hr.

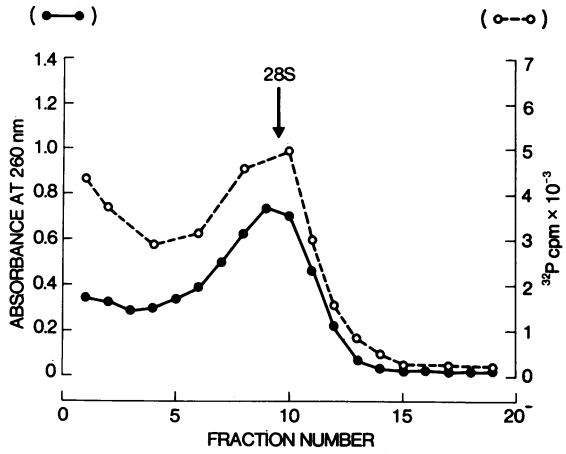

FIG. 6. Analysis of labeled 35S RNA of avian myeloblastosis virus on sucrose–dimethyl sulfoxide gradients. For experimental details see the legend to Fig. 2.

position indicating 9–10 negative charges (Fig. 5). This oligonucleotide was reported to have the sequence pAAAUUG (14), which is consistent with formulation of the oligonucleotide we were dealing with as ^{32}ppAAAUUG.

High-molecular-weight RNA (70S) from avian myeloblastosis virus was subjected to the labeling procedure, and analysis of the reaction products yielded labeled and virtually intact 35S RNA, while the labeled 70S-associated 4S RNAs stayed near the top of the gradient (Fig. 6). The ^{32}P-label was shown to be introduced exclusively onto the 5′-end. The phosphorylated 5′-terminal group of the 35S RNA was identified as ppA and pppA prior to the labeling reaction (E. Rapaport, unpublished data).

Molecular weight calculations of labeled tRNAs yielded varying values of 30,000–50,000, based on the specific activity of the [^{32}P]orthophosphate used as starting material. These values may indicate varying chemical yields of 50–85% in the labeling reaction. The molecular weight of labeled 35S RNA was calculated as 2.3×10^6, based on the specific activity of a tRNA (molecular weight 25,000) that was labeled with the same [^{32}P]diimidazolidate of orthophosphate.

DISCUSSION

The new 5′-end labeling procedure after isolation of RNA is a purely chemical technique which is complementary to the phosphatase-polynucleotide kinase method and permits the introduction of a radioactive label of higher specific activity (using $^{32}P_i$ instead of [γ-^{32}P]ATP). It introduces a ^{32}P-label onto a primary phosphate ester that is situated at the 5′-end of the RNA molecule. The ^{32}P-label after isolation was shown to be removed by the action of alkaline phosphatase.

The usefulness of the new technique as far as 5′-end group analysis or 5′-terminal oligonucleotide analysis is concerned has been demonstrated. The procedure can be applied even to partially degraded high molecular weight RNA. Breaks or "nicks" in the primary structure of high molecular weight RNA result in a 5′-OH end and a 2′(3′)-phosphate or 2′,3′-cyclic phosphate group at the site of the chain break (15). The reaction with [^{32}P]diimidazolidate of orthophosphate does not introduce a label at the 5′-OH ends of the nicks. It does label the 2′(3′)-phosphate end of the breaks. Here however, the β-phosphate of the pyrophosphate group that is produced is labile due to the neighboring 2′(3′)-OH group of the ribosyl moiety (16). Another consideration is that in the case of random breaks along the RNA chain, the only appreciably large population of molecules of a single size is the labeled intact RNA molecule. It is essential for our labeling procedure that the 5′-end of the molecule end in a phosphate, since the diimidazolidate intermediate does not react with a free hydroxyl group. All the processed RNAs whose sequences have been completely or partially determined (tRNAs, mRNAs, rRNAs) contain a primary phosphate group at their 5′-ends (17). Viral or phage high-molecular-weight RNA also generally contains a primary phosphate at the 5′-end (although it may be part of a triphosphate or diphosphate sequence) (17). There is one report, however, of an animal virus (Rous sarcoma virus) RNA containing 5′-OH ends (18). Whereas, the phosphatase-polynucleotide kinase procedure permits the differentiation between 5′-OH and 5′-phosphorylated ends of RNAs, it is not suitable for the differentiations between mono-, di-, or triphosphorylated 5′-ends of RNAs. The new technique is suitable for such an analysis, yielding a different 5′-end product for each species.

We feel that the main virtue of our new technique is its capability of labeling a very sensitive high-molecular-weight viral RNA of undetermined secondary structure, which is very susceptible to hydrolytic and enzymatic degradations in aqueous solutions. By carrying out the labeling reaction in a nonaqueous medium we eliminate the possible action of nucleases and aqueous chemical hydrolytic action. The nonaqueous medium also provides the dissociating environment for an RNA complex like the 70S RNA of avian myeloblastosis virus (19). This labeling technique may, in addition, be useful in cases where the 5′-end of the RNA is part of a double strand. Difficulties have been encountered with the enzymatic method (20), while our procedure, which is carried out in anhydrous media in which the double strand would dissociate, would not be expected to suffer from the same steric hindrance.

Molecular weight per phosphorylated 5′-end of high-molecular-weight viral RNA can be estimated by subjecting tRNA of known molecular weight to the same [^{32}P]diimidazolidate of orthophosphate solution under the same conditions and thus minimizing the inaccuracy that may arise from a chemical yield of less than 100%. It is assumed that in dimethyl formamide, where the RNA is devoid of secondary structure, the chemical yield would be determined by the 5′-

primary phosphate group and not by the molecular weight of the rest of the molecule. The value obtained for the 35S RNA of avian myeloblastosis virus (2.3×10^6) is in good agreement with values reported earlier (15). However, molecular weight values obtained by correlating the specific activity of the [^{32}P]diimidazolidate of orthophosphate to the ^{32}P-label of the 35S RNA were often higher. This may be attributed either to low chemical yields of the labeling reaction or to the presence of a very sizable population of 5′-OH ends.

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