EVIDENCE THAT THE POLYADENYLIC ACID SEGMENT OF "35S" RNA OF AVIAN MYELOBLASTOSIS VIRUS IS LOCATED AT THE 3'-OH TERMINUS

Mary L. Stephenson, Jesse F. Scott and Paul C. Zamecnik

The John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston, Massachusetts 02114

Received September 7, 1973

Summary. By means of a periodate oxidation-[^{3}H]borohydride labeling technique, evidence has been obtained which indicates that most of the 3'-OH ends of the high molecular weight RNA of avian myeloblastosis virus are occupied by polyadenylic acid segments, approximately 30 residues long. Of the polyadenylic acid sequences released by endonucleolytic digestion of this high molecular weight RNA, at least 90 per cent have a 3'-OH terminus, and are thus terminally, and not internally, located.

It has been demonstrated or suggested by a number of groups that the viral RNA from Rauscher, avian myeloblastosis, feline leukemia (1,2), Moloney sarcoma (3), Rous sarcoma (4), visna, Mason-Pfizer agent (5), reo (6), eastern equine encephalitis, polio (7) and Sinbis (8) contain sequences of poly(A). Others have shown that mRNA, both viral (9-11) and non-viral (12-15), contains poly(A) segments, and evidence has been presented that the poly(A) of some of these mRNAs contains the 3'-OH terminus of the molecule (16,17). In the cases of the RNAs from poliovirus (18) and reovirus (19) there is evidence that the poly(A) segments also contain the 3'-OH termini. Until the present report, however, there has been no definitive evidence that high molecular weight oncogenic viral RNA terminated in a 3'-OH poly(A) sequence.

We have used a periodate-[^{3}H]borohydride labeling technique on both the total nucleoside of RNA hydrolysates and the 3'-OH terminus of the RNA in order to get ratios of the number and kind of total nucleosides compared with those at the 3'-OH terminus of the same molecule. We have previously reported (20) that the 3'-OH end of the "35s" RNA released from the 70s RNA isolated from AMV* contained in the plasma of infected chicks was largely adenosine. We

*Abbreviations: Me_2SO, dimethylsulfoxide; AMV, avian myeloblastosis virus; SDS, sodium dodecylsulfate.

here present evidence that all this adenosine is the 3'-OH terminus of a poly(A) segment and that at least 90% of the poly(A) segments isolated have a 3'-OH terminus.

MATERIALS AND METHODS

Avian myeloblastosis virus BAI Strain A was obtained through the courtesy of Dr. and Mrs. Joseph Beard via the Virus Cancer Program. Virus from approximately 500 ml of fresh unfrozen plasma from infected chicks was partially purified by differential centrifugation and the RNA was extracted by modifications of the method of Duesberg (21,22) as described by us earlier (20). A particular preparation described in detail here (540 ml) yielded a total of 86 A_{260nm} units. Viral 70s RNA was isolated from 5-30% sucrose gradients using a Spinco SW 41 Rotor. The 70s material from 6 gradients was pooled and reprecipitated. The yield of RNA was 25 A_{260nm} units. The 70s RNA (21 A_{260nm}) was dissolved in 80% Me_2SO containing 0.05 M Tris, pH 7.5, .005 M EDTA and applied to 80% Me_2SO, 0-20% sucrose gradients in order to isolate the "35s" material and free it of the 70s-associated 4s RNA. The Spinco SW 41 Rotor was used at 40,000 rpm for 48 hours at 25°. External markers of 28, 18 and 4s were used to estimate the s values of the viral RNA. All of the material was pooled except that traveling in the 4s region of the gradient. The pooled material did not give a sharp peak at 35s but consisted of a broad peak ranging from about 10-35s. The pooled samples from four gradients containing 2 µl diethylpyrocarbonate per ml were made 1 M in LiCl by addition of 4 M LiCl, and were precipitated by the addition of 2.5 volumes of 95% ethanol. The RNA was dissolved and reprecipitated as before. Yield: 11 A_{260nm} units. For brevity the pooled RNA sample has been termed "35s" RNA, but it should be emphasized that all the RNA from the 70s material except that in the 4s region has been included (whether or not it is nicked "35s" RNA or other viral RNA), since Monier *et al.* (23) have reported runs of poly(A) in AMV in the more slowly sedimenting areas of sucrose gradients. Poly(A) was isolated from the viral RNA fraction by the method of Mendecki *et al.* (16) with minor modifications. Both T_1 RNase, 140 units (Worthington) and pancreatic RNase, 20 µg (Worthington) were used per ml incubation mixture. The cloudy suspension obtained by the SDS extraction of the RNA retained by the filter was centrifuged for 10 min at 12,000 x g, and the clear supernatant containing the poly(A) segments was removed. Carrier DNA free of RNA was added (200 µg) in order to coprecipitate nucleic acids, which were precipitated by the addition of 2 volumes of alcohol. The poly(A):DNA precipitate was dissolved in 0.5 ml of 0.2 M NaCl and reprecipitated using 2.5 volumes alcohol. The final pellet was lyophilized to remove traces of alcohol and was then dissolved in 250 µl H_2O. A solution of DNA (200 µg in 250 µl H_2O) was carried through all subsequent operations as a "blank" control.

To determine the 3'-OH terminal nucleoside of the viral RNA we have used the periodate oxidation-[^{3}H]borohydride reduction procedures perfected for microanalytical analysis by the Randeraths (24-26) following earlier procedures (27-29) which have also recently been modified by others (30,31). The exposed cis-hydroxyl groups on the 3'-OH terminal nucleoside of an RNA chain are oxidized to dialdehydes with periodate and subsequently reduced with tritium-labeled borohydride, yielding an RNA containing labeled 3'-OH termini. Piperidine hydrolysis of the intact RNA yields the terminal nucleoside as a labeled trialcohol, thus identifying the terminal 3'-OH residue of the chain. By this procedure we have shown the major 3'-OH residue of "35s" RNA of AMV to be adenosine (20). The labeled nucleoside trialcohols of adenosine, cytidine, guanosine, and uridine are isolated by thin layer chromatography on cellulose plates, using unlabeled carrier trialcohols, while nucleotides

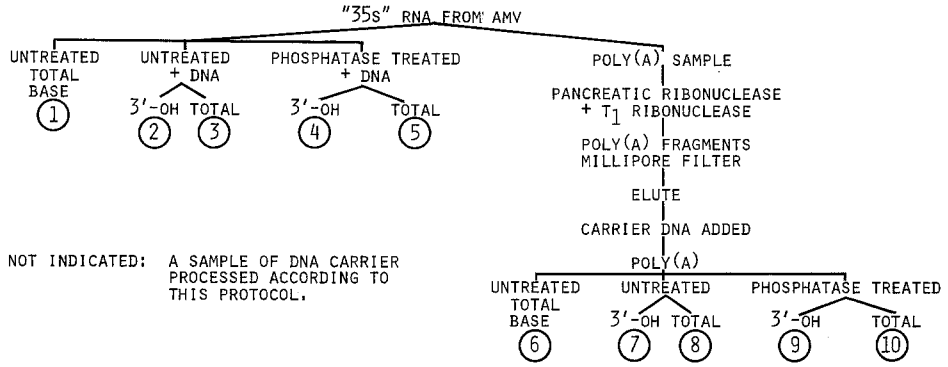

Figure 1. Schematic diagram of experimental plan to determine amount of poly(A) in 35s RNA from AMV.

remain at the origin under the conditions used. For the current studies we have used these conditions with the following modifications: DNA carrier was present in the RNA fraction, the periodate concentration was .005 M, and the phosphate concentration was reduced from 0.1 to 0.015 M following addition of the [^{3}H]borohydride (Amersham 6.4 Ci/mmole).

Determination of total nucleosides in the RNA utilized this same method of the Randeraths (25,26). In this case the RNA was hydrolyzed enzymatically to nucleosides prior to labeling by the periodate-borohydride reaction. We have used this method recently to determine the minor base constituency of the tRNA of AMV (32,33).

In order to estimate the 3'-PO_4 termini, the RNA sample (ca. 2 A_{260nm} units) was incubated for 30 min at 37° with E. coli alkaline phosphatase, 2 µg, in 0.02 M ammonium acetate at pH 7.4, 0.001 M $MgCl_2$ in a total volume of 110 µl. This dephosphorylated sample was then treated as described above for the determination of 3'-OH termini and total base.

RESULTS AND DISCUSSION

This experiment described in detail is one of three experiments on high molecular weight RNA of AMV, all of which indicate the presence of poly(A) segments at the 3'-OH terminus. Figure 1 outlines the experimental plan by which the data given below were obtained. Table I indicates the results of such an experiment, giving the radioactivity found in the total nucleoside and the radioactivity in the 3'-OH terminal nucleoside of a separate aliquot of the same sample. The per cent of radioactivity in each nucleoside is also given. The samples actually counted were the nucleoside trialcohols. Table II utilizes the data from Table I to indicate some of the properties of the isolated poly(A) fragments.

The "35s" pooled sample has adenosine as the predominant 3'-OH terminal nucleoside. This is in agreement with our previously published results (20) and those of Glitz (34).

With regard to the poly(A) segments, it can be calculated from Table I that the poly(A) contains 0.65 per cent of the total "35s" nucleotides; and 2.5 per cent of the adenosine in "35s" is found in the poly(A). This amount of poly(A) is in moderate agreement with that found by Gillespie et al. using another technique (1). Our data show in particular additionally that poly(A) is attached to the 3'-OH end of the molecule and that most of the poly(A) in the "35s" RNA contains a 3'-OH terminus (Table II, 2C).

Table II gives an indication of the average size of the RNA molecules calculated from the ratios of 3'-OH ends to total nucleotides. The original control (II, 1A) has a calculated molecular weight of 1.75×10^6; however, following phosphatase treatment (II, 1B), the calculated molecular weight dropped to about 777,000, indicating that the "35s" RNA molecules as isolated had a few breaks. Since mainly U and C are liberated after treatment with phosphatase (Table I), the limited degradation of "35s" RNA is probably due to a RNase of the same specificity as that of RNase A. The broad peak isolated from the Me_2SO sucrose gradients had an apparent molecular weight in the vicinity of 800,000 and agrees with the estimation of 1.25 times as many phosphate termini as 3'-OH termini (Table II, 1C). Since we (unpublished data) and others (35,36) have obtained sharp 35s peaks using radioactive RNA precursors and brief incubation times of AMV myeloblasts in vitro, it is our suspicion that the 3'-PO_4 ends mentioned here represent breaks or scissions in 35s chains occurring during the life span of the virion in the chick plus subsequent handling procedures. The poly(A) segments are about 30 nucleotides long (Table II, 2A,B). We also arrived at a value of 42 nucleotides in poly(A) using data derived from independent chromatograms from which the percentages of total A in the "35s" and in poly(A) were obtained and multiplied by the average number of residues on the 35s RNA, assuming a molecular weight of 2.2×10^6.

Table I

Comparison of total and 3'-OH terminal nucleosides from AMV "35s" RNA and poly(A) fractions

RNA Sample		Untreated				Phosphatase Treated			
		Total		3'-OH End		Total		3'-OH End	
		CPM	Per Cent	CPM	Per Cent	CPM	Per Cent	CPM	Per Cent
"35s" RNA		③		②		⑤		④	
	A	53,500,000	26.9	33,600	84.3	56,800,000	28.5	58,200	64.8
	C	52,700,000	26.5	1,150*	2.9	44,500,000	22.4	16,700	18.6
	G	52,100,000	26.2	2,540	6.4	50,700,000	25.5	6,390	7.1
	U	40,500,000	20.4	2,560	6.4	46,900,000	23.6	8,510	9.5
	Total	199,000,000		39,900		199,000,000		89,800	
Poly(A) from "35s" RNA		⑧		⑦		⑩		⑨	
	A	1,310,000	100.0	44,900	98.8	1,290,000	98.5	42,500	89.9
	C	0*	0	0*	0	0*	0	1,580	3.4
	G	0*	0	537*	1.2	20,300	1.5	1,180*	2.5
	U	0*	0	0*	0	0*	0	1,190	4.2
	Total	1,310,000		45,400		1,310,000		46,500	

Certain points may be emphasized with regard to the data presented. First, they are consistent with the conclusion that all "35s" molecules of AMV RNA terminate at the 3'-OH end in a poly(A) segment. This conclusion is consonant with the observations of others (see Introduction) with regard to the location of the poly(A) segments in certain mRNAs and with the findings of Yogo and Wimmer (18) for poliovirus RNA and of Nichols et al. (19) for reovirus RNA. They are also consistent with our finding (unpublished) that the terminal adenosine residue labeled by the periodate-[^{3}H]borohydride treatment of the 35s RNA is not released as a nucleoside trialcohol by either RNase A or RNase T_1 and excludes one of the possibilities we considered earlier (20) that the molecule might end in a .....CpCpA$_{OH}$ sequence.

The second point is that the average poly(A) segment is short. The size of approximately 30 residues which we have determined is less than any so far reported (see Introduction) for poly(A) segments from viral RNA. It should be noted in this regard that inasmuch as the function of the poly(A)

Legend to Table I:

The counts indicated were obtained from individual two dimensional cellulose plates separating the four nucleoside trialcohols indicated by added carrier "cold" trialcohols visualized in UV light. Because of high blanks and spurious "hot" spots, great care was taken to cut out all the surrounding areas in addition to the known trialcohols. Approx. 120 samples were cut from each plate in order to determine the cpm in the trialcohols. The samples marked with * were too close to the average surrounding background samples for significance. Samples not so marked have an overall counting error of $\leq \pm 5\%$.

All counts have been corrected for the DNA blanks which were worked up in an identical manner using individual plates. These controls introduced corrections small but essential for estimating in the poly(A) samples the presence or absence of nucleosides other than adenosine. Aliquot sizes have been taken into account and also all cpm normalized to the total base #1 and #6 indicated in Fig. 1, which were from samples not precipitated following borohydride treatment. All other samples were precipitated from alcohol 5 times in order to wash the RNA free of [^{3}H]borohydride used for the 3'-OH determinations (20). The multiplication factors used to normalize these samples varied as follows: 2 and 3, 1.40; 4 and 5, 0.87; 7 and 8, 11.2; and 9 and 10, 2.5. The normalization factors were applied due to losses incurred during the reprecipitations. Circled numbers in this and in Table II indicate the samples from which the data were obtained, as shown in Fig. 1. Each circled number represents a separate experimental work-up from periodate oxidation through chromatographic and counting procedures.

Table II

Approximate size of the "35s" RNA and the poly(A) isolated from it
(Data taken from Table I)

1 Original "35s"

A. Control

$$\frac{\text{Total nucleotides } ③}{\text{Total ends } ②} = \frac{199,000,000}{39,000} = 4,990 \text{ nucleotides x } 350 \text{ (ave. wt. per nucleotide)} = 1.75 \times 10^6 \text{ ave. mol. wt.)}$$

B. Phosphatase treated control

$$\frac{\text{Total nucleotides } ⑤}{\text{Total ends } ④} = \frac{199,000,000}{89,800} = 2,220 \text{ nucleotides x } 350 = 777,000 \text{ ave. mol. wt.}$$

C. Since phosphatase-treated ends contain the control ends also:

$$\frac{\text{Phosphatase-treated ends } ④ - \text{Control ends } ②}{\text{Control ends } ②} = \frac{89,800 - 39,900}{39,900} = 1.25$$

$$\frac{\text{Phosphatase-treated terminal A } ④ - \text{Control terminal A } ②}{\text{Control terminal A } ②} =$$

$$\frac{58,200 - 33,600}{33,600} = 0.74$$

2 Poly(A) Isolated from "35s"

A. Control

$$\frac{\text{Total nucleotides } ⑧}{\text{Total ends } ⑦} = \frac{1,310,000}{45,400} = 28.9 \text{ nucleotides x } 350 = 10,100 \text{ ave. mol. wt.}$$

$$\frac{\text{Total adenosine } ⑧}{\text{Total terminal adenosine } ⑦} = \frac{1,310,000}{44,900} = 29.2 \text{ nucleotides x } 350 = 10,200 \text{ ave. mol. wt.}$$

B. Phosphatase treated poly(A)

$$\frac{\text{Total nucleoside } ⑩}{\text{Total ends } ⑨} = \frac{1,310,000}{46,500} = 28.1 \text{ nucleotides x } 350 = 9,860 \text{ ave. mol. wt.}$$

$$\frac{\text{Total adenosine } ⑩}{\text{Total terminal adenosine } ⑨} = \frac{1,290,000}{42,500} = 30.3 \text{ nucleotides x } 350 = 10,600 \text{ ave. mol. wt.}$$

C. Since phosphatase-treated ends contain the control ends also:

$$\frac{\text{Phosphatase ends } ⑨ - \text{Control ends } ⑦}{\text{Control ends } ⑦} = \frac{46,500 - 45,400}{45,400} = .02$$

$$\frac{\text{Phosphatase terminal A } ⑨ - \text{Control terminal A } ⑦}{\text{Control terminal A } ⑦} = \frac{42,500 - 44,900}{44,900} = -.05$$

remains to be defined, the basis for differences in estimates of length must await further study (5).

The third point is that less than 10% of poly(A) segments of a size retained by the Millipore filter technique are internal (i.e., 3'-PO_4 terminated). Nakazato et al. (13) have described a short poly(A) segment from heterogeneous nuclear RNA from HeLa which is 3'-PO_4 terminated, and Horst et al. (37) have produced evidence that the poly(A) segment of Rous sarcoma virus RNA is interrupted by non-A residues. Since phosphatase treatment of the poly(A) fraction we obtained did not change the apparent length of the segment, it would appear that the length we obtain is not related to either of the above observations. We cannot comment on the apparent low level of residues other than A which we found as part of the poly(A) segment because of the low level of radioactivity in those regions of the chromatograms.

Finally, this report is the first of which we know to provide evidence that the poly(A) segment of the high molecular weight RNA of an oncogenic virus (AMV) is located at the 3'-OH end of the molecule.

Acknowledgments. We are grateful to Dr. and Mrs. Joseph Beard and to The Virus Cancer Program of the NIH for providing the AMV. We wish to thank Dr. LeRoy Wirthlin for valuable discussions and Miss Sandra Svihovec and Miss Virginia Kuhns for capable assistance.

This work was supported by the National Cancer Institute Contract #NO1-CP-33366, Cancer Training Grant CA05018 of the National Institutes of Health, and Contract AT(30-1)2643 of the U.S. Atomic Energy Commission.

This is publication No. 1449 of the Cancer Commission of Harvard University.

REFERENCES

1. Gillespie, D., Marshall, S., and Gallo, R.C. (1972) Nature New Biol. 236, 227-231.
2. Verma, I.M., Meuth, N.L., and Baltimore, D. (1972) J. Virol. 10, 622-627.
3. Green, M., and Cartas, M. (1972) Proc. Nat. Acad. Sci. US 69, 791-794.
4. Lai, M.M.C., and Duesberg, P.H. (1972) Nature 235, 383-386.
5. Gillespie, D., Takemoto, K., Robert, M., and Gallo, R.C. (1973) Science 179, 1328-1330.
6. Shatkin, A.J., and Sipe, J.D. (1968) Proc. Nat. Acad. Sci. US 59, 246-253.
7. Armstrong, J., Edmonds, M., Nakazato, H., Phillips, B., and Vaughan, M. (1972) Science 176, 526-528.
8. Johnson, R.E., and Bose, H.R. (1972) Biochem. Biophys. Res. Commun. 46, 712-718.
9. Kates, J., and Beeson, J. (1970) J. Mol. Biol. 50, 19-33.
10. Philipson, L., Wall, R., Glickman, G., and Darnell, J.E. (1971) Proc. Nat. Acad. Sci. US 68, 2806-2809.

11. Soria, M., and Huang, A.S. (1973) J. Mol. Biol. 77, 449-455.
12. Lim, L., and Canellakis, E.S. (1970) Nature 227, 710-712.
13. Edmonds, M., Vaughan, M.H., and Nakazato, H. (1971) Proc. Nat. Acad. Sci. US 68, 1336-1340.
14. Darnell, J.E., Wall, R., and Tushsinski, R.J. (1971) Proc. Nat. Acad. Sci. US 68, 1321-1325.
15. Lee, Y., Mendecki, J., and Brawerman, G. (1971) Proc. Nat. Acad. Sci. US 68, 1331-1335.
16. Mendecki, J., Lee, Y., and Brawerman, G. (1972) Biochem. 11, 792-798.
17. Nakazato, H., Kopp, D.W., and Edmonds, M. (1973) J. Biol. Chem. 248, 1472-1476.
18. Yogo, Y., and Wimmer, E. (1972) Proc. Nat. Acad. Sci. US 69, 1877-1882.
19. Nichols, J.L., Bellamy, A.R., and Joklik, W.K. (1972) Virology 49, 562-572.
20. Stephenson, M.L., Wirthlin, LeR.S., Scott, J.F., and Zamecnik, P.C. (1972) Proc. Nat. Acad. Sci. US 69, 1176-1180.
21. Duesberg, P.H., and Robinson, W.H. (1965) Proc. Nat. Acad. Sci. US 54, 794-800.
22. Duesberg, P.H. (1968) Proc. Nat. Acad. Sci. US 59, 930-937.
23. Monier, F., Iida, K., and Harel, J. (1972) Biochimie 54, 1237-1244.
24. Randerath, K., and Randerath, E. (1969) Anal. Biochem. 28, 110-118.
25. Randerath, K., and Randerath, E. (1971) in Procedures in Nucleic Acid Research, Vol. II, pp. 796-812, Cantoni, G.L. and Davies, E.R., eds., Harper and Row, New York.
26. Randerath, K., Yu, C.-T., and Randerath, E. (1972) Anal. Biochem. 48,172-198.
27. Whitfeld, P.R., and Markham, R. (1953) Nature 171, 1151-1152.
28. Yu, C.-T., and Zamecnik, P.C. (1960) Biochim. Biophys. Acta 45, 148-154.
29. Khym, J.X., and Cohn, W.E. (1960) J. Am. Chem. Soc. 82, 6380-6386.
30. Rajbhandary, U.L., Stuart, A., Faulkner, R.D., Chang, S.H., and Khorana, H.G. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 425-434.
31. Leppla, S.F., Bjoraker, B., and Bock, R.M. (1968) Methods Enzymol. Part B 12, 236-240.
32. Randerath, K., Rosenthal, L.J., and Zamecnik, P.C. (1971) Proc. Nat. Acad. Sci. US 68, 3233-3237.
33. Rosenthal, L.J., and Zamecnik, P.C. (1973) Proc. Nat. Acad. Sci. US 70, 865-869.
34. Ahmad, M.S., Markham, P.D., and Glitz, D.G. (1972) Biochim. Biophys. Acta 281, 554-563.
35. Erikson, R.L. (1969) Virology 37, 124-131.
36. Canaani, E., and Duesberg, P. (1972) J. Virol. 10, 23-31.
37. Horst, J., Keith, J., and Fraenkel-Conrat, H. (1972) Nature New Biol. 240, 105-109.