STUDIES OF THE ROUS SARCOMA VIRUS RNA: CHARACTERIZATION OF THE 5'-TERMINUS*

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Received November 14,1972

SUMMARY

The 5' terminus of the Rous Sarcoma Viral 30-40S RNA was characterized as follows: Unlabeled RNA was treated with polynucleotide kinase and (γ-^{32}P) ATP. Degradation of the 5'-(^{32}P) RNA with alkali yielded labeled pAp while degradation with venom phosphodiesterase yielded labeled 5'-AMP. Dephosphorylation with alkaline phosphatase was unnecessary for the RNA to accept ^{32}P indicating the presence of 5'-OH ends. This establishes that the base at the 5' end of Rous Sarcoma Viral 30-40S RNA is adenine.

INTRODUCTION

The genetic information of avian tumor viruses is present in a high molecular weight RNA with a sedimentation velocity between 60-70S (1,2). Upon treatment with heat or denaturing agents this material is converted to subunits sedimenting at approximately 30-40S and some small heterogeneous RNAs (2,3). Relatively little is known concerning the number of subunits, their structure or degree of homogeneity (3). Several recent investigations have dealt with the nature of the 3' terminal nucleoside of Rous Sarcoma Virus and Avian Myeloblastosis Virus RNA (4-6). The studies reported below determine the 5' terminal nucleoside of the Prague strain of Rous Sarcoma Virus RNA.

MATERIALS AND METHODS

Unlabeled ribonucleotides were purchased from Schwarz-Mann. γ-^{32}P-labeled ATP was supplied by the New England Nuclear Corp. The following enzymes were obtained from Worthington Biochemical Corp.: Bacterial alkaline phosphatase, venom phosphodiesterase and micrococcal nuclease. Polynucleotide kinase, purified as described elsewhere (7), had no detectable RNase.

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* This work was supported by grants from the Special Virus Cancer Program of the National Cancer Institute, National Institutes of Health and the American Cancer Society.

Purification of Viral RNA - The results shown below were obtained in four series of separate experiments using three different lots of virus. The Prague strain of Rous sarcoma virus of subgroup C (8) was used in all experiments. The virus was originally cloned by Dr. P.K. Vogt (USC, Los Angeles) and propagated on ordinary C/O or C/B (8) chick embryo fibroblasts (Kimber Farms, Berkeley, Cal.). For virus purification, media were harvested from infected cultures at intervals of 3-5 hrs. Procedures for purifying the virus and extraction of viral RNA by the phenol-sodium dodecylsulfate (SDS) method have been described previously (2). After centrifugation in a 15-30% gradient of sucrose containing 0.1 M NaCl, 0.05 M Tris-HCl pH 7.5, 10^{-3} M EDTA (STE) and 0.1% SDS for 110 min at 41,000 rpm in a SW41 rotor, the fractions containing 60-70S RNA were pooled; the RNA was precipitated with 2 volumes of 95% ethanol, dissolved in STE and heated at 100° for 45 sec. The material was centrifuged in the gradient described above for 200 min at 41,000 rpm and the 30-40S RNA recovered by precipitation with ethanol. The ratio of a to b subunits (3) in the 60-70 S RNA of the virus preparations used was $\geq$ 2:1 (Duesberg and Vogt, unpublished observations), implying that the results described below apply mostly to the a subunit of viral RNA.

Preparation of ^{32}P-labeled RNA - For alkaline phosphatase treatment, the reaction mixture (0.1 ml) contained 1.5 A_{260} units of RNA, 10 μmoles of Tris-HCl buffer pH 7.5, and .3 units of bacterial alkaline phosphatase. After incubation at 38°C for 30 min, alkaline phosphatase activity was inhibited by the addition of 2 μmoles of 2-mercaptoethanol and 0.2 μmole of sodium phosphate buffer, pH 6.9 (9); 1 μmole $MgCl_2$, 0.1 μmole γ-^{32}P- ATP (33,000 cpm/pmole) and 5 units of polynucleotide kinase were added at this time after which the incubation was carried out for 20 min at 37°. An additional 3 units of polynucleotide kinase was added and the mixture was incubated at 30°C for another 20 min period. In experiments where 30-40S RNA was not treated with alkaline phosphatase the same amount of ^{32}P was incorporated at the 5' terminus. The 5'-^{32}P-labeled 30-40S RNA was isolated by centrifugation through a 5-20% sucrose-STE gradient containing 2 mM sodium phosphate buffer pH 6.9, for 160 min at 48,000 rpm in a SW 50.1 rotor. About 80% of the RNA applied to the gradient sedimented at 30-40S. Fractions containing the 30-40S RNA were pooled and precipitated with ethanol after the addition of 3 A_{260} units of cold t-RNA. The precipitate was dissolved in STE and again centrifuged in a 5-20% sucrose-STE phosphate gradient as above to further purify the 30-40S RNA. Tubes 1-10 (Fig. 1) were pooled, filtered through Sephadex G-25 which had been equilibrated with H_2O. The RNA was concentrated and aliquots subjected to either snake venom phosphodiesterase

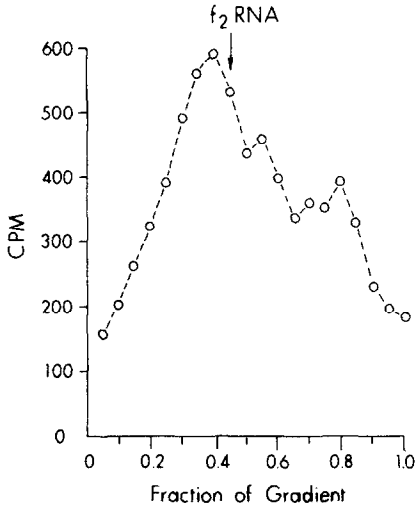

Fig. 1. Sedimentation pattern of 30-40S 5'-^{32}P-Rous Sarcoma Virus RNA. A solution containing 1 OD_{260} of heat-denatured RSV 5'-^{32}P RNA (120,000 cpm) in STE buffer which had been treated sequentially with bacterial alkaline phosphatase and polynucleotide kinase was added to 5.2 ml of 5-20% sucrose-NaCl-TRIS-EDTA-sodium phosphate gradient which was centrifuged at 48,000 rpm for 160 min at 4° in a SW 50.1 rotor. Fractions were collected and (0.02 ml) aliquots counted in Bray's scintillation fluid. A marker of f_2 RNA measured by A_{260} was included and its position in the gradient is indicated by the arrow.

for complete degradation to 5' -mononucleotides as described in Fig. 2 or degradation to the 5'-^{32}P- terminal nucleoside diphosphate with KOH followed by chromatography on Dowex 1-formate (10) which resolved the 4 ribonucleoside diphosphates (as detailed in Fig. 3). Although the data shown were obtained with material from the leading front of the labeled RNA obtained in the sucrose gradient (Fig. 1) similar results were obtained with material from tubes 10-16.

RESULTS

Purification of Labeled RNA - The sedimentation of the 5'-^{32}P-labeled RSV RNA (after denaturation) is shown in Fig. 1. The RNA sediments with a greater velocity than the 30S f_2 RNA used as a marker. Based on their relative sedimentation velocities, a value of 37S was calculated for the Rous Sarcoma Virus RNA subunits which agrees with previous estimates (3). This value indicated that the alkaline phosphatase and polynucleotide kinase treatments did not cause appreciable breakdown of the RNA, although a small peak in the upper third of the gradient may represent degraded material. The latter fractions were discarded. The molecular weight calculated from the number of termini labeled with ^{32}P and the concentration of 30-40S RNA

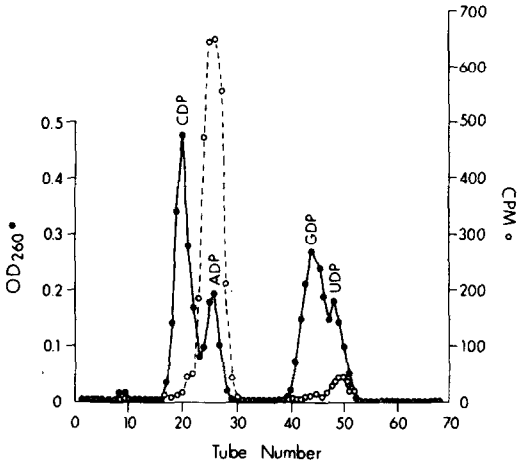

Fig. 2. Electrophoretic separation of venom phosphodiesterase digest of 5'-^{32}P-labeled 30-40S RSV RNA. An aliquot of material prepared as described in Fig. 1 was dissolved in 0.1 ml of 0.1 M TRIS-HCl buffer, pH 7.5 containing 1 μmole $MgCl_2$ and 10 μg of venom diesterase. The mixture was incubated for 30 min at 37°. The digestion of the RNA was monitored with 5'-nucleotidase and considered complete when all the radioactivity was susceptible to this enzyme. The mixture was then spotted on Whatman No. 3MM paper and electrophoresed at 5000V for 2 hrs in the pyridine-acetic acid pH 3.5 buffer system (7).

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used for phosphorylation was 2.3 x 10^6 daltons (assuming an extinction coefficient of 7 for 10^{-3}M nucleotides as 30-40S RNA).

Determination of 5' Terminus Nucleotide - The products of the venom diesterase digestion of the 5'-^{32}P-labeled 30-40S RNA are shown in Fig. 2 and Table 1. These results indicate that A is the predominant base at the 5' terminus. Similar findings were obtained when alkaline hydrolysis was used and the terminus isolated as ^{32}pAp (Fig. 3). The 5' terminus of TMV RNA and f_2 RNA were determined in our laboratory by the methods employed above and found to be respectively A and G in agreement with previously reported results (11-13).

DISCUSSION

The results presented above indicate that the 5'-terminus of the 30-40S RNA segments of RSV RNA is almost exclusively adenosine. The presence of A at the 5' -terminus of RSV RNA is in keeping with the finding of this base at the 5' end of all single stranded RNA's of eukaryotic viruses (14). At present there are conflicting reports (4-6) concerning the nature of the 3-OH end which need to be resolved.

Our interests in the nature of the 5'-terminus stem from our desire to determine whether 30-40S subunits, which hydrogen bonded together con-

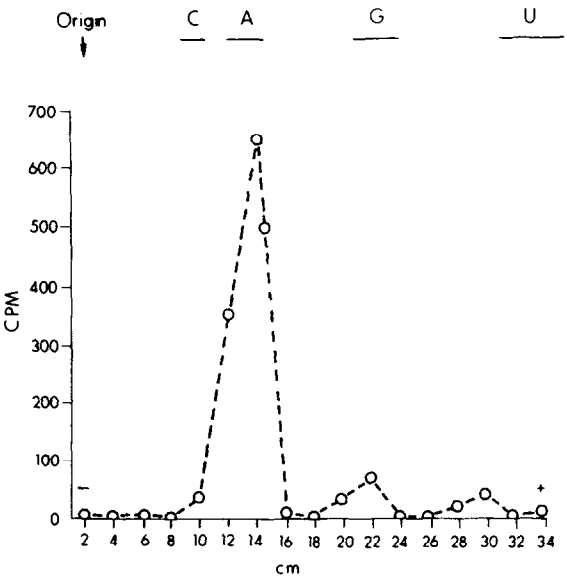

Fig. 3. Chromatography of alkaline hydrolysis products of 5'-^{32}P-labeled RSV RNA. To an aliquot of RNA (containing 0.3 pmole of termini) prepared as described in Fig. 1 dissolved in 0.5 ml H_2O, an equal volume of 1 M KOH was added and the mixture incubated at 37°C for 15 hrs. The KOH was neutralized with 1 M perchloric acid in the cold. Unlabeled nucleoside diphosphates of C, A, G and U (0.5 μmole of each) were added as markers and the mixture chromatographed on a 0.6 x 15 cm column of Dowex 1 formate. Material was eluted with a gradient of 0.01 N HCl to 0.25 M NaCl in 0.01 N HCl (60 ml of each). Fractions (1.6 ml) were collected and 1 ml aliquots counted in Bray's solution. The recovery of ^{32}P applied to the column was quantitative.

stitute the majority of the 60-70S RNA, are redundant. The answer to this important question will determine the total genetic information present in 60-70S RNA. If, for example, the 60-70S RNA is made up of approximately 4 30-40S segments, all heterogeneous, the information available can dictate the synthesis of approximately 50 polypeptide chains of 20,000 daltons.

By virtue of labeling the 5'-terminus of RSV RNA, we have attempted to answer the question of heterogeneity at this site. No evidence of heterogeneity was found for the terminal nucleotide. Preliminary experiments in which oligonucleotides prepared with RNase T1 from 5'-^{32}P labeled RSV RNA were separated on DEAE cellulose on the basis of charge, indicate that over 50% of the radioactivity was recovered in a peak corresponding to ^{32}pApGp thereby indicating that G is the predominant penultimate base. Further work is needed to establish whether heterogeneity exists at this site.

TABLE I

BASE ANALYSIS OF 5' TERMINUS OF RSV RNA

BASE ANALYZED	VENOM DIESTERASE % as 5'-XMP		ALKALINE HYDROLYSIS % as pXp	
	Experiment Number			
	1	2	3	4
C	3	4	1	<1
A	86	81	92	97
G	6	9	1	1
U	5	6	6	2

TABLE 1. Comparison of 5' terminus base analysis as determined by venom diesterase and alkaline hydrolysis. The results presented above were from two independently isolated RSV RNA preparations. In each case, experiments were performed at least twice with each RNA preparation.

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