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Properties and Location of Poly(A) in Rous Sarcoma Virus RNA

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The poly(A) sequence of 30 to 40*S* Rous sarcoma virus RNA, prepared by digestion of the RNA with RNase T_1, showed a rather homogenous electrophoretic distribution in formamide-polyacrylamide gels. Its size was estimated to be about 200 AMP residues. The poly(A) appears to be located at or near the 3′ end of the 30 to 40*S* RNA because: (i) it contained one adenosine per 180 AMP residues, and because (ii) incubation of 30 to 40*S* RNA with bacterial RNase H in the presence of poly(dT) removed its poly(A) without significantly affecting its hydrodynamic or electrophoretic properties in denaturing solvents. The viral 60 to 70*S* RNA complex was found to consist of 30 to 40*S* subunits both with (65%) and without (approximately 30%) poly(A). The heteropolymeric sequences of these two species of 30 to 40*S* subunits have the same RNase T_1-resistant oligonucleotide composition. Some, perhaps all, RNase T_1-resistant oligonucleotides of 30 to 40*S* Rous sarcoma virus RNA appear to have a unique location relative to the poly(A) sequence, because the complexity of poly(A)-tagged fragments of 30 to 40*S* RNA decreased with decreasing size of the fragment. Two RNase T_1-resistant oligonucleotides which distinguish sarcoma virus Prague B RNA from that of a transformation-defective deletion mutant of the same virus appear to be associated with an 11*S* poly(A)-tagged fragment of Prague B RNA. Thus RNA sequences concerned with cell transformation seem to be located within 5 to 10% of the 3′ terminus of Prague B RNA.

Poly(A) stretches have been found in the RNA of tumor viruses and many cytocidal viruses as well as in many cellular messenger and heterogenous nuclear RNAs (1, 3, 7, 13, 14, 16-18, 21-23, 26, 27, 33, 35, 37, 45, 47). Although the function of poly(A) in these RNAs is unknown, it has been suggested that poly(A) is added to mRNAs post-transcriptionally (7, 34). Consistent with this notion, the poly(A) stretches have been found at the 3′ end of a number of mRNAs (31, 32, 40).

Based on endgroup-labeling techniques tumor virus RNA was reported to terminate at the 3′ end with a poly(A) segment about 30 residues long in the case of an avian virus (43) and with a poly(A) segment of 190 residues in the case of murine sarcoma-leukemia virus (38).

In this report we present evidence in agreement with earlier studies (23) that the poly(A) sequence of Rous sarcoma virus (RSV) is about 180 nucleotides long and confirm its location at or near the 3′ end by two methods: (i) poly(A) prepared enzymatically from RSV RNA contained one adenosine per 180 AMP residues. (ii) Removal of the poly(A) stretch from RSV RNA by digestion with bacterial RNase H in the presence of poly(dT) resulted in 30 to 40*S* RNA which was only marginally smaller than 30 to 40*S* RNA containing poly(A). Further, no internal poly(A) sequences were found in 30 to 40*S* RSV RNA.

In agreement with earlier observations on RSV RNA (23) and with a recent report on murine tumor virus RNA (20), we found that about 30% of the 30 to 40*S* RNAs of RSV had no poly(A). Finger print analyses of 30 to 40*S* RSV RNAs with and without poly(A) indicated that the two species are indistinguishable with regard to their heteropolymeric sequences.

Moreover, fingerprint analyses of poly(A)-tagged fragments of 30 to 40*S* RSV RNA showed a decreasing complexity with decreasing size. This indicates that locations of some, perhaps all, RNase T_1-resistant oligonucleotides and of poly(A) are the same on all 30 to 40*S* RNAs.

MATERIALS AND METHODS

Reagents. The following reagents were purchased. [^{3}H]uridine (40 Ci/mmol) was from New England Nuclear Corp.; [^{3}H]adenosine (10 Ci/mmol or 16 Ci/mmol was from New England Nuclear Corp. or Schwarz/Mann Research; Carrier-free ^{32}P was from ICN; [^{3}H]poly(A) (73 μCi/μmol of phosphate) and poly(dT) were from Miles Laboratories; oligo(dT)-

cellulose was from Collaborative Research Inc.; RNase A, RNase T_1, DNase I, and *E. coli* alkaline phosphatase were from Worthington Biochemical Corp.; and RNase T_2 was from Calbiochem. *E. coli* RNase H was prepared as described previously (24).

Virus. Prague RSV of subgroup B (PR RSV-B) was used in all of these studies. It was propagated and purified according to published procedures (10, 11).

RNA preparations. Tobacco mosaic virus (TMV) RNA, [^{3}H]uridine, [^{3}H]adenosine, or ^{32}P-labeled 60 to 70*S* RSV RNAs as well as 30 to 40*S* subunits were prepared according to published procedures (9, 11). All RSV RNAs used in these studies were isolated from radioactive virus harvested from infected cultures at 3-h intervals.

[^{3}H]uridine or ^{32}P-labeled chick cell 4, 18, and 28*S* RNAs were prepared as follows. Primary chick embryo fibroblast cells were seeded at a concentration of 4×10^6 cells per 10-cm petri dish and were grown in medium 199 supplemented with 2% tryptose phosphate broth (TPB), 1% calf serum, 1% chick serum, 1% dimethyl sulfoxide, 0.05% glucose, 100 units per ml of penicillin, 50 μg per ml of streptomycin, and 0.5 μg per ml of fungizone. Twelve hours later, medium was changed to 8-ml per dish of Dulbecco modified Eagle medium with the same supplements as described above except that TPB was omitted and dialyzed calf and chick sera were used. A 200-μCi amount of [^{3}H]uridine or 1 mCi of ^{32}P was added per dish; generally, four dishes were labeled at one time. After 10 to 12 h of incubation at 42 C, dishes were removed from the incubator and placed on an ice bath. Medium was removed and the cells were washed twice with Tris saline; then, 1.5 ml of hypotonic buffer containing 0.01 M Tris-hydrochloride, pH 7.4, 0.01 M NaCl, 2mM EDTA, and 0.05% Triton X-100 was added to each dish. After sitting for 5 min on ice, cells were harvested with a rubber policeman and pipetted into a Dounce homogenizer. Cells were broken up with six to seven strokes and under these conditions, very few nuclei were broken. Nuclei were pelleted by centrifugation at $600 \times g$ for 5 min in a Sorvall centrifuge. The supernatant was then diluted with standard buffer (0.01 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, and 1 mM EDTA), and the RNA extracted as described above for viral RNA. After precipitation the RNA was pelleted and washed twice with 75% ethanol and redissolved in 0.3 ml of standard buffer containing 0.2% sodium dodecyl sulfate (SDS). The solution was heated at 100 C for 1 min, quickly chilled, and was then layered on a 5-ml 10 to 25% linear sucrose gradient containing standard buffer plus 0.1% SDS. Sedimentation was in a Spinco SW65 rotor at 65,000 rpm for 2 h at 20 C. Fractions (0.3 ml) were collected and a small portion of each fraction was counted in 3 ml of toluene-based scintillation fluid containing 10% NCS (Nuclear Chicago). The sedimentation profile showed three distinct peaks representing 4, 18, and 28*S* RNAs. Peak fractions of each RNA were pooled, ethanol precipitated, and redissolved in buffer containing 0.01 M Tris-hydrochloride, pH 7.4, 10 mM NaCl, and 1 mM EDTA. The purified RNA samples were stored at −70 C.

Conditions for enzyme reactions. DNase I: 0.01 M Tris-hydrochloride, pH 7.2, 4 mM $MgCl_2$ and 20 μg per ml of DNase, incubated at 38 C for 30 min. Combined digestion with RNase A and RNase T_1: 0.01 M Tris-hydrochloride, pH 7.2, 0.3 M NaCl, 1 mM EDTA, 20 μg per ml of RNase A, and 150 units per ml of RNase T_1, incubated at 38 C for 30 min. RNase T_1 alone: 0.01 M Tris-hydrochloride, pH 7.2, 0.15 M NaCl, 1 mM EDTA, and 150 units per ml of enzyme incubated at 38 C for 60 min. RNase T_2 alone: 0.04 M ammonium acetate, pH 4.4, 1 mM EDTA, 5 units per ml of enzyme, incubated at 38 C for 2 h. Combined digestion with RNase A, T_1 and T_2: same conditions as for RNase T_2 alone plus 20 μg per ml of RNase A, and 150 units per ml of RNase T_1, incubation at 38 C for 2 h. *E. coli* RNase H (24): reaction mixture (100 or 150 μliters) contained 0.02 M Tris-hydrochloride, pH 8.0, 10 mM $MgCl_2$, 6 mM dithiothreitol, 25 units per ml of enzyme and 350 pmol ($\sim 1.85 \times 10^4$ counts/min) of [^{3}H]poly(A) plus 175 pmol of poly(dT). When RSV RNA ($\sim$0.4 μg) was used as substrate, 0.25 μg of poly(dT) was added, whereas [^{3}H]poly(A)·poly(dT) was omitted. The reaction was carried out at 38 C for 1 h. Dephosphorylation and fractionation of commercial [^{3}H]poly(A): 25 μg (3.7×10^6 counts/min) of [^{3}H]poly(A) (Miles) was treated with *E. coli* alkaline phosphatase in a 1-ml solution containing 0.05 M Tris-hydrochloride, pH 8.2, and 2 units of enzyme. After incubation at 38 C for 30 min, it was diluted to 4 ml with standard buffer containing 0.1% SDS and phenol-extracted as described for viral RNA. After ethanol precipitation, the poly(A) was pelleted and washed three times with 75% ethanol. It was redissolved in 0.5 ml 0.01 M Tris-hydrochloride, pH 7.2. For further fractionation a portion of purified poly(A) was heat denatured (100 C 1 min) in 0.3 ml of standard buffer containing 0.2% SDS and was sedimented through a 5-ml 10 to 25% sucrose gradient as described above. Sedimentation was carried out in a Spinco SW50.1 rotor at 49,000 rpm for 12 h at 20 C. Peak fractions of poly(A) were pooled, ethanol precipitated, and resedimented similarly. Three subsequent sedimentations were performed to prepare poly(A) with a uniform sedimentation profile and a peak at about 8*S*.

Isolation of [^{3}H]adenosine or ^{32}P-labeled poly(A) from RSV RNA. Purified 60–70*S* RSV RNA in ($\leq$0.2 ml) 0.01 M Tris, pH 7.2, 1 mM EDTA was heat denatured at 100 C for 1 min and digested either with RNase A plus RNase T_1 or RNase T_1 alone as described above. After digestion, the reaction mixture was diluted with standard buffer to 4 to 5 ml after addition of 20 μg of carrier TMV RNA and SDS to 0.1% the solution was phenol-extracted three times. The RNA was ethanol precipitated twice to remove all soluble nucleotides. Such poly(A) was either used directly for end-group analysis or further purified by heating (100 C, 1 min) followed by sucrose gradient sedimentation in an SW50.1 rotor at 49,000 rpm for 10 h at 20 C. Peak fractions representing more than 80% of the total radioactivity were pooled, and poly(A) was then ethanol precipitated twice and used for end-group analysis.

End-group analysis. Purified commercial (Miles, Inc.) 7.8*S*, [^{3}H]poly(A) (0.7 to 0.9 μg) or [^{3}H]adenosine labeled poly(A) from (0.5 $\sim$1.5 μg) 60 to 70*S* RSV RNA was completely digested to monoculeotides with

RNase T_2 in a volume of 0.2 ml (see above). The reaction mixture was then lyophilized and redissolved in 30 μliters of water. A 200-μg amount of an equimolar mixture of each of the four ribonucleoside monophosphates and adenosine in 12 μliters buffer was added to it as internal standards. The mixture was subjected to electrophoresis on a Whatman 3 MM paper (12 by 56 cm) according to published procedures (23) except that electrophoresis was at 1,500 V for 2.5 h. After the paper had been air dried the spots representing adenosine and the four nucleotides were located by exposing the paper to UV light in a dark room. Each spot as well as regions between spots were cut out separately. The nucleoside or nucleotides were eluted from the paper with 0.4 ml of water. The eluted radioactivity was measured in 15 ml of toluene-based scintillation fluid containing 20% NCS and counted in a Packard liquid scintillation counter. After elution, the paper was tested for any remaining radioactivity by the same method. Since no radioactivity could be detected on the eluted paper, the recovery appeared to be complete.

Oligo(dT)-cellulose column fractionation of poly(A)-containing RNA. The procedures were a modification of the method of Aviv and Leder (4). One-half gram (dry weight) of oligo(dT)-cellulose was washed and soaked in 0.01 M Tris-hydrochloride (pH 7.4) buffer for several hours at room temperature. After decanting the fine particles several times, it was packed into a column (0.7 by 3 cm). Before each run, the column was washed with 2 ml of 0.1 M KOH and subsequently washed with ice-cold buffer I (0.5 M LiCl, 0.01 M Tris, pH 7.4, 0.05% SDS) until the pH was neutral. An RNA sample in 0.2 ml of cold buffer I was applied to the top of the column and allowed to enter the column. Fractionation was carried out by stepwise elution with 2-ml batches of buffer I, buffer II containing 0.1 M LiCl, but otherwise the same as buffer I, and buffer III containing 0.01 M Tris-hydrochloride and 0.05% SDS. Fractions (0.5 ml) were collected. The RNA in each fraction was determined by its radioactivity.

Millipore binding and elution. The procedure is a slight modification of that described by Mendecki et al. (31). Briefly, the RNA was dissolved in/or diluted at least 20-fold with buffer containing 0.5 M KCl, 0.01 M Tris-hydrochloride (pH 7.4), and 1 mM $MgCl_2$ and filtered slowly by gravity (approximately 6 ml/h) through a Millipore filter previously soaked in the same buffer for 30 min. After washing three times with 1-ml portions of the same buffer, the RNA bound was recovered by eluting with three 0.5-ml portions of buffer containing 0.1 M Tris-hydrochloride (pH 9.0) and 0.5% SDS.

RESULTS

Size of poly(A) in RSV RNA. Earlier studies in this (23) and other laboratories (16, 17) have estimated the size of the poly(A) stretch of tumor virus RNA to be around 60,000 daltons. However, more recently the poly(A) stretch of avian myeloblastosis virus RNA was reported to be only 9,000 daltons (43). Since both the size and the absolute amount of poly(A) in viral RNA must be known to distinguish between several relatively small or one relatively large poly(A) stretch, we have redetermined the size of the poly(A) in RSV RNA.

Previous size estimates were done with poly(A) prepared from avian tumor virus RNA by RNase A and RNase T_1 digestion at high ionic strength (see above references). Although poly(A) is known to be relatively resistant to RNase A at high ionic strength (5), it is conceivable that the enzyme may introduce some nicks in this condition. This was indicated by experiments in which poly(A) prepared from viral RNA by digestion with RNase T_1, which is specific for G, and with a combination of RNase T_1 and RNase A were compared. It was found that after electrophoresis in formamide-polyacrylamide gels the RNase T_1-prepared poly(A) was much more homogenous than that prepared earlier by RNase A (Fig. 1, ref. 23). If commercial poly(A) was treated under the same conditions as used to prepare viral poly(A) with RNase T_1, its electrophoretic distribution was the same as that of an untreated control. However, commercial poly(A) was rendered heterogenous if treated with RNase A and RNase T_1 (not shown). We conclude that RNase A at high ionic strength nicks viral and other poly(A) sequences. Nicking during isolation by RNase A may be the reason for the size difference between the poly(A) prepared by us and that described by Stephenson et al. (43) and may also account for the heterogeneity of A-rich fragments ranging from poly(A) to oligo(A) described by Horst et al. (19).

The molecular weights of commercial 7.8S poly(A) and of the poly(A) of RSV RNA prepared by RNase T_1 were estimated from their electrophoretic mobilities in formamide-polyacrylamide gels to be 116,000 and 70,000, respectively (Fig. 1 insert), using 18S rRNA (0.7×10^6 [42]) and tRNA (2.5×10^4 [42]) as standards and assuming a linear inverse relationship between the logarithm of the molecular weight of a polynucleotide and its electrophoretic mobility (42). However, this size estimate of poly(A) may be subject to 10 to 20% error since deviations from a linear log molecular weight-mobility relationship have been observed under similar conditions (12).

Further it is conceivable that viral poly(A) prepared by RNase T_1 contains at its ends a stretch of nucleotides containing U, A, and C which would not be digested by RNase T_1. This may affect slightly the molecular weight of the poly(A) prepared by RNase T_1. However, base analyses of viral poly(A) prepared with

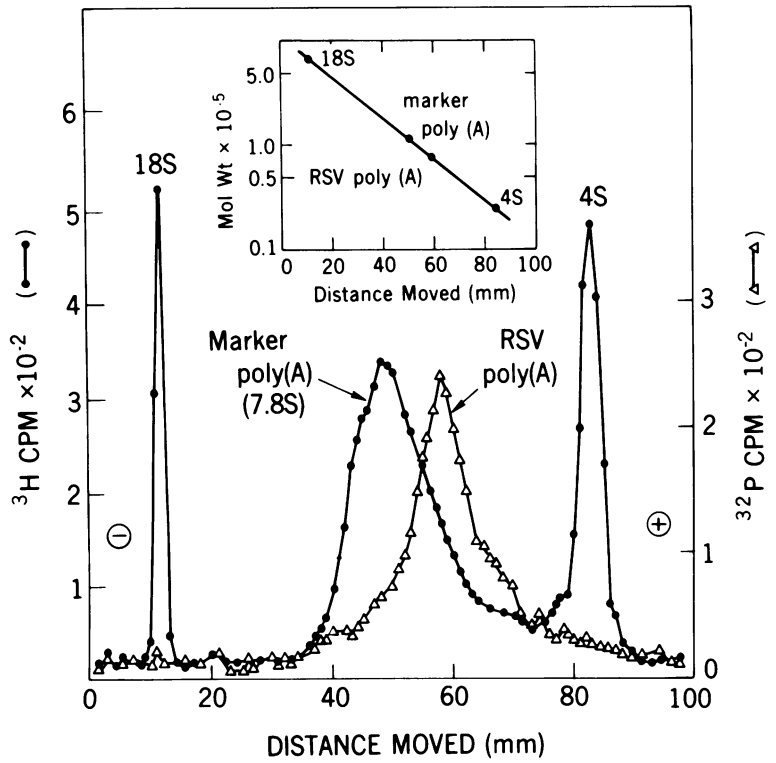

FIG. 1. *Determination of the size of marker 7.8S poly(A) and RSV poly(A) by formamide polyacrylamide gel electrophoresis. RSV poly(A) was isolated from 30 to 40S [^{32}P]RNA subunits by RNase T_1 digestion. The digest was phenol-extracted three times and ethanol-precipitated in the presence of 30 μg of TMV RNA. The RNA recovered was then passed through an oligo(dT)-cellulose column (Fig. 3). The poly(A) peak eluted with buffer III and was divided into two portions. One portion was ethanol-precipitated for analysis of its base composition as described for Table 1. The other portion was mixed with ^{3}H-labeled 4S, 18S RNAs, and 7.8S poly(A). After ethanol precipitation the mixture was dissolved in buffered formamide and subjected to electrophoresis in a 5% polyacrylamide gel containing 98% formamide at 100 V for 7 h as described previously (12). The insert shows a log molecular weight/electrophoretic mobility plot (42) used to estimate the molecular weight of RSV poly(A).*

RNase T_1 and a combination of RNase A and RNase T_1 were very similar and suggested that very few (<3%) bases other than A were present in our poly(A) (not shown). We assume that some of those bases come from the 5 end of RNase T_1-prepared viral poly(A). Thus it appears that our size estimate of viral poly(A) was little, at most 3%, affected by bases other than A. Moreover, we may conclude that, in contrast to an earlier report (19), the poly(A) of RSV RNA is free of interspersed G-residues sensitive to digestion by RNase T_1, since it is not degraded by this enzyme.

Location of the poly(A) at or near the 3′ end of RSV RNA. There is suggestive evidence from end group analyses that the poly(A) of tumor virus RNA, like the poly(A) of other viral or cellular mRNAs is located at the 3′ end of the RNA molecule (2, 38, 43, and J. Keith, M. Gleason, and H. Fraenkel-Conrat, Proc. Nat. Acad. Sci. U.S.A., in press). One method used to determine the 3′ terminal location of poly(A) was based on oxidation and reduction of the vicinal 2′ and 3′ OH groups at the 3′ terminus of the RNA (38, 43). However this method has led to contradictory results when applied to tumor virus RNA (15, 25, 30).

Another method was based on the ratio of nucleosides to nucleotides after complete hydrolysis of radioactive RNA or poly(A) (2, 38, and J. Keith, M. Gleason, and H. Fraenkel-Conrat, Proc. Nat. Acad. Sci. U.S.A., in press). However, the accuracy of this method suffers from the low nucleoside/nucleotide ratios of RSV RNA (1:200 for poly(A), 1:10,000 for 30 to 40S RNA). We describe below two approaches to locate the poly(A) stretch in RSV RNA.

End-group analysis of viral poly(A). First we determined the end-group of biologically labeled poly(A) from 30 to 40S RSV RNA. If poly(A) is at the 3′ terminus of the RNA it

should, after complete digestion to mononucleotides, contain one adenosine per about 200 AMP residues, assuming our size estimate was correct. We have chosen enzymatic digestion of poly(A) instead of digestion by alkali, because pilot experiments had shown that approximately 50% of the ^3H of poly(A) was rendered volatile during lyophylization after KOH hydrolysis, presumable due to isotope exchange with water (39, 46). Exhaustive digestion with RNase A also proved unsatisfactory, as the digestion product was mostly ApAp rather than AMP (not shown). Complete digestion of viral, as well as commercial poly(A) to mononucleotides was obtained with RNase T_2 (Table 1).

It is shown in Table 1 that the poly(A) from RSV RNA contained one adenosine per 180 AMP residues and that commercial poly(A) contained one per 320. This is compatible with our size estimate of about 200 nucleotides for viral poly(A) and with the size estimate of 116,000 for commercial 7.8*S* poly(A).

No significant radioactivity was detectable at the origin of the pherogram used to resolve the digest (Table 1), neither was there radioactivity between the spots of the four UV-absorbing monophosphate ribonucleotide markers used to monitor the electrophoresis. However, there were small amounts of radioactivity in the CMP spot if viral or commercial poly(A) were analyzed (experiments 1 and 2, Table 1). This was most likely due to slight trailing of AMP, because after prolonged electrophoresis (experiment 3, Table 1) no radioactivity was seen in the CMP position. Significant amounts of radioactivity migrated with the GMP spot, when the poly(A) of RSV was analyzed, especially in experiment 3 (Table 1). This could be due to residual heteropolymeric sequences associated with this preparation of viral poly(A) or less likely (see above; 38) due to RNase T_1-resistant G residues interspersed into poly(A). The radioactivity in GMP may have originated from cellular conversion of [^3H]adenosine into [^3H]GMP or from [^3H]guanosine contaminating the [^3H]adenosine used by us (from New England Nuclear Corp., experiments 1 and 2, Table 1; from Schwarz-Mann, experiment 3, Table 1). The amount of ^3H electrophoresing with GMP varied relative to that found in AMP in the experiments described in Table 1. By contrast, the radioactivity associated with the adenosine spot. relative to that in the AMP spot remained constant (.55%) in all experiments with poly(A) from RSV (Table 1).

Control experiments using 28*S* [^{32}P]rRNA (1.25×10^5 counts/min) showed that <0.04% (<50 counts/min) of the mononucleotides had been dephosphorylated under our conditions of enzymatic digestion using RNase T_2, RNase T_1, and RNase A (Fig. 2). This may be a problem to be considered if alkali is used to digest RNA (28). Therefore, we believe that the adenosine residues detected by complete enzymatic digestion of viral poly(A) were derived from the 3′ end of the RNA, and consequently that

TABLE 1. *End-group analysis of marker and RSV poly(A)*

Poly(A) tested experiments	Marker [^3H]poly(A)[a]						[^3H]poly(A) from 60 to 70*S* RSV RNA[b]					
	I		II		III		I		II		III	
	Counts/min[c]	%	Counts/min	%	Counts/min	%	Counts/min	%	Counts/min	%	Counts/min	%
Adenosine	400	0.31	328	0.32	400	0.31	314	0.59	144	0.55	430	0.52
CMP	246	0.18	145	0.14	0	0	162	0.30	46	0.19	0	0
AMP	128,780	99.43	103,410	99.44	129,900	99.66	52,890	98.65	24,190	98.58	80,000	97.29
GMP	72	0.06	70	0.07	36	0.03	218	0.41	144	0.55	1,800	2.19
UMP	32	0.04	36	0.04	0	0	31	0.06	13	0.05	0	0
Total	129,530	100	103,990	100	130,340	100	53,165	100	24,540		82,230	100
AMP/adenosine	322:1		315:1		325:1		170:1		168:1		186:1	
Avg of AMP/adenosine			320 ± 5						178 ± 8			

[a] Complete digestion of poly(A) by RNase T_2 was described in Materials and Methods. In experiments I and II, dephosphorylated and ethanol-precipitated Miles poly(A) was used; in experiment III poly(A) had been further purified by three subsequent sedimentations.

[b] [^3H]adenosine-labeled poly(A) from RSV RNA was isolated by digestion with RNases T_1 and A as described in Materials and Methods. In experiments I and II, poly(A) isolated from same RNA preparation was used directly without further purification by sedimentation. In experiment III, poly(A) isolated from a different RNA preparation was further purified by sedimentation to remove the oligonucleotides that might have co-precipitated with poly(A). Paper electrophoresis of experiment III was run for 3 h instead of 2.5 h.

[c] Thirty counts per minute of background eluted from interspot region has been subtracted.

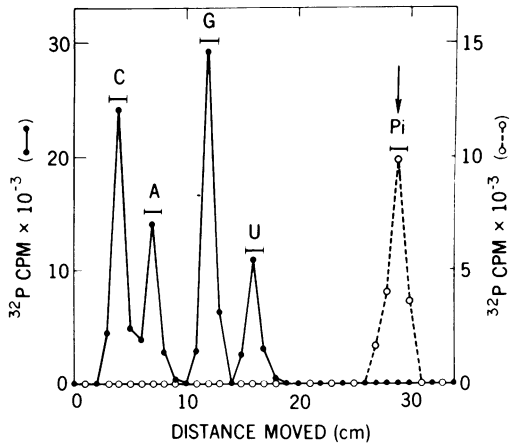

FIG. 2. *Electrophoresis of the ^{32}P-mononucleotides produced by complete enzymatic digestion of 28S chick rRNA. Approximately 1 μg of [^{32}P]28S rRNA (1.2 × 10^5 counts/min) was digested with RNases A, T_1, and T_2 in a total volume of 0.2 ml at 38 C for 2 h. The digest was analyzed by paper electrophoresis. A 20-μliter amount of [^{3}H]$^{32}PO_4$ (2 × 10^4 counts/min) was spotted on the same paper and run in parallel as a PO_4^{3-} marker. The ^{32}P nucleotides and PO_4^{3-} marker were detected by determining the radioactivity of 1-cm strips of the pherogram in toluene-based scintillation fluid.*

poly(A) appears to be located at the 3′ end of viral RNA. However, our experiment does not rule out the possibility that the penultimate base(s) of viral poly(A) are not A but a few U, C, G residues resistant to digestion with RNases T_1 and A used to prepare our poly(A). Further, it may be argued that our evidence for the 3′ location of poly(A) is not compelling, since it is based entirely on the end-group of the viral poly(A) which represents only 0.5% of the radioactivity associated with the poly(A) molecule.

30 to 40S RSV before and after removal of poly(A) with RNase H of E. coli. Therefore, independent evidence was sought to locate the position of poly(A) on viral 30 to 40*S* RNA. Using RNase H in the presence of poly(dT) to digest specifically the poly(A), but not the heteropolymeric sequences of viral RNA, it should be possible to determine the location of poly(A) on the RNA by analysis of the RNase H-resistant heteropolymeric sequences of the RNA. An RNase H which is free of other nucleolytic activities is essential for this experiment. Starting material for these experiments was RSV RNA, [^{3}H]adenosine or ^{32}P-labeled, selected for an intact poly(A) stretch by binding and subsequent elution from oligo(dT)-cellulose at low ionic strength (fraction III, Fig. 3). This RNA was incubated with *E. coli* RNase H in the presence of poly(dT) (24) (see Materials and Methods). The RNase H-treated RNA was then compared to untreated RNA with regard to its Millipore filter binding capacity, its poly(A) content and size.

Analysis of 30 to 40*S* RSV RNA before and after digestion with RNase H and in the presence of poly(dT) indicated that 80% of the RNase A- and RNase T_1-resistant, viral poly(A) can be digested by RNase H (Table 2). It appears then that most or all poly(A) (about 15 to 20 pmol) of about 1,200 pmol of viral RNA (specific activity ~10^5 counts/min/μg) was digested in our conditions. Control experiments using the same amount of enzyme, indicated that 110 to 140 pmol of [^{3}H]poly(A)·poly(dT) were digested in the same conditions. Thus, digestion of the viral poly(A) was at enzyme excess (Table 2).

Moreover Millipore binding capacity of 30 to 40*S* RSV RNA was reduced from about 90 to 10% by treatment with RNase H (Table 2). The enzyme/substrate ratios in these experiments were about the same as those used above {see control experiments with [^{3}H]poly(A)·poly(dT), Table 2}.

The size of the RNase H-treated 30 to 40*S* RNA was indistinguishable from that of untreated RNA on the basis of sedimentation in formaldehyde-sucrose gradients (Fig. 4) but was found to be slightly smaller on the basis of its

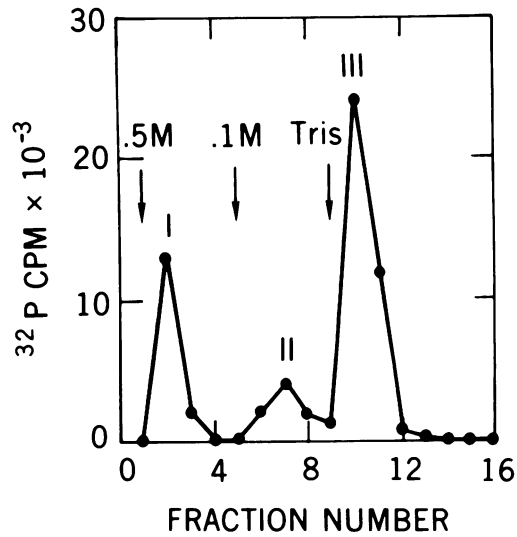

FIG. 3. *Oligo(dT)-cellulose chromatography of 30-40S RSV RNA. Purified 30 to 40S [^{32}P]RNA was dissolved in 0.2 ml of buffer I and heated at 100 C for 1 min. Fractionation was as described in Materials and Methods. Arrows indicate where the elution with different buffers (I, II, and III) started. Fractions (0.5 ml) were collected and 5-μliter portions of each fraction were counted in 3 ml of toluene-based scintillation fluid containing 10% NCS.*

TABLE 2. *30 to 40S RSV RNA[a] and poly(A) before and after digestion with RNase H*

Total 30 to 40S RNA			Fraction resistant to RNases A + T_1 after:[b]					
			poly(dT)		RNase H		RNase H + poly(dT)	
Expt	Counts/min	Label	Counts/min[c]	%	Counts/min	%	Counts/min	%
I	3710	[^{3}H]A	235	6.1			44	1.2
II	4200	[^{3}H]A	240	5.7	220	5.2	40	0.9
III	4100	[^{3}H]A	260	6.1	245	6.0	75	1.7
IV	21,000	^{32}P	345	1.6			95	0.5

Total [^{3}H]poly(A)			Fraction digested by RNase H with poly(dT)		
Expt	Counts/min	pmol	Counts/min	pmol	%
V	18,700	353	7,200	136	38
VI	18,500	349	5,800	109	31

Expt	Substance	Millipore binding capacity[d]							
		-RNase H			+RNase H				
		Counts/min applied	Counts/min bound	%	Counts/min applied	pmol	Counts/min bound	pmol	%
VII	[^{32}P]30 to 40S RSV RNA	3,788	3,297	87.3	5,200		574		11.0
VIII	[^{3}H]poly(A)[e]				13,800	260	5500	122	47.0

[a] Poly(A)-containing 30 to 40S RNA pooled from fraction no. 9 to 11 shown in Fig. 3.

[b] RNase H digestion was carried out as described in Materials and Methods using approximately 0.4 μg of [^{3}H]adenosine (1.5×10^4 counts/min) or ^{32}P (7×10^4 counts/min) labeled RSV RNA in each assay. Control tubes either minus RNase H or minus poly(dT) (0.25 μg) but with RNase H were incubated in parallel. At the end of reaction, duplicate portions of reaction mixture from each tube were taken separately. They were incubated with DNase to remove poly(dT) after adjusting their volume to 100 μliters and the salt conditions to those described in Materials and Methods for digestion with DNase. Subsequently they were adjusted to 0.5 ml per tube and subjected to RNase A plus RNase T_1 digestion for the assay of the remaining poly(A) sequences. At the end of digestion by RNases, 100 μg of yeast tRNA was added and trichloroacetic acid precipitable material was determined (44). Each number represents the average of duplicate assays. Fifteen counts per minute of background had been subtracted.

[c] The amount of RNA resistant to RNases.

[d] RNase H digestion was carried out as described in [b]. A control without RNase H was incubated in parallel. Each tube contained 1.5×10^4 counts/min of ^{32}P-labeled poly(A)-containing 30 to 40S RNA. After incubation each reaction mixture was adjusted to 0.1 M NaCl and 1 mM EDTA and a 100-fold excess of cold poly(A) (25 μg) was added to prevent poly(dT) from complexing to RSV RNA. They were then heat-denatured at 100 C for 40 s and chilled. Each reaction mixture was adjusted to a total volume of 0.3 ml with SDS standard buffer and sedimented through a 5 ml 10 to 25% linear SDS sucrose gradient after adding 18S [^{3}H]rRNA as an internal marker (Materials and Methods). After sedimentation, 30 to 40S RSV RNAs were pooled separately, ethanol-precipitated and subjected to Millipore binding (Materials and Methods).

[e] Standard assays of RNase H with [^{3}H]poly(A) and poly(dT) were performed in parallel and carried out as described in Materials and Methods. Each number represents the average of duplicate assays.

electrophoretic mobility in formamide-polyacrylamide gels (Fig. 5). This suggests that the poly(A) segment is located at or near a terminus of the RNA, since its removal did not significantly affect the size of the RNA. Given a terminal location of the poly(A), we would expect at most a small increase in electrophoretic mobility after removal of poly(A), since the poly(A) segment represents only about 1.5% of the 30 to 40S RSV RNA. Further, the rather homogenous distribution of RNase H-treated 30 to 40S RSV RNA in formaldehyde gradients or formamide gels indicates that the RNase H used by us was free of detectable endonuclease. The second minor peak of RNA observed both in the RNase H-treated and untreated RNA

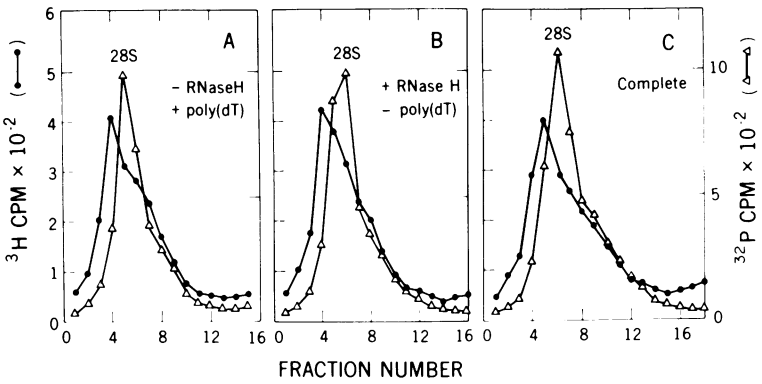

FIG. 4. *Formaldehyde sucrose gradient sedimentation of 30 to 40S RSV RNA before and after treatment with RNase H. Poly(A)-containing [^{3}H]adenosine-labeled 30 to 40S RSV RNA was prepared as described for Fig. 3. Three identical portions of 30 to 40S RNA were incubated with poly(dT) (A), RNase H (B), poly(dT), and RNase H (C) as described in Table 2. Subsequently, each sample was diluted with standard buffer to 2 ml after adding to each 10 μliters of 0.2 M EDTA and 30 μg of TMV RNA. After phenol extraction 28S [^{32}P]rRNA was added to each sample; the RNAs were then ethanol precipitated. After pelleting and washing twice with 75% ethanol, the RNA in each tube was redissolved in 0.3 ml of buffer containing 0.1 M NaCl, 2 mM EDTA, pH 7.2, 0.1% SDS, and 1.1 M formaldehyde. After incubation at 65 C for 15 min each RNA sample was layered on the top of a 5-ml 10 to 20% sucrose gradient containing the same salts and formaldehyde as the buffer described above. Sedimentation was in parallel in a Spinco SW 65 rotor at 65,000 rpm for 3 h at 20 C. Fractions (0.3 ml) were collected through the bottom of the tube, and 100-μliter portions of each fraction were measured for radioactivity.*

control seen in the formamide-polyacrylamide gels (Fig. 5) is thought to be 30 to 40*S* RNA of size class *b*, derived from transformation-defective virus which segregates spontaneously from stocks of nondefective avian sarcoma virus (29).

We conclude that RNase H treatment effectively removes all or most of the poly(A) associated with viral RNA without significantly reducing its size. It follows that the poly(A) is located near a terminus and that there are no large poly(A) stretches located within the 30 to 40*S* RNA chain.

Not all 30 to 40S RNA subunits of 60 to 70S RSV RNA contain poly(A). During this and earlier (23) studies, it was repeatedly observed that heat-denatured 60 to 70*S* RNA or purified 30 to 40*S* RNA had a much lower binding capacity to either Millipore filters or oligo(dT)-cellulose than intact 60 to 70*S* RNA. This raised the question whether all subunits in 60 to 70*S* RNA contain a poly(A) sequence. It seemed plausible that if there exist some 30 to 40*S* subunits which do not contain poly(A) sequences, they could bind to oligo(dT)-cellulose only if complexed to other, poly(A)-containing subunits within the same 60 to 70*S* RNA complex. Therefore, the oligo(dT)-cellulose binding capacity of purified 60 to 70*S* and 30 to 40*S* RNAs were compared at different ionic strengths. The results, summarized in Table 3, indicate that 90% of the 60 to 70*S* but only 65% of the 30 to 40*S* RNA bind to oligo(dT)-cellulose at high ionic strength, implying that about 1 out of 3 30 to 40*S* RNA species is devoid of a poly(A) stretch that confers binding capacity for oligo(dT)-cellulose. This is in agreement with a recent report by Ihle et al. (20) that only two-thirds of purified 30 to 40*S* RNA of mouse leukemia virus RNA binds to poly(U)-sepharose. As expected, most of an 18*S* ribosomal RNA used as internal control in the binding studies was incapable of binding to an oligo(dT)-cellulose (Table 3).

Direct analyses of the poly(A) content of fractions of 60 to 70*S* and 30 to 40*S* RNA, distinguished by their differential binding capacities to oligo(dT)-cellulose, confirmed the above conclusions. It was found that after complete enzymatic digestion of heteropolymeric sequences with RNase A and RNase T_1 (Materials and Methods), 30 to 40*S* and 60 to 70*S* RNA eluting from oligo(dT)-cellulose at high ionic strength (fraction I, Fig. 3, Table 3) were devoid of enzyme-resistant RNA and therefore did not contain poly(A) (not shown). However, 60 to 70*S* and 30 to 40*S* RNA eluting at low ionic strength (fraction III, Fig. 3, Table 3) contained 1.5% resistant RNA, if labeled with ^{32}P, and 6% if labeled with [^{3}H]adenosine as expected for a poly(A) containing RNA (Table 2). The RNA species which eluted at intermediate ionic strength contained 1 to 1.5% resistant

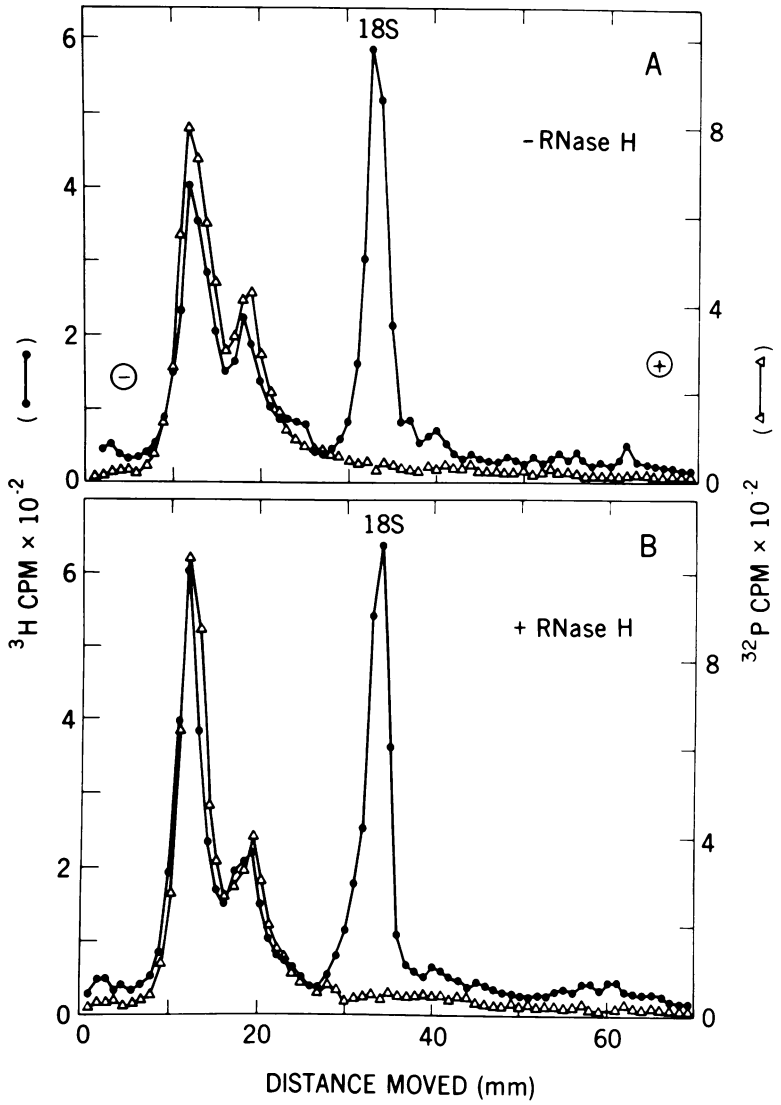

FIG. 5. *Formamide polyacrylamide gel electrophoresis of 30 to 40S RSV RNA before (A) and after (B) treatment with RNase H. Poly(A)-containing ^{32}P-labeled 30 to 40S RSV RNAs eluting at low ionic strength from oligo(dT)-cellulose (fraction III shown in Fig. 3) was incubated with RNase H as described for Fig. 4. After phenol extraction, [^{3}H]adenosine-labeled poly(A) containing 30 to 40S RNA and 18S rRNA were added as internal markers. The RNAs were ethanol precipitated. Electrophoresis was in polyacrylamide gels containing 98% formamide at 100 V for 12 h as described (12).*

RNA, if labeled with [^{3}H]adenosine (not shown). The degree of RNase resistance in this RNA species varied with different preparations, so this fraction of RNA is likely to include RNA species carrying poly(A) segments which are much shorter than those of the RNA fraction eluting from oligo(dT)-cellulose only at low ionic strength (fraction III, Fig. 3). We conclude that 60 to 70*S* viral RNA contains 30 to 40*S* RNA subunits with poly(A) (~65%), some without poly(A) (~30%) and a few subunits (5 to 10%) with short segments of poly(A).

The sizes of 30 to 40*S* viral RNA subunits which lack poly(A) (fraction I, Table 3, Fig. 5) were compared to the 30 to 40*S* RNA subunits which contain poly(A) (fraction III, Table 3, Fig. 3) by simultaneous electrophoresis in formamide-polyacrylamide gels. It can be seen in Fig. 6A and B that 30 to 40*S* [^{3}H]- or [^{32}P]RNA with poly(A) migrated a little slower than 30 to

TABLE 3. *Oligo(dT)-cellulose column binding of RSV RNA*[a]

Experiments and RNA preparations	Labeling	60 to 70S RNA			30 to 40S RNA			18S rRNA[b]		
		% Eluted			% Eluted			% Eluted		
		I	II	III	I	II	III	I	II	III
I[c]	[^{3}H]U	5.7	4.3	90.0	18.0	24.0	58.0	86.0	5.6	8.4
	^{32}P	5.0	4.0	91.0	14.0	18.0	68.0	87.0	5.8	7.2
II	[^{3}H]A	5.4	3.6	91.0	17.6	15.7	67.0			
III	[^{3}H]A				29.5	8.5	62.0			
IV	^{32}P				25.0	13.0	62.0			
V[d]	^{32}P				21.8	5.2	73.0			
Average		5.4	4.0	90.7	21.0	14.1	65.3	86.5	5.7	7.8

[a] Purified RSV 60 to 70S or 30 to 40S RNAs were subjected to oligo(dT)-cellulose column fractionation as described in Materials and Methods and for Fig. 3. An elution profile of 30 to 40S is shown in Fig. 3. Percentages of radioactivity eluting at high, intermediate, and low ionic strength were termed I, II, and III, respectively, and correspond to the elution profile shown in Fig. 3.

[b] [^{3}H]uridine or ^{32}P-labeled 18S rRNAs were used as internal controls in the elution of 60 to 70S RSV RNA in Exp. 1.

[c] [^{3}H]uridine and ^{32}P were added to different dishes of the same virus-infected culture and the RNAs were isolated in parallel. These differentially labeled RNAs were used for comparison of their size (Fig. 4 and 5).

[d] Fractionated 30 to 40S RNAs of this experiment were used for fingerprint analysis (Fig. 7).

40S [^{3}H]- or [^{32}P]RNA without poly(A). An 18S rRNA marker is included in these experiments; in addition, a shoulder is seen at the leading edge of each peak, this is thought to be 30 to 40S RNA of size class *b* (see above). A control experiment shows that poly(A) containing 30 to 40S [^{3}H]- and [^{32}P]RNA coincide upon electrophoresis in the same conditions (Fig. 6C). It appears then, that in agreement with the experiments described above in which poly(A) was removed enzymatically from 30 to 40S RNA, the presence of poly(A) reduces slightly the electrophoretic mobility of 30 to 40S RNA.

The existence of 30 to 40S RNA subunits with and without poly(A) in 60 to 70S tumor virus RNA observed here and by others (20, 23), raises the question of whether poly(A)-containing and nonpoly(A)-containing RNAs represent genetically different fractions of the RNA. To test this the fingerprint patterns of RNase T_1-resistant oligonucleotides of the two types of 30 to 40S RNA were compared. It can be seen in Fig. 7 that, except for the absence of the poly(A) spot (lower right corner in Fig. 7A) in the poly(A) lacking 30 to 40S RNA, the fingerprints of poly(A)-containing and poly(A)-lacking 30 to 40S RNA species are identical. Therefore, we conclude that the 30 to 40S RNA species with and without poly(A) stretch are genetically identical. This conclusion is compatible with the finding that the complexity of avian tumor virus RNA is on the order of 3.5×10^6 daltons, which is the approximate complexity of one 30 to 40S subunit only (K. Beemon, P. Duesberg, and P. Vogt, Proc. Nat. Acad. Sci. U.S.A., in press).

Locations of some RNase T_1-resistant oligonucleotides and of poly(A) appear to be the same in all 30 to 40S RNAs. The above experiments and those of others (43) indicate that poly(A) is located at or near the 3' end of 30 to 40S RSV RNA. If the heteropolymeric sequences of 30 to 40S RNA all had the same location on the polynucleotide relative to poly(A), instead of being circularly permuted, poly(A)-tagged natural or artifically produced fragments would be very useful for sequence analyses of viral RNAs. It would be expected, for example, that if the heteropolymeric sequences of all 30 to 40S RNAs are located identically relative to poly(A), the complexity of a poly(A)-tagged fragment of 30 to 40S RNA should be directly proportional to the size of that fragment. However, if the RNA sequences are extensively permuted the complexity of a poly(A)-tagged fragment must not be less than that of the total RNA. To distinguish between these alternatives we have alkali-degraded 30 to 40S [^{32}P]RNA into fragments sedimenting broadly with a peak of 11S (36), corresponding to an average size of about 240,000 daltons (41). The poly(A) containing fragments of the degraded RNA were isolated by binding and subsequent elution from Millipore filters (Materials and Methods). The recoveries ranged between 5 to 10% in different experiments. This is expected if one considers that an 11S fragment of 240,000 daltons corresponds roughly to 5 to

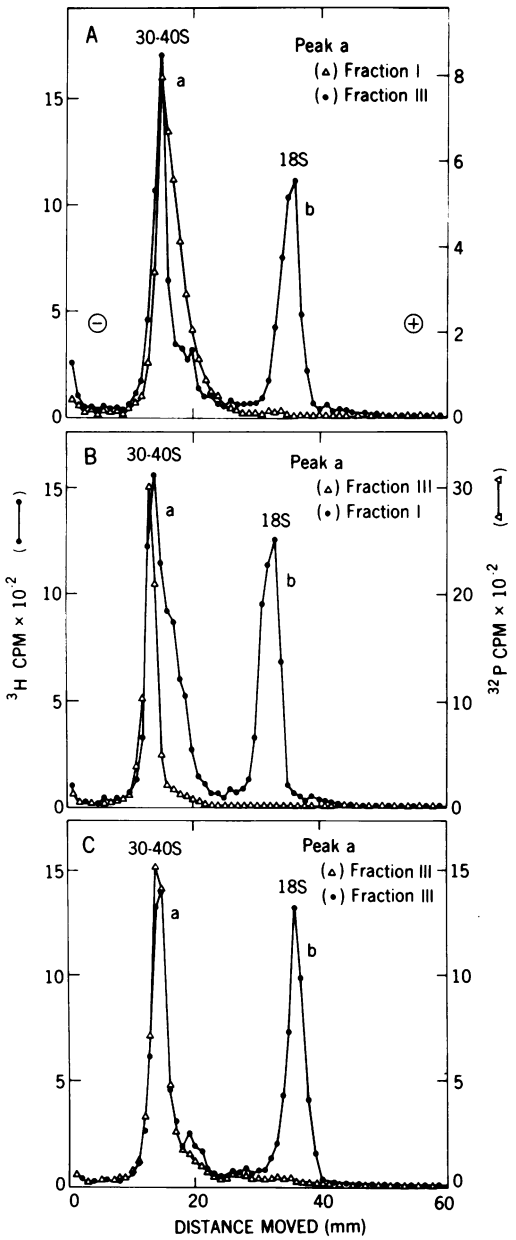

FIG. 6. *Comparison of the size of poly(A)- and non-poly(A)-containing 30 to 40S RSV RNA isolated from 60 to 70S viral RNA by formamide gel electrophoresis. 30 to 40S RNA without poly(A) was prepared by elution from oligo(dT)-cellulose at high ionic strength (fraction I, Fig. 3) and 30 to 40S with poly(A) was that which eluted at low ionic strength (fraction III, Fig. 3). Portions of 30 to 40S RNA from oligo(dT)-cellulose fractions I and III were mixed together with 18S rRNA and analyzed by formamide gel electrophoresis as described for Fig. 5. (A) 30 to 40S RSV RNA without poly(A) (fraction I) and 30 to 40S RNA with poly(A) (fraction III). (B) Different preparations of the same two species of 30 to 40S RSV RNA as in (A). (C) Two preparations of 30 to 40S RSV RNA with poly(A). Peak a represents 30 to 40S RSV RNA. Peak b is the 18S [^{3}H]rRNA added as an internal standard.*

10% of a 30 to 40S RNA of 3.5×10^6 daltons.

We have compared the complexity of these poly(A)-tagged fragments of 30 to 40S RNA to that of undegraded 30 to 40S RNA by fingerprinting. It can be seen in Fig. 7C that the number of RNase T_1-resistant oligonucleotides, and thus the complexity of poly(A) containing 11S fragments of PR-B RNA, is significantly lower than that of 30 to 40S RNA (Fig. 7A). Many large oligonucleotides seen in the fingerprint of 30 to 40S RNA are completely absent from the pattern of poly(A)-tagged 11S RNA fragments. These are thought to be oligonucleotides located most distantly from the poly(A) stretch on the 30 to 40S RNA. Some oligonucleotides marked by letters in Fig. 7A and C are present at high and some at intermediate concentrations relative to the total counts per minute on the fingerprint or to the counts per minute of the poly(A) spot (Table 4). These are thought to be located at or near the poly(A) stretch of 30 to 40S RNA. Varying relative concentrations of oligonucleotides are expected from the heterogenous size distribution of the poly(A)-tagged fragments used to prepare the fingerprint pattern. Since fragments of larger size would contribute oligonucleotides not present in smaller fragments. We conclude that some, perhaps all, RNase T_1-resistant sequences of tumor virus RNA have the same location relative to the poly(A).

It should then be possible to map the position of large oligonucleotides relative to that of poly(A) on the 30 to 40S polynucleotide by comparing the mass ratios of a given oligonucleotide to the total RNA or to that of poly(A) in the fingerprint patterns derived from intact poly(A)-tagged 30 to 40S RNA and poly(A)-tagged fragments of RNA. For example, it can be deduced from the data in Table 4 that spot a and b must be more distant from the poly(A) stretch than spot c.

DISCUSSION

The experiments described here confirm and extend earlier studies on poly(A) in tumor virus RNA (16, 17, 23) and suggest that two-thirds of the 30 to 40S subunits of 60 to 70S RSV RNA contain a poly(A) stretch consisting of about 200 nucleotides at or near the 3' end of the RNA.

The finding that some 30 to 40S RNA sub-

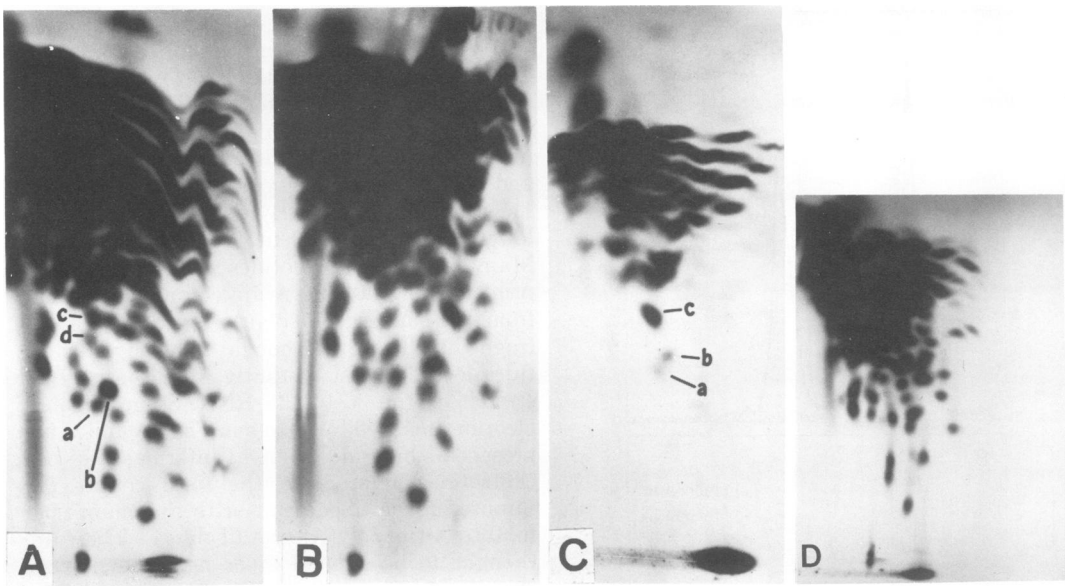

FIG. 7. *(A-O) Fingerprint analyses of 30 to 40S RSV RNA containing poly(A), (A); of 30 to 40S RSV RNA without poly(A), (B); and of poly(A)-tagged 11S fragments of 30 to 40S RSV RNA, (C); and of the 30 to 40S RNA of a transformation-defective PR RSV-B (D). 30 to 40S RSV RNA without poly(A) was prepared by elution from oligo(dT)-cellulose at high ionic strength and 30 to 40S RSV RNA with poly(A) by elution at low ionic strength (see Fig. 3). The RNAs were exhaustively digested with RNase T_1 and the digests were analyzed by two-dimensional fingerprint analysis as described (K. Beemon, P. Duesberg, and P. Vogt, Proc. Nat. Acad. Sci. U.S.A., in press). An 11S, poly(A)-containing fragment of 30 to 40S RNA was prepared by incubating 30 to 40S RSV RNA in 50 μliters 0.05 M Na_2CO_3 (pH 10) at 50 C for 6 min (36). The solution was then mixed with 300 μliters of standard buffer containing 0.2% SDS and sedimented in the presence of an 18S [^3H]rRNA standard. A symmetrical peak sedimenting at 11S was obtained and ethanol precipitated. The pellet was redissolved and passed through a Millipore filter. Millipore-bound 11S RNA (5 to 10% of the 11S RNA applied) was eluted as described in Materials and Methods and fingerprinted as described for (A).*

TABLE 4. *Radioactivity[a] of certain oligonucleotides and of the total RNA from which it was derived after fingerprinting as shown in Fig. 7a and c*

Part of digest analyzed	60 to 70S RNA (Fig. 7a)		Poly(A) fragment (10S)		Poly(A) fragment (11S) (Fig. 7c)	
	Counts/min	%	Counts/min	%	Counts/min	%
Total	1.17×10^6	100	48,750	100	70,400	100
Poly(A)	15,560	1.33	8,140	16.7	9,190	13.1
Spot a	1,470	0.13	31	0.06	79	0.11
Spot b	2,900	0.25	48	0.10	108	0.15
Spot c	1,730	0.15	391	0.8	570	0.81
Spot d[b]	1,550	0.13				

[a] Quantitations were done according to the procedures described elsewhere (K. Beemon, P. Duesberg, and P. Vogt, Proc. Nat. Acad. Sci. U.S.A., in press).

[b] Another possible spot to corresponding spot c of Fig. 7c.

units exist which contain poly(A) while others lack poly(A), but appear otherwise identical, could reflect either heterogeneity of the 60 to 70S RNA population extracted from our virus preparation or subunit differences within the 60 to 70S complex. Since binding of RNA, both to Millipore filters and to oligo(dT)-cellulose, is thought to involve poly(A) sequences, and since over 90% of the 60 to 70S RNA binds to these substrates, it appears likely that most of the 30 to 40S RNA species without poly(A) are part of a 60 to 70S complex which includes other

subunits containing poly(A). If correct, this would be an independent argument in favor of the subunit structure of 60 to 70*S* RNA; it would imply that 60 to 70*S* complexes exist between poly(A)-containing and poly(A)-free 30 to 40*S* subunits. The phenomenon could not be explained in terms of a hypothesis which assumes that the 60 to 70*S* RNA is a conformational isomer of 30 to 40*S* RNA.

Results of three different experiments now suggest that the poly(A) of tumor virus RNA is at or near the 3′ end of the RNA. It was shown by two different end-group methods that adenosine is the 3′ terminal nucleotide of poly(A) as well as of viral RNA (2, 38, 43, and J. Keith, M. Gleason, and H. Fraenkel-Conrat, Proc. Nat. Acad. Sci. U.S.A., in press). Nevertheless, end-group analyses of macromolecules are subject to error, because only a very small fraction of the molecule is analyzed. However, these results are complemented by our observation that RNase H removes poly(A) from viral RNA without significantly reducing its size. The sum of these experiments suggests strongly that the poly(A) of 30 to 40*S* tumor virus RNA is at the 3′ end of the polynucleotide, as with other poly(A)-containing RNAs (31, 32, 40). There is no evidence for internal poly(A), because (i) one poly-(A) stretch of the size found in this study can account for all poly(A) associated with an average 30 to 40*S* RNA, (ii) 30 to 40*S* RNAs with and without poly(A) (either naturally occurring or after treatment with RNase H) have approximately the same size, (iii) the poly(A) of 30 to 40*S* tumor virus RNA was found to reside at a unique location relative to other heteropolymeric sequences of the RNA. However, preliminary experiments suggest that A-rich runs, other than the poly(A) of 200 nucleotides described above, exist in the RNA. This suggestion is based on the observation that 20 to 25% of all 11*S* fragments of 30 to 40*S* RNA were found to bind to oligo(dT)-cellulose, whereas only 5 to 10% of such fragments would be expected to bind if binding were solely due to poly(A)-tagged fragments of an RNA with an original size of around 3.5×10^6 (12).

Some, perhaps all, individual RNase T_1-resistant oligonucleotides appear to have the same relative location on all the 30 to 40*S* RNA molecules with respect to the position of poly(A), suggesting that individual 30 to 40*S* RNAs are not permuted with regard to sequences of the RNA. This is compatible with genetic experiments which indicate that certain markers of different avian tumor virus strains recombine at stable frequencies (J. Wyke, J. Bell, and J. Beamand, Cold Spring Harbor Symp. Quant. Biol., in press; P. K. Vogt, personal communication). Thus, the poly(A) should prove a useful marker for mapping oligonucleotides on the RNA.

Given a complexity of only 3.5×10^6 daltons for RSV RNA (K. Beemon, P. Duesberg, and P. Vogt, Proc. Nat. Acad. Sci. U.S.A., in press) and several (two to three) 30 to 40*S* subunits (H. Delius, P. Duesberg, and W. Mangel, Cold Spring Harbor Symp. Quant. Biol., in press) for 60 to 70S RNA it may be argued that the different sequences are distributed unevenly over several subunits. In this case one subunit may contain only one-half of all different sequences, but all of these sequences would be represented twice per 30 to 40*S* polynucleotide. The complexity of poly(A)-tagged fragments of 30 to 40*S* RNA would then not only be a function of their size but also of the number of subunits over which the different sequences of RSV RNA are distributed. The low complexity of the 10 to 11*S* fragments analyzed here (containing one to three large oligonucleotides out of about 20 to 30 per 30 to 40*S* RNA) argues against the possibility that the different sequences of RSV RNA are distributed repetitively over many subunits. Although our evidence does not exclude the possibility that sequences of RSV RNA are unequally distributed over two subunits, this is considered unlikely for other biological reasons. Nondefective sarcoma viruses containing 30 to 40*S* RNA of size class *a* segregate transformation-defective viruses which contain only 30 to 40*S* RNA of size class *b* at rather high frequencies (11, 29). If the sequences of RSV RNA were distributed over several different 30 to 40*S* subunits transformation defectives would be expected which contain both class *a* and class *b* RNA. This was never observed in many analyses of transformation-defective viruses (11; P. Duesberg and P. Vogt, unpublished observations).

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ADDENDUM IN PROOF

It was shown recently that the fingerprint pattern of the transformation-defective deletion mutant (11, 12, 29) of PR RSV-B lacks the two oligonucleotide

spots termed a and b in Fig. 7 but is otherwise indistinguishable from that of PR RSV-B RNA (Fig. 7; P. Duesberg and P. Vogt, unpublished observations). Moreover, the RNA of this deletion mutant, like that of other transformation-defective mutants (11, 12, 29) is about 12 to 15% shorter than that of the wild-type PR RSV-B (P. Duesberg and P. Vogt, unpublished observations). Thus, it appears that RNA sequences concerned with cell transformation and lacking in this transformation-defective mutant are located within 5 to 10% of the 3′ terminus of PR RSV-B RNA.

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