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The Homopolyadenylate and Adjacent Nucleotides at the 3′-Terminus of 30–40S RNA Subunits in the Genome of Murine Sarcoma-Leukemia Virus

(polyadenylate/oncornavirus)

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ABSTRACT **Adenosine is the major 3′OH-terminal nucleoside of the 60–70S RNA genome of the murine sarcoma-leukemia virus, its 30–40S RNA subunits, and the poly(A) segments derived by RNase treatment of both RNA species, as determined by periodate oxidation–[^{3}H]-borohydride reduction. The binding of 30–40S RNA to oligo(dT)–cellulose suggests that most viral RNA subunits contain poly(A). The molecular weight of poly(A) derived from viral RNA by digestion with RNase and purified by affinity chromatography is 64,000–68,000, as determined by gel electrophoresis. From the size of poly(A) and the poly-(A) content of viral RNA (1.6%), it is estimated that there is about one poly(A) segment for each viral 30–40S RNA subunit. The results of 3′-termini labeling with [^{3}H]borohydride, *in vivo* labeling with [^{3}H]adenosine, and base composition of [^{32}P]poly(A) indicate that a homo-poly(A) segment is located at the 3′-end of a 30–40S RNA subunit. The homogeneous poly(A) segments isolated from RNase T1 digests of 60–70S [^{32}P]RNA consist of one cytidylate, one uridylate, and about 190 adenylate residues, while those isolated from RNase A digests consist exclusively of adenylate residues. These results indicate that —G(C,U)$A_{190}A_{OH}$ is the 3′-terminal nucleotide sequence of the viral 30–40S RNA subunits.**

The 60–70S RNA genome of oncornaviruses (RNA tumor viruses) dissociates under denaturing conditions to three or four 30–40S RNA subunits containing polyadenylate [poly-(A)] and small amounts of low-molecular-weight nucleic acid molecules (1–8). Poly(A) segments are present at the 3′-terminus of mammalian heterogeneous nuclear RNA (HnRNA) and mRNA (9–13), vaccinia virus mRNA (14), and poliovirus RNA (15). Recent investigations have reported either uridine (16–18) or adenosine (19, 20) at the 3′-terminus of the genome of oncornaviruses. Poly(A) in the RNA of Rous sarcoma virus was reported to possess interpolated G, U, and C residues (21). Stephenson *et al.* showed that most of the 35S RNA of avian myeloblastosis virus contain a poly(A) segment at the 3′-OH terminus (22).

We have used several procedures to characterize the structure and location of poly(A) segments in 60–70S RNA and 30–40S RNA subunits of murine sarcoma-leukemia virus [MSV(MLV)]. Our results indicate that each 30–40S subunit of the viral genome is terminated with the sequence —G-(C,U)$A_{190}A_{OH}$.

MATERIALS AND METHODS

Materials. KB^3H_4 (7.2 Ci/mmol) was purchased from Amershan/Searle; [^{3}H]poly(A) and *Escherichia coli* tRNA from Miles Laboratories; [^{3}H]uridine (42 Ci/mmol), [^{3}H]-adenosine (40 Ci/mmol), and carrier-free [^{32}P]phosphate from New England Nuclear Corp.; RNase A (X-A), alkaline phosphatase, DNase I, and ribonucleosides from Sigma Chemical Co.; RNase T1, T2, and U2 from Calbiochem; and poly(C) from PL Biochemicals.

Purification of Virus and Viral RNA. The MSV-transformed rat-cell line, 78A1, which produces the Moloney strain of MSV(MLV), was grown in suspension culture in Eagle's minimal essential medium supplemented with 10% fetal-calf serum (6). Virus was purified from culture medium collected at 1, 3, or 12 hr after complete renewal of medium (6, 23). Purified virus in NTE buffer (0.1 M NaCl–10 mM Tris·-HCl–1 mM EDTA, pH 7.4) was lysed by addition of one-tenth volume each of 10% dithiothreitol, 5% Na dodecyl sarcosinate, and phenol–chloroform (1:1) (23). The mixture was extracted three times with phenol–chloroform. Viral 60–70S RNA was isolated by zonal centrifugation in a 10–30% sucrose gradient in NTE buffer containing 0.5% Na dodecyl sarcosinate for 2.5 hr at 40,000 rpm in a Spinco SW41 rotor at 4° (Fig. 1*A*). For isolation of 30–40S RNA, virion 60–70S RNA was heated in NTE buffer at 70° for 3 min and centrifuged as described above for 5 hr (Fig. 1*B*).

^{3}H-Labeling of the 3′-Termini of 60–70S and 30–40S RNA. Viral 60–70S RNA (150 μg) and 30–40S RNA (25 μg) were oxidized with $NaIO_4$ and reduced with KB^3H_4 to label the 2′,3′-terminal glycol group of RNA (20, 24). Labeled RNA (200 μl) was purified on a Sephadex G-25 column (2.5 × 30-cm) and by three ethanol precipitations.

Chromatography of RNase Digests on DEAE-Sephadex. Terminally labeled 60–70S RNA (50 μg) or 30–40S RNA (10 μg) was treated with RNase T1 (10 units/ml) plus RNase A (2 μg/ml) in 10 mM Tris·HCl (pH 7.4)–1 mM EDTA–0.3 M NaCl for 1 hr at 37°. The digest (0.2 ml) was mixed with 1.8 ml of 0.3 M NaCl–5 mM Tris·HCl (pH 7.5)–7 M urea and applied to a DEAE-Sephadex A-25 column (1 × 1-cm) equilibrated with the same buffer. Elution was performed with a 50-ml linear gradient of 0.3–1.0 M NaCl in 5 mM Tris·HCl (pH 7.5)–7 M urea (15).

Isolation of Poly(A) Segments by Oligo(dT)–Cellulose Chromatography. RNase digests (0.2 ml) of terminally labeled 60–70S or 30–40S viral RNA were diluted to 3 ml in 10 mM Tris·HCl (pH 7.5)–0.5 M KCl and applied to oligo(dT)–cellulose columns (0.6 × 3-cm) equilibrated with the same buffer as described (25, 26).

Abbreviations: HnRNA, heterogeneous nuclear ribonucleic acid; rRNA, ribosomal RNA; MSV(MLV), murine sarcoma-leukemia virus.

Identification of 3'-Labeled Nucleosides. RNA terminally labeled with [^{3}H]borohydride was hydrolyzed with piperidine (20) or KOH (17). The trialcohols in the hydrolysate, together with the four unlabeled nucleoside trialcohol markers, were resolved on cellulose thin-layer plates (Eastman no. 60604) by both one- (solvent E) and two-dimensional chromatography (24). To estimate the specific activity of labeled 3'-terminal nucleosides derived from viral RNA, we prepared labeled trialcohols of nucleosides with each batch of KB^3H_4 (20).

Radioactive Labeling of Viral RNA. Viral RNA was isolated from virus produced in cells labeled for 12-hr periods with carrier-free [^{32}P]phosphate (50 μCi/ml) in buffered, phosphate-free medium (27) or with [^{3}H]adenosine (10 μCi/ml) in standard growth medium. For the rapid harvest procedure, monolayer cultures were labeled with [^{3}H]uridine (20 μCi/ml) for 12 hr, unlabeled medium was added, and virus-containing medium was harvested and replaced with fresh medium every hour.

Binding of 30–40S RNA to Oligo(dT)–Cellulose. The 60–70S RNA from virus labeled with [^{3}H]uridine for 1 hr was denatured at 100° for 45 sec in NTE buffer, adjusted to 0.5 M KCl, and processed on oligo(dT)–cellulose columns as described above.

Purification of Poly(A) for Determination of Nucleotide Composition and Molecular Weight. Nuclease digestion of 60–70S [^{32}P]RNA was carried out at 37° for 60 min in NTE buffer with RNase A (2 μg/ml) or T1 RNase (10 units/ml). Alkylated DNase I (10 units/ml) (28) was included to remove possible contamination with DNA. Digests were adjusted to 0.5 M NaCl and processed on a poly(U)–Sepharose column (13). Poly(A) from the RNase T1 digest [RNase T1-Poly(A)] was eluted from the column with a formamide gradient, 0–90%, in NTE–0.2% dodecyl sarcosinate (NTES), while poly(A) from the RNase A digest [RNase A-poly(A)] was eluted with 90% formamide–10% NTES after the column was washed. Column-purified poly(A) was further purified on 15% polyacrylamide gels containing 7 M urea (13). ^{32}P radioactivity was monitored by Cerenkov radiation and eluted from the gel slices by shaking in NTES buffer at 45°. Eluted poly(A) was precipitated with ethanol and further purified on poly(U)–Sepharose or oligo(dT)–cellulose by washing and batchwise elution. Purified poly(A) was subjected to base-ratio analysis as described (29), and to gel electrophoresis for determination of molecular weight (6).

The 60–70S RNA labeled with [^{3}H]adenosine was digested with RNase T1, and poly(A) was purified as described above. [^{3}H]Poly(A) was digested with 20 units/ml of RNase U2 in 50 mM sodium acetate (pH 4.5)–2 mM EDTA for 12 hr at 37°, and further digested with 20 units/ml of RNase T2 for 12 hr. Labeled adenosine and AMP were resolved and quantitated by high-voltage paper electrophoresis (29).

Polyacrylamide Gel Electrophoresis. The size distribution of unbound and retained-eluted RNA from oligo(dT)–cellulose columns was determined by electrophoresis in 3% polyacrylamide–0.1% Na dodecyl sulfate gels (30). Poly(A) purified on 15% polyacrylamide–7 M urea gels and by affinity chromatography (13) was analyzed on 10% polyacrylamide–7 M urea gels (6). The following markers were used: [^{3}H]uridine-labeled 28S, 18S, and 5S rRNA, and 4S RNA isolated from human KB cells, and 5.9S poly(C) labeled by methylation with [^{3}H]dimethyl sulfate (31).

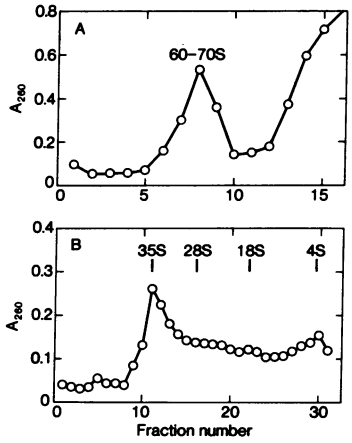

FIG. 1. Purification of MSV(MLV) 60–70S and 30–40S RNA by zonal centrifugation in sucrose density gradients. (*A*) MSV-(MLV) RNA extracted from virus particles was centrifuged for 2.5 hr, and A_{260} was measured. (*B*) The pooled 60–70S RNA peak from (*A*) was heated at 70° and centrifuged for 5 hr.

RESULTS

Identification of the 3'-Terminal Nucleosides of MSV(MLV) 60–70S and 30–40S RNA. The major difficulty in determining the nucleoside at the 3'-terminus of oncornavirus RNA has been the heterogeneity of RNA after isolation and denaturation, due mainly to degradation which introduces additional 3'-termini, particularly after prolonged periods of virions in cell culture. To minimize degradation, virus was purified from culture supernatants of MSV(MLV)-producing transformed rat cells (78A1) that were harvested every 3 hr. Viral 60–70S and 30–40S RNA were isolated, terminally labeled by periodate oxidation–[^{3}H]borohydride reduction, and purified by exclusion chromatography on Sephadex G-25 and alcohol precipitation. Table 1 summarizes the results of terminal nucleoside analysis by one- and two-dimensional chromatography of labeled trialcohols. Control *E. coli* tRNA had 94% adenosine at the 3'-terminus while 4S RNA from virions had 73% adenosine. The major 3'-termini (67–76%) of 30–40S and 60–70S RNA was adenosine (Table 1). The molecular weights calculated from the pmoles of trialcohol formed per unit mass of 30–40S RNA was 2.0 to

TABLE 1. *Analysis of alkaline digests of 3'-terminally labeled viral RNA*

Source of RNA	One- or two-dimensional thin-layer chromatography	% cpm in trialcohols*			
		A'	G'	C'	U'
MSV(MLV) 60–70S	One	76	11	0	13
MSV(MLV) 60–70S	Two	69	12	0	20
MSV(MLV) 30–40S	One	75	3	10	11
MSV(MLV) 30–40S	Two	67	25	0	8
MSV(MLV) ~ 4S	Two	73	3	12	9
E. coli tRNA	One	94	0.4	5	0.5
E. coli tRNA	Two	94	1	3	4

* Average of two independent experiments.

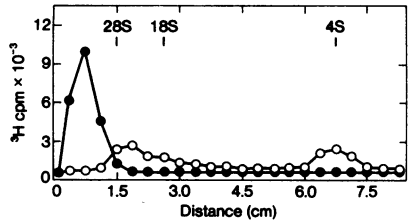

FIG. 2. Gel electrophoresis of unbound and retained-eluted RNA from oligo(dT)-cellulose chromatography. Viral 60–70S RNA, labeled for 1 hr with [^{3}H]uridine, was denatured and fractionated on an oligo(dT)-cellulose column. The size of unbound RNA (○) and retained-eluted RNA (●) was analyzed on 3% polyacrylamide–0.1% Na dodecyl sulfate gels. Values for cpm have been multiplied by 10^{-3}.

2.5×10^6, consistent with published estimates of about 2.5 to 3×10^6 for 30–40S RNA (1–4).

Binding of 30–40S RNA to Oligo(dT)-Cellulose. Viral 60–70S RNA was labeled with [^{3}H]uridine for 1 hr, denatured, and chromatographed on an oligo(dT)-cellulose column. Molecules containing poly(A) sequences are preferentially retained on the column in high salt and are eluted by low salt concentration (e.g., see Fig. 3*A*). The size of the unbound and retained-eluted RNA fractions was determined by electrophoresis in 3% polyacrylamide–0.1% Na dodecyl sulfate gels. The retained-eluted RNA was mainly 30–40 S (Fig. 2). The unbound RNA was heterogeneous, mainly smaller than 28 S, and probably consists of the 10–15% 7S and 4–5S RNA found associated with MSV(MLV) 60–70S RNA (32) as well as degradation products of 30–40S RNA. These results suggest that most 30–40S RNA molecules contain poly(A) segments.

Location of Poly(A) at the 3′-OH Terminus. Viral 60–70S and 30–40S RNA isolated from 3-hr virus harvests were terminally labeled with KB^{3}H$_4$ and digested with RNase A plus RNase T1. Under these conditions, only poly(A) sequences will remain intact, and poly(A) located at the 3′-end should be terminally labeled. Poly(A) was purified from the RNA digests by either oligo(dT)-cellulose or DEAE-Sephadex chromatography. [^{14}C]rRNA was not retained, while synthetic [^{3}H]poly(A) was bound to oligo(dT)-cellulose and was eluted with 10 mM Tris·HCl (Fig. 3*A*). The poly(A)

TABLE 2. *Analysis of alkaline digests of poly(A) segments isolated from terminally labeled 60–70S and 30–40S RNA*

Isolated from:	Purified by:	One- or two-dimensional thin-layer chromatography	% cpm in trialcohols*			
			A′	G′	C′	U′
60–70S RNA	Oligo(dT)-cellulose	One	79	8.3	8.3	4.2
	Oligo(dT)-cellulose	Two	92	0	5	2.5
30–40S RNA	Oligo(dT)-cellulose	One	87	10	0.7	2
	DEAE-Sephadex	Two	88	0	12	0

* Average of two independent experiments.

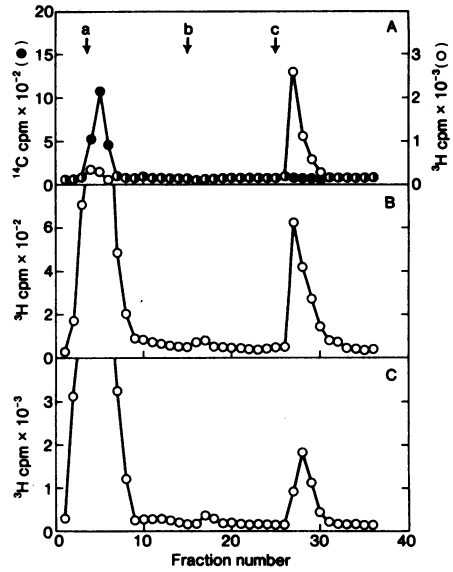

FIG. 3. Chromatography of RNase A plus RNase T1 digests of terminally labeled MSV(MLV) RNA on oligo(dT)-cellulose. The following RNA preparations were fractionated by washing with 0.5 M KCl–10 mM Tris·HCl (pH 7.5) at (*a*) and with 10 mM KCl–10 mM Tris·HCl (pH 7.5) at (*b*), and then eluting with 10 mM Tris·HCl (pH 7.5) at (*c*). (*A*) Control mixture of [^{14}C]rRNA (●) and [^{3}H]poly(A) (○); (*B*) RNase digests of terminally labeled 60–70S RNA; (*C*) RNase digests of terminally labeled 30–40S RNA.

from RNase digests of terminally labeled 60–70S and 30–40S RNA eluted at the same position as synthetic [^{3}H]poly(A) (Fig. 3*B* and *C*). Terminally labeled poly(A) purified on oligo(dT)-cellulose was hydrolyzed and analyzed by one- and two-dimensional chromatography. 79–92% of the 3′-termini of poly(A) was adenosine (Table 2).

The profiles on DEAE-Sephxdex in 7 M urea of RNase digests of 3′-terminally labeled 60–70S RNA (Fig. 4*A*, *open circle*) and 30–40S RNA (Fig. 4*B*) show the elution of ter-

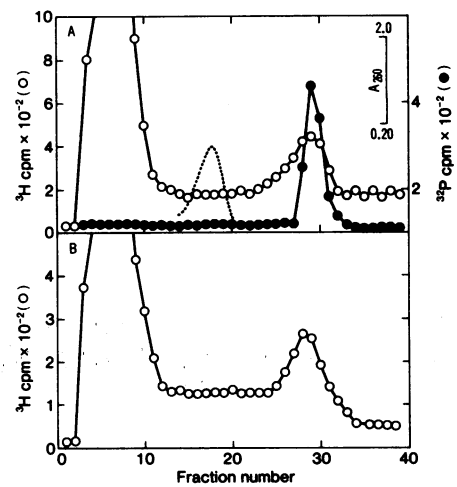

FIG. 4. Chromatography of RNase A plus RNase T1 digests of terminally labeled MSV(MLV) RNA on DEAE-Sephadex in 7 M urea. Elution profile of RNase digest of terminally labeled 60–70S RNA (*A*), and 30–40S RNA (*B*). Absorbance of marker tRNA (– – –); elution pattern of marker [^{32}P]poly(A) isolated from 60–70S [^{32}P]RNA by RNase digestion and oligo-(dT)-cellulose column chromatography (●).

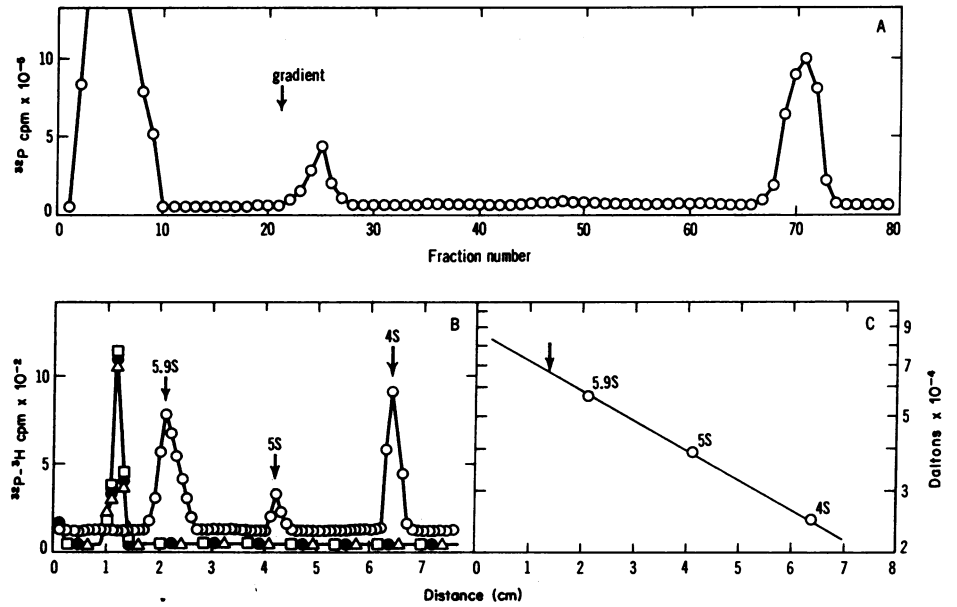

FIG. 5. Size of poly(A) derived from viral 60 to 70S RNA. (*A*) Poly(A) was purified from an RNase T1 digest of viral 60–70S [^{32}P]-RNA by poly(U)–Sepharose chromatography. (*B*) The digests of 60–70S [^{32}P]RNA with RNase T1 (●), RNase A (△), and RNase T1 plus RNase A (□) were fractionated on 10% polyacrylamide–7 M urea gels (5 hr at 5 mA per gel). Molecular mass values of 2.4, 3.9, and 5.9 × 10^{4} were assumed for 4S RNA, 5S RNA, and 5.9S poly(C) markers, respectively, run on parallel gels. (*C*) Calibration of molecular mass against S values by use of the markers in *B*.

minally labeled poly(A) at the same position as [^{32}P]poly(A) derived from 60 to 70S RNA. The poly(A) recovered from the column fractions was analyzed for the 3'-terminal [^{3}H] nucleoside. More than 85% of the radioactivity coincided with adenosine (Table 2).

In addition, poly(A) was isolated from 60–70S RNA that was labeled with [^{3}H]adenosine and purified on poly(U)–Sepharose. [^{3}H]Adenosine-labeled poly(A) contained approximately one adenosine (73 cpm) per 190 adenylate residues (13,716 cpm), as determined by digestion with RNase U2 and RNase T2 and high-voltage electrophoresis. These results provide additional evidence that poly(A) is located at the 3'-terminus of 30–40S RNA.

Length of Poly(A) Segments in Viral RNA. Viral [^{32}P]RNA was digested with RNase T1, RNase A, or RNase T1 plus RNase A, purified by poly(U)–Sepharose chromatography (Fig. 5*A*) and by gel electrophoresis, and analyzed by electrophoresis in 10% polyacrylamide gels containing 7 M urea. Fig. 5*A* is a typical profile of an RNase T1 digest of 60–70S [^{32}P]RNA on poly(U)–Sepharose. A sharp peak of poly(A) is seen in fraction 70. The poly(A) from the various RNase digestions migrated more slowly than 5.9S RNA and appeared to be very homogeneous (Fig. 5*B*). The molecular weight of the three preparations of poly(A) was estimated graphically (Fig. 5*C*) to be 64,000–68,000, corresponding to about 190 nucleotides.

Content of Poly(A) in Viral RNA. To determine the number of poly(A) segments in viral RNA, we labeled with 60–70S RNA with [^{32}P]phosphate, digested it with RNase T1, and purified it by poly(U)–Sepharose chromatography (Fig. 5*A*). The average poly(A) content in three experiments was 1.6% (2 to 3 × 10^{7} cpm were analyzed each time). The oligo(A) eluted at the beginning of the gradient (see Fig. 5*A*), probably derived from internal sequences, was about 0.5% of the total counts.

The molecular weights of 60–70S RNA and 30–40S RNA subunits are estimated at 10 to 12 × 10^{6} and 2.5 to 3 × 10^{6}, respectively (33). Since poly(A) from 60 to 70S RNA has a molecular weight of 64,000–68,000 (Fig. 5*C*) and represents 1.6% of 60–70S RNA, we estimate that each 30–40S RNA subunit contains close to one poly(A) segment.

Nucleotide Sequence of Poly(A) Segment. Viral 60–70S [^{32}P]RNA was digested with RNase T1 or RNase A, and poly(A) was purified by poly(U)–Sepharose chromatography followed by polyacrylamide gel electrophoresis. RNase T1 hydrolyzes phosphodiester bonds between guanylate residues and adjacent 3'-nucleotides, while RNase A cleaves bonds between pyrimidine residues and adjacent 3'-nucleotides. The base composition of RNase A-poly(A) and RNase T1-poly(A) was determined (Table 3). The RNase T1-poly(A) contained 1.2% CMP, 98.3% AMP, and 0.4 UMP, suggesting a structure of —G(C,C,C,U)A_{190}. The absence of GMP in RNase T1-poly(A) is consistent with data that poly(A) is located at the 3'-end. The RNase A-poly(A) contained 0.8% CMP-like material and 99.2% AMP. Molloy and Darnell (13) showed that CMP-like material in alkaline hydrolysates of RNase A-poly(A) from HeLa cell mRNA and HnRNA resulted from an unknown chemical modification of about 1% of AMP. The possible presence of CMP at the 3'-end of RNase A-poly(A) was examined by treatment of ^{32}P labeled RNase A-poly(A) with alkaline phosphatase before alkaline hydrolysis. No change in base composition was observed (Table 3). We therefore subtracted two CMP residues from the tabulated base composition of RNase A-poly(A) and RNase T1-poly(A) since base analysis of RNase A-poly(A) shows that the equivalent of two CMP-like residues is formed by chemical modification (13). These results support the conclusion that the poly(A) in 30–40S RNA is composed entirely of adenylate residues with pyrimidine residues at the 5'-end of poly(A). The 3'-terminal sequence of the 30–40S RNA is, therefore, —G(C,U)$A_{190}A_{OH}$.

TABLE 3. *Base composition [%(cpm)] of poly(A) segments derived from MSV(MLV) 60–70S [^{32}P]RNA*

	Exp.	CMP	AMP	GMP	UMP
RNase T1-poly(A) segments	1	1.2(634)	98.3(43340)	0(23)	0.4(191)
	2	1.2(458)	98.3(37360)	0(36)	0.4(136)
RNase A-poly(A) segments	1	0.8(366)	99.2(46410)	0(24)	0 (15)
+ alkaline phosphatase*	2	0.8(356)	99.2(41320)	0(38)	0 (20)

* The RNase A-poly(A) was treated with alkaline phosphatase (2 units/ml) in 15 mM Tris·HCl (pH 8.0) for 30 min at 37° before alkaline digestion.

DISCUSSION

We showed by periodate oxidation-[^{3}H]borohydride reduction that the major 3′-terminal nucleoside of 60–70S RNA, 30–40S RNA, and the poly(A) segments derived from both RNA species is adenosine. Our results differ from reports that 60–70S RNA of MSV(MLV) (18) and of several other RNA tumor viruses (16, 17) contain uridine as the major 3′-OH nucleoside, but are consistent with the analysis of the RNA of avian myeloblastosis virus reported by Zamecnik's laboratory (20). The differences between laboratories are probably due to the difficulty in using borohydride, which reacts with many compounds (10, 15, 20, 24) and necessitates extreme caution in purifying labeled RNA and resolving the derived trialcohols.

The results of base-composition analysis indicate that the poly(A) segments of MSV(MLV) are composed entirely of adenylate residues and terminated with 3′OH adenosine. We have not detected interpolated bases within the 190-nucleotidelong stretch of poly(A) derived from MSV(MLV) 60–70S RNA.

The 3′-terminus of both 30–40S and 60–70S RNA is $-G(C,U)A_{190}A_{OH}$. Darnell *et al.* (13, 35) found a similar sequence, $-G(C,C,U)A_{200}$, in HnRNA and mRNA of HeLa cells. Brownlee *et al.* (34) reported a similar composition for the poly(A) of mRNA for the immunoglobulin light chain. These findings suggest that similar recognition sites and mechanisms for poly(A) addition may occur in the processing of cellular HnRNA and oncornavirus RNA sequences. This seems reasonable in view of the integrated state of oncornavirus DNA in the cellular genome (33).

It has been proposed that poly(A) in mammalian HnRNA and mRNA plays a role in mRNA processing and transport or in the cytoplasmic function of mRNA (35). Whether similar functions are involved in oncornavirus replication is not clear. Parental viral RNA is found in the polyribosomes in an EDTA-releasable form early after infection of mouse cells with MSV(MLV) (Robin, Salzberg, and Green, in manuscript), but its role as mRNA has not been established. Viral RNA of the same size and sequence content as MSV(MLV) 30–40S RNA subunits are found in free and membrane-bound polyribosomes of MSV(MLV)-producing rat cells (32, 36, 37) that synthesize viral polypeptides.

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