NUCLEOTIDE SEQUENCES IN THE RNA OF MAMMALIAN LEUKEMIA AND SARCOMA VIRUSES

Roy H. L. Pang and Leo A. Phillips

Viral Leukemia and Lymphoma Branch, National Cancer Institute,
National Institutes of Health, Bethesda, Maryland 20014

Received September 22,1975

SUMMARY: The nucleotide sequences in viral RNA from purified murine sarcoma and hamster leukemia viruses (S+H+) from HTG-1 cells and Rauscher leukemia virus (RLV) from JLS-V 9 cells have been examined by polynucleotide agarose affinity chromatography. There is at least one copy of poly(A) sequences per genomic viral RNA molecule. After heat denaturation of genomic viral RNA (S+H+), there are two types of viral subunits for 34S and 28S species: one that contains poly(A) sequences and one that does not. There are no detectable poly(U) tracts in the viral RNA. However, poly(C) sequences and poly(G) tracts were detected in viral RNA, although less poly(G) than poly(C) tracts were observed. In addition, heat-denatured genomic viral RNA has a greater affinity for poly(G) agarose column than native genomic viral RNA.

Poly(A) sequences have been detected in the viral RNA of a large number of viruses (1-10). In addition, poly(C) tracts have been reported in encephalomyocarditis virus (11) and foot and mouth disease virus (12). Marshall and Gillespie reported the absence of poly(U) sequences in oncornavirus RNA by hybridization (13). In addition, Gillespie et al. detected little or no poly(G) tracts in the RNA of oncornaviruses (14). The present investigation was undertaken to detect the presence of polynucleotide sequences in the viral RNA of mammalian oncornaviruses by agarose affinity chromatography. In this communication, we present evidence for the presence of poly(A), poly(C), and poly(G) sequences in the viral RNA of murine sarcoma and hamster leukemia viruses (S+H+) from HTG-1 cells and Rauscher leukemia virus (RLV) from JLS-V 9 cells.

MATERIALS AND METHODS

Cell lines. HTG-1 cells, producing S+H+ viruses and JLS-V 9 cells, infected with RLV, were used for the present studies. Cells were maintained as described (7).

Radioactive labeling of viruses. Viruses were labeled with either [^{3}H]-nucleotides as indicated or with $H_3{}^{32}PO_4$ (25-50 μCi/ml) for 3 hr and purified as described (7).

Extraction of high molecular weight viral RNA. Labeled viral RNA was extracted with sodium dodecyl sulphate and phenol-chloroform mixtures as described (10). High molecular weight viral RNA was fractionated in 15 to 30% isokinetic sucrose gradients (7). Specific radioactivity of tritiated viral RNA from S+H+ viruses and RLV was ca. 2000 cpm/µg and 1000 cpm/µg respectively.

Viral RNA dissociation and enzymatic digestion. Genomic viral RNA was dissociated into subunits by incubation at 75°C for 3 min. For enzymatic digestion, genomic viral RNA was incubated with either RNase T1 at a final concentration of 20 units/ml or RNase A at a final concentration of 25 µg/ml at 37°C for 30 min.

Detection of polynucleotide sequences by agarose affinity chromotography. The procedures of Lindberg and Persson were followed (15, 16). Two cm high polynucleotide agarose (P/L Biochemicals, Inc.) were packed in 15.5 x 0.8 cm glass columns. The agarose was first washed with 2 x 5 ml elution buffer (EB), consisting of 90% formamide, 0.01 M EDTA and 2% sarkosyl NL-97 (Geigy Industrial Chemicals) in 0.05 M potassium phosphate buffer, pH 7.5. Then, the column was washed with 2 x 5 ml concentrated salt buffer (CSB), consisting of 25% formamide, 0.01 M EDTA and 0.7 sodium chloride in 0.05 M Tris-HCl, pH 7.5. Samples for analysis consisted of RNA as indicated, 0.1 ml sample solution (1% sarkosyl NL-97 and 0.03 M EDTA), 0.8 ml CSB and 1 x STE buffer, consisting of 0.1 M NaCl, 0.001 M EDTA and 0.05 M Tris-HCl, pH 6.8 (7), if necessary, to a final volume of 1 ml. For the preparation of polynucleotide samples, the appropriate amount of [^{3}H]-or [^{32}P]-polynucleotide and 10 µg respective cold polynucleotide in 0.2 ml distilled water was mixed with 0.8 ml CSB. Fifty to 100 µl of sample were used to determine the total radioactivity. The rest was allowed to pass through polynucleotide agarose column. The column was first washed with 6 x 1 ml CSB and then with 6 x 1 ml EB. Radioactive samples were either counted directly in 15 ml Aquasol (New England Nuclear) or CCl_3COOH precipitated, millipored and counted in 10 ml Butyl-PBD-PBBO (7). A minimum of 5000 cpm was used for each experiment. Chromatography was carried out at either 4°C or 25°C. There was no detectable difference in the binding of viral RNA to polynucleotide agarose at the two temperatures indicated. Over 90% of the viral RNAs applied to the columns was recovered. All data shown are the averages of at least three experiments.

RESULTS

Sizes of genomic viral RNA and viral RNA subunits. The profiles of genomic and subunit viral RNA from purified S+H+ viruses from HTG-1 cells were obtained by rate zonal sedimentation in 15 to 30% isokinetic sucrose gradients as indicated (Fig. 1). Genomic viral RNA peaked at 50±6S; whereas subunits peaked at 28±4S. However, the single 28±4S peak observed in these profiles could be resolved into three peaks of 34S, 28S, and 5S after longer centrifugation (Fig. 3).

Specificity of polynucleotide agarose. The specificity of polynucleotide agarose was tested by passing [^{3}H]polynucleotides through the agarose columns (Table 1). Over 90% of [^{3}H]poly(A), poly(C), poly(G), and poly(U) bound to

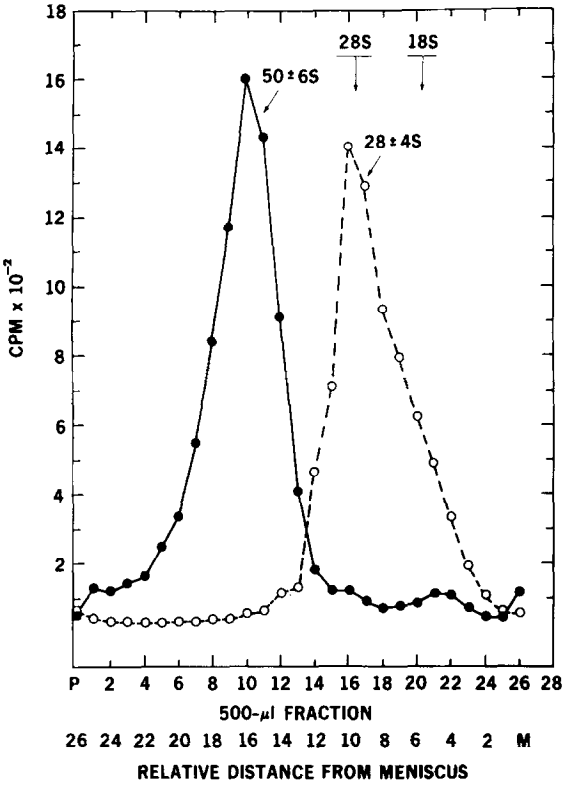

Figure 1. Sedimentation of genomic S+H+ viral RNA (●——●) and subunits (○——○), obtained by heat denaturation, in 15 to 30% isokinetic sucrose gradients. Centrifugation was carried out in SW 41 at 12°C for 210 min. at 40,000 rpm. Cytoplasmic [^{32}P]RNA from 3T3 cells served as markers.

Table 1. Specificity of polynucleotide agarose affinity columns

	Percent binding[1]			
Agarose	Poly(A)	Poly(C)	Poly(G)	Poly(U)
Poly(A)	0.5	0.6	2.5	97.0
Poly(C)	0.9	0.2	91.9[2]	0.3
Poly(G)	2.5	98.2[2]	2.3[3]	0.5
Poly(U)	93.1	0.2	1.1	0.4

[1][^{3}H]nucleotides was allowed to pass through various polynucleotide agarose columns and the amount of labels bound to the respective columns was determined as described in the text.

[2]The percent binding represented the amount of labels that could not be washed from the polynucleotide agarose columns by CSB.

[3][^{32}P]poly(G), synthesized as described previously (19), was used for binding study.

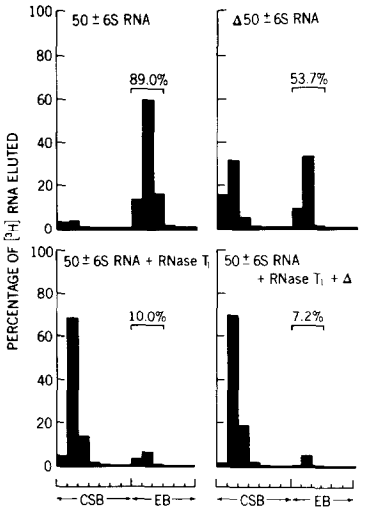

Figure 2. Genomic viral RNA (S+H+) and subunits obtained as indicated, were chromatogrammed by poly(U) agarose columns as described in the text.

poly(U), poly(G), poly(C), and poly(A) agarose respectively. However, only 50% of poly(G) and poly(C) could be eluted from poly(C) and poly(G) agarose columns respectively even at 90°C. Other polynucleotides did not have any appreciable binding to their respective polynucleotide agarose columns, indicating specific binding of the polynucleotide agarose for the substrate.

Detection of poly(A) sequences. In order to detect the presence of poly(A) sequences, viral RNA was allowed to pass through poly(U) agarose columns. Ninety percent of [2,8 ^{3}H-adenosine]labeled viral RNA from S+H+ viruses bound to poly(U) agarose, indicating that there is at least one copy of poly(A) sequences per genomic viral RNA molecule (Fig. 2). After incubation at 75°C for 3 min, 54% of the label bound to the poly(U) agarose columns; whereas after RNase T_1 treatment, only 10% bound to the columns. Degradation of genomic viral RNA was not observed after passing through poly(U) agarose columns at 4°C.

Determination of sizes of viral subunits. The sizes of viral RNA subunits that did and did not bind to poly(U) agarose columns were determined in 15 to 30% isokinetic sucrose gradients (Fig. 3). There were three major peaks of 34S, 28S, and 5S viral RNA after heat denaturation of genomic

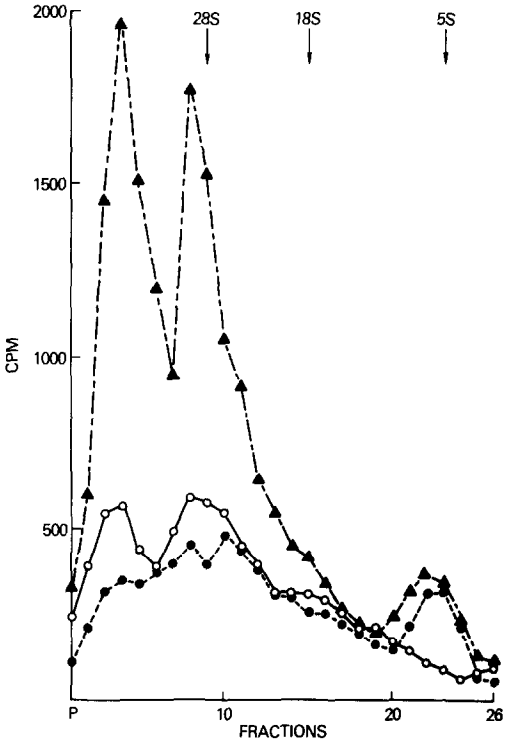

Figure 3. Sedimentation of heat-denatured S+H+ viral RNA (▲——▲) and poly(U) agarose fractionated heat-denatured viral RNA: CSB fraction (●——●) and EB fraction (○——○) in 15 to 30% isokinetic sucrose gradients. Centrifugation was carried out in SW41 rotor at 12°C for 16 hr at 27,000 rpm. Cytoplasmic [^{32}P]RNA from 3T3 cells served as markers.

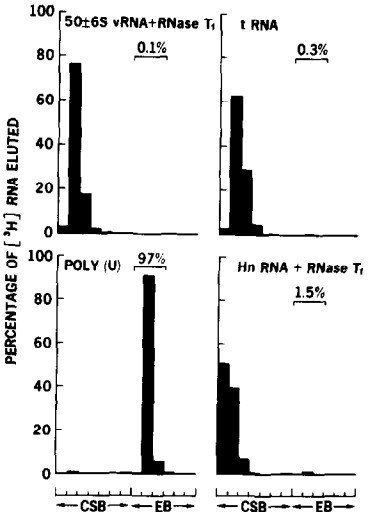

Figure 4. S+H+ viral RNA, labeled with [^{3}H]uridine, was chromatogrammed by poly(A) agarose as described in the text. [^{3}H]poly (U) and HnRNA served as positive controls. A minimum of 20,000 cpm was used in each experiment.

Table 2. Poly(G) agarose chromatography of viral RNA[1]

Nucleic acid	Treatment[2]	Percent binding ^{32}P	Percent binding ^{3}H
50±6S vRNA (S+H+)	None	11.8	18.5
50±6S vRNA (S+H+)	Δ	16.3	28.8
50±6S vRNA (S+H+)	RNase T_1	15.3	11.2
50±6S vRNA (S+H+)	RNase A	4.8	1.0
5S±cytoplasmic RNA (3T3-IC-19)	None	-[3]	1.7
t-RNA (*E. coli*)	None	-	2.0

[1]Viral RNA was chromatogrammed by Poly(G) agarose columns as described in the text.

[2]Viral RNA labeled with either $H_3{}^{32}PO_4$ or [2,8-^{3}H-adenosine] was incubated at 75°C for 3 min. or with either RNase T_1 at a final concentration of 20 units/ml or RNase A at a final concentration of 25 µg/ml at 37°C for 30 min. as indicated.

[3]Not done.

viral RNA. Both fractions that did and did not bind to poly(U) agarose contained 34S and 28S subunits, indicating that there are two types of 34S and 28S viral RNA subunits; one that contains poly(A) sequences and one that does not. However, 5S RNA fragments were only detected in the fraction which did not bind to poly(U) agarose column, indicating that the 5S fragments detected after heat denaturation of genomic viral RNA were not poly(A) sequences cleaved from viral RNA subunits.

Absence of poly(U) sequences in viral RNA. After RNase T_1 digestion, genomic viral RNA, labeled with [^{3}H]uridine, did not bind to poly(A) agarose (Fig. 4). On the other hand, after RNase T_1 treatment, heterogenous nuclear RNA (HnRNA), which contains oligo(U)-rich regions (17), bound to the poly(A) agarose columns. In addition, neither native nor heat-denatured genomic viral RNA, not shown, bound to the columns.

Detection of poly(C) sequences in viral RNA. Genomic viral RNA and viral RNA subunits bound to poly(G) agarose columns, regardless of the labels used (Table 2). In addition, in both instances using [^{32}P] labeled or [^{3}H] labeled RNA, heat-denatured viral RNA subunits have a greater affinity for poly(G) agarose columns than native genomic viral RNA. For example, 11.8%

Table 3 Poly(C) agarose chromatography of viral RNA[1]

Nucleic acid	Treatment	Percent binding
50±6X vRNA (S+H+)	None	8.6
50±6S vRNA (S+H+)	Δ	4.4
50±6S vRNA (S+H+)	RNase T_1	0.3
t-RNA (*E. coli*)	None	0.9

[1]Viral RNA was chromatogrammed by poly(C) agarose columns as described in the text.

[2]Viral RNA, pulsed-labeled with [2,8-^{3}H-adenosine] for 3 hr, was heat-denatured at 75°C for 3 min. or incubated with RNase T_1 at a final concentration of 20 units/ml for 30 min. at 37°C as indicated.

genomic viral [^{3}H]RNA bound to poly(G) agarose; whereas 16.3% heat-denatured viral RNA bound to poly(G) agarose. After RNase T_1 treatment, [^{3}H]- and [^{32}P] labeled viral RNA bound to poly(G) agarose columns, 11.2% and 15.3% respectively. However, after RNase A digestion, the binding to poly(G) agarose is negligible. Degradation of viral RNA was observed after passing through poly(G) agarose.

Detection of poly(G) sequences in viral RNA. About 8.6% genomic viral RNA and 4.4% subunit viral RNA, obtained by heat denaturation, bound to poly(C) agarose columns, indicating the presence of poly(G) sequences or (G)-rich regions (Table 3). After digestion by RNase T_1, viral RNA did not bind to poly(G) agarose columns.

Detection of Polynucleotide Sequences in RLV RNA. In order to test that the detection of poly(A), poly(C), and poly(G) sequences is not unique to viral RNA from purified S+H+ viruses from HTG-1 cells, [^{3}H] labeled viral RNA from purified RLV from JLS-V 9 cells was chromatogrammed by polynucleotide agarose columns (Table 4). Both genomic and subunit viral RNA bound to poly(C), poly(G), and poly(U) agarose columns, indicating the presence of poly(G), poly(C), and poly(A) sequences. In addition, as in the case of viral RNA from S+H+ viruses, viral RNA subunits of RLV, obtained by heat denaturation, had a greater binding affinity for poly(G) agarose than genomic viral RNA, 34.2% and 25.2% respectively. Chromatograms

Table 4. Polynucleotide agarose chromatography of RLV(JLS-V9) viral RNA[1]

Agarose	Percent binding			
	Genomic	Δ	RNase T_1	RNase A
Poly(A)	1.9	1.2	0.7	-[1]
Poly(C)	22.4	12.4	2.0	-
Poly(G)	25.2	34.8	11.9	1.8
Poly(U)	81.7	35.7	5.0	-

[1][2,8-^{3}H-adenosine] labeled viral RNA was chromatogrammed as described in the text. Treatments of viral RNA were the same as described in Table 3.
[2]Not done.

of [^{32}P] labeled viral RNA, not shown, indicated comparable results.

DISCUSSION

Both S+H+ viruses from HTG-1 cells and RLV from JLS-V 9 cells contain poly(A) sequences in the viral RNA as determined by polynucleotide agarose affinity chromatography. The detection of 34S and 28S viral RNA subunits in fractions which did and did not bind to poly(U) agarose columns indicates that there are two types of viral RNA subunits for 34S and 28S species: one that contains poly(A) sequences and one that does not contain poly(A) sequences. Ihle et al. also reported the detection of 34S subunits which contained no poly(A) regions higher than 20 nucleotides in addition to poly(A)-containing subunits in RNA tumor virus from spontaneously activated AKR cells, Moloney leukemia virus, and RD-114 virus (19). The fact that 5S poly(A) fragments could not be detected suggests that the detection of nonpoly(A) containing 28S and 34S subunits may not result from the cleavage of poly(A) sequences from poly(A) containing subunits by heat denaturation. However, we have not determined whether or not there are homologous sequences in the two RNA subunit species by hybridization.

Clearly, in S+H+ viruses from HTG-1 cells and RLV from JLS-V 9 cells, there is no detectable poly(U) sequences. This is consistent with the previous report that poly(U) tracts were not detected in the RNA of reovirus,

oncogenic viruses and nononcogenic viruses by hybridization (13). This also rules out the possibility of the contribution of poly(A):poly(U) bonding to the secondary structure in viral RNA. The fact that binding to poly(C) and poly(G) agarose columns is labile after RNase digestion indicates that poly(C) and poly(C) tracts are present in the viral RNA. In addition, the presence of both poly(C) and poly(G) tracts was further substantiated by binding to poly(G) and poly(C) glass filters respectively. We have not, however, rules out completely the possibility that we are detecting (C)-rich and (G)-rich regions. Nevertheless, we have shown that viral RNA subunits, obtained by heat denaturation or enzymatic digestion of genomic viral RNA, can be fractionated in ng amount by polynucleotide agarose affinity chromatography. This will facilitate further studies of the viral RNA subunits and fragments. Investigation is currently underway to determine the size of poly(C) and poly(G) sequences.

We have observed (unpublished data) that poly(U) agarose columns could detect riboadenylates with chain lengths between $A_{\overline{5}}$ and $A_{\overline{25}}$ and that thermal elution profiles of [^{32}P]riboadenylates from poly(U) agarose columns indicated higher Tm with increasing nucleotide lengths. This may explain why only 50% of [^{3}H]poly(C) and [^{3}H]poly(G) could be eluted from poly(G) and poly(C) agarose respectively even at 90^{o}C; whereas over 90% of viral RNA, containing poly(C) and poly(G) sequences, could be eluted from the same agarose columns at much lower temperature. Presumably, the nucleotide lengths of poly(C) and poly(G) detected in the viral RNA are much shorter than those used in the control experiments.

The detection of both poly(C) and poly(G) sequences and the fact that heat-denatured viral RNA has a greater affinity to bind to poly(G) agarose columns than genomic viral RNA indicate that interstrand or intrastrand poly(C):poly(G) bonding may play a role in the secondary structure of the genomic viral RNA.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Warren Jelinek of the Rockfeller Univeristy, New York, for the donation of tritiated HnRNA; Dr. Wen K. Yang of Oak Ridge National Laboratories, Oak Ridge, Tenn., for the supply of tritiated mammalian t-RNA; Dr. Howard Coyer of P/L Biochemicals, Inc., for technical data on polynucleotide agarose; Dr. Daniel K. Haapala for stimulating discussion; Dr. Peter J. Fischinger for critical evaluation of the manuscript and last but not least, James J. Park for excellent technical assistance. The initial stocks of poly(A), poly(C), and poly(G) agarose were gifts from P/L Biochemicals, Inc.

REFERENCES:

1. Yogo, Y., and Wimmer, E. (1973) Nature New Biol., 242, 171-174.
2. Johnson, R. E., and Base, H. R. (1972) Proc. Natl. Acad. Sci. USA, 69, 1514-1516.
3. Donaghue, T. P., and Faulkner, P. (1973) Nature New Biol., 246, 168-176.
4. Green, M., and Cartas, M. (1972) Proc. Natl. Acad. Sci. USA, 69, 791-794.
5. Lai, M. M. C., and Duesberg, P. H. (1972) Nature, 235, 383-386.
6. Ross, J., Tronick, S. R., and Scolnick, E. M. (1972) Virology, 49, 230-235.
7. Phillips, L. A., Hollis, V. W., Jr., Bassin, R. H., and Fischinger, P. J. (1973) Proc. Natl. Acad. Sci. USA, 91, 3002-3006.
8. Kates, J. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 743-752.
9. Nichols, J., Bellamy, A. R., and Joklik, W. K. (1972) Virology, 49, 562-572.
10. Phillips, L. A., Park, J. J., and Hollis, V. W., Jr. (1974) Proc. Natl. Acad. Sci. USA, 71, 4366-4370.
11. Porter, A., Carey, N., and Fellner, P. (1974) Nature, 248, 675-678.
12. Brown, F., Newman, J., Stott, J., Porter, A., Friby, D., Newton, C., Carey, N., and Fellner, P. (1974) Nature, 251, 342-344.
13. Marshall, S., and Gillespie, D. (1972) Nature New Biol., 240, 43-45.
14. Gillespie, D., Marshall, S., and Gallo, R. C. (1972) Nature New Biol., 236, 227-231.
15. Lindberg, U., and Persson, T. (1972) Eur J. Biochem., 31, 246-254.
16. Lindberg, U., Persson, T., and Phillipson, L. (1972) J. Virol., 10, 909-919.
17. Schmukler, M., Jewett, P. B., and Levy, C. C. (1975) J. Biol. Chem., 250, 2206-2212.
18. Molloy, G. R., Thomas, W. L., and Darnell, J. E. (1972) Proc. Natl. Acad. Sci. USA, 69, 3684-3688.
19. Ihle, J. N., Lee, K., and Kenny, F. T. (1974) J. Biol. Chem., 249, 38-42.