*THE NUCLEIC ACID FROM AVIAN MYELOBLASTOSIS VIRUS COMPARED WITH THE RNA FROM THE BRYAN STRAIN OF ROUS SARCOMA VIRUS**

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This paper describes properties of the nucleic acid isolated from the avian myeloblastosis virus (AMV) and compares this nucleic acid with the RNA from the Bryan strain of Rous sarcoma virus (RSV) and its helper virus, Rous associated virus (RAV). The Bryan strain of RSV is defective[1] and infected cells do not produce mature RSV unless simultaneously infected with an avian leukosis virus such as Rous associated virus (RAV) which functions as helper virus and presumably provides a protein coat for RSV.[2] For this reason all preparations of RSV contain a helper virus which is usually present in 4–10 times higher concentration[1, 3, 4] of infectious particles than RSV and which has coat properties indistinguishable from those of RSV. Because of the similar properties, RSV has not been separated from its helper virus.[4] A recent report from this laboratory[5] describes the purification of the mixture designated RSV + RAV and the isolation and preliminary characterization of the viral RNA.

Avian myeloblastosis virus, like RAV, is an avian leukosis virus and the two are closely related biologically. Both will function as helper viruses for RSV,[2, 6] interfere with RSV,[4] and produce tumors in chickens. They differ in specific antigens, and only AMV produces acute myeloblastic leukemia.[7] The intact virus particles have been shown to have similar morphology, chemical composition, and physical properties.[7, 8] Because of the close relation, it might be expected that the two would have similar nucleic acids. However, previous reports[9, 10] describe only low-molecular-weight RNA isolated from AMV, and each of several reports[11–13] reveals a different RNA base composition. In the present study we find the nucleic acids of AMV and RSV + RAV to be single-stranded RNA, indistinguishable in sedimentation behavior and change in conformation with change in salt concentration and very similar in base composition.

Methods and Materials.—Viruses: An AMV strain, kindly supplied by Dr. P. K. Vogt, belonging to the BAI strain A and to the B classification of Vogt,[7] and RSV + RAV-8[6] were used.

Production of AMV: Chick embryos on the 12th day of incubation were inoculated intravenously[14] with 10^4 converting units[15] of AMV and allowed to hatch. The chicks that showed a very high leukemic cell count (5×10^5 or more per mm^3) in the peripheral blood 10 days after hatching were exsanguinated by cardiac puncture. The blood was collected into heparin and centrifuged at low speed to pack the cells. The leukemic myeloblasts forming the upper two thirds of the packed cell layer were distinctly separated from the lower layer of erythrocytes. The leukemic cells were isolated and resuspended at a concentration of $1–5 \times 10^6$ cells per ml in nutrient medium.[15] Ten ml of cell suspension in a 100-mm plastic tissue culture dish was incubated at 40°C in a humidified incubator flushed with a CO_2-air mixture to maintain the culture pH around 7.3. The cells did not attach to the floor of the plate. At approximately 12-hr intervals for 48–96 hr the cells were removed from the medium by low-speed centrifugation and resuspended in fresh medium. The medium recovered after each period of incubation was centrifuged a second time at $2{,}000 \times g$ for 15 min to remove cell debris and was then frozen at −80°C until used for virus purification at a later time. Such medium contained about 10^5 converting units per ml.

Labeling AMV with P^{32} and uridine H^3 (H^3-UR): From 1 to 5 × 10^7 myeloblasts washed 3 times with Tris buffered saline (pH 7.4) containing 5% dialyzed calf serum were suspended for incubation in 10-ml nutrient medium low in phosphate. The medium contained phosphate-free M-199 77% by volume, tryptose broth 10%, dialyzed calf serum 5%, dialyzed chicken serum 5% $NaHCO_3$ (2.8% solution) 3%, and carrier-free P^{32} 500 μc. At 12-hr intervals the cells were removed from the medium by centrifugation, and the medium containing P^{32}-labeled virus was stored at −80°C. The cells were resuspended in fresh P^{32}-containing medium, and their incubation was continued.

Avian myeloblastosis virus was labeled with H^3-UR by incubating myeloblasts in modified Eagle's medium[16] containing dialyzed serum, $NaHCO_3$, and H^3-UR (specific activity 5 mc per μmole) 200 μc per 10 ml medium.

Production of RSV + RAV: Growth of RSV + RAV in chick embryo cell cultures and labeling of the virus with H^3-UR has been described.[5, 17]

Titration of virus infectivity: The infectious titer of AMV was determined using conversion of yolk sac cultures.[15] Serial 10-fold dilution steps of the virus suspensions were made and 4 assay plates were used per dilution. RSV infectivity was determined by focus formation.[18]

Virus purification: In view of the structural and biological similarity of AMV and RSV + RAV, AMV was purified by the procedure previously used for RSV + RAV. The two viruses were indistinguishable in their behavior during purification. The method of purification of H^3-UR or P^{32}-labeled virus from small quantities of tissue culture medium (5–100 ml) by ammonium sulfate precipitation and density gradient centrifugation has been described.[5]

Purification of both viruses from large volumes of tissue culture medium (1–10 liters) was carried out in 4 steps after centrifugation of the medium at 2,000 rpm for 10 min to remove cell debris. Each step was carried out in the cold (0–4°C). First, virus was precipitated by addition of solid ammonium sulfate to achieve 50% of saturation. Neutral pH was maintained by addition of Tris·HCl buffer. After stirring for 15 min the precipitate was collected by centrifugation and redissolved in buffer containing NaCl 0.05 *M*, Tris·HCl (pH 7.4) 0.01 *M*, and EDTA 0.001 *M* to make a final volume 1/20 that of the original medium. Second, 30 ml of a solution of the redissolved precipitate from the previous step was layered over 5 ml of a buffer solution containing sucrose 60% (w/v) and RbCl 15% (w/v) to form a sharp interface in 35-ml centrifuge tubes and centrifuged in the Spinco model L30 rotor at 30,000 rpm for 1 hr. Cell debris was seen as a pellet on the wall of the tube. Virus appearing in a band at the density interface in each tube was collected after puncturing the bottom of each tube. Third, to remove sucrose and RbCl the virus solution was diluted with an equal volume of buffer containing Tris·HCl (pH 7.4) 0.01 *M*, EDTA 0.001 *M*, and the virus was precipitated with ammonium sulfate and redissolved in buffer as described in step 2. Fourth, 2–5 ml of this virus solution was layered over a 24-ml preformed gradient of sucrose (15–60%) containing NaCl 0.05 *M*, Tris·HCl (pH 7.4) 0.01 *M*, and EDTA 0.001 *M* and centrifuged at 25,000 rpm in the Spinco SW25 rotor for 2 hr. The dense virus band visible at the midportion of the tube was recovered by collecting fractions after puncturing the bottom of the tube.

Isolation of nucleic acid: The method of nucleic acid extraction from P^{32}-labeled virus using carrier TMV-RNA and from large quantities of unlabeled virus has been described.[5, 19]

Counting radioactive samples: Radioactive samples were counted as previously described.[5, 19]

Materials: Tobacco mosaic virus (TMV) was the gift of Dr. C. Arthur Knight, and TMV-RNA was prepared by phenol extraction with SDS as described previously.[5]

Results.—Virus purification: Recovery of viral infectivity during purification has been measured in the case of RSV + RAV. In a typical experiment, starting with 5 liters of tissue culture medium, recovery of RSV infectivity with respect to the total starting infectivity during purification as described in *Methods* was 90 per cent after the initial ammonium sulfate precipitation, 80 per cent after centrifugation to a density interface, 40 per cent after the second ammonium sulfate precipitation, and 31 per cent over all 4 steps of purification (determined after the final sucrose gradient centrifugation).

Recovery of RSV infectivity after the two-step purification of virus from small

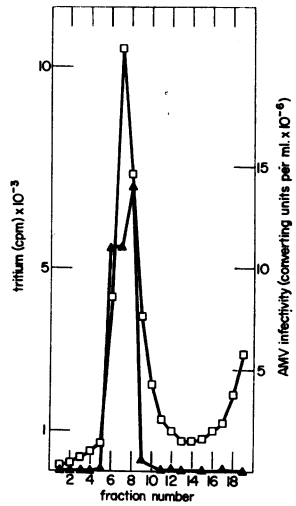

FIG. 1.—Purification of uridine-H^3 labeled AMV in a preformed sucrose density gradient. After precipitation of labeled AMV from tissue culture medium (20 ml) with ammonium sulfate at 50% of saturation the virus solution (1.0 ml) was layered over a 4.2 ml sucrose gradient (15–60% sucrose w/v) containing Tris·HCl (pH 7.4) 0.01 *M* and EDTA 0.001 *M* and centrifuged in the Spinco SW39 rotor at 36,000 rpm and 4°C for two hr. Fractions were collected from the bottom of the tube and each fraction was assayed for AMV infectivity (closed triangles) and radioactivity (open squares).

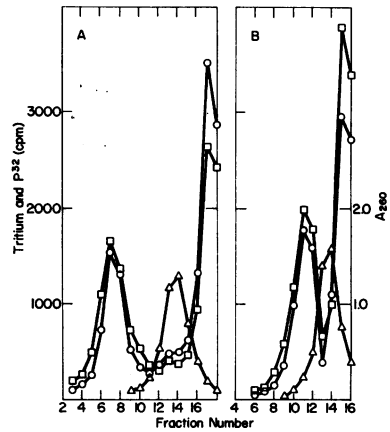

FIG. 2.—Sucrose gradient centrifugation of RSV + RAV nucleic acid labeled with P^{32} and AMV nucleic acid labeled with uridine-H^3 at two different salt concentrations. Solutions containing P^{32}-labeled RSV + RAV nucleic acid, tritium-labeled AMV nucleic acid, and carrier TMV-RNA 100 μg in 0.2 ml were layered on top of two 5.0-ml linear sucrose gradients (5–20% sucrose) and centrifuged at 36,000 rpm for 2.25 hr at 4°C in the Spinco SW39 rotor. The nucleic acid solution and the sucrose gradient in experiment *B* contained only EDTA 0.001 M, and in experiment *A* NaCl 0.10 *M*, Tris·HCl (pH 7.4) 0.01 *M*, and EDTA 0.001 *M*. After the centrifugation fractions were collected from the bottom of each tube and all fractions were analyzed for absorbancy at 260 mμ (open triangles) and for tritium (open squares) and P^{32} (open circles) in a Packard Tricarb scintillation spectrometer.

volumes of medium (see *Methods*) has been shown to be 80–100 per cent,[5] and the same recovery was obtained with AMV.

Results of the final step of purification of AMV labeled with uridine-H^3 by zone centrifugation in a preformed density gradient of sucrose are shown in Figure 1. Viral infectivity can be seen to coincide with radioactivity, and in such a gradient AMV was found to have a buoyant density of 1.15 gm per ml. The same results have been obtained for RSV + RAV.[5, 17]

Nucleic acid from radioactively labeled virus: The nucleic acid isolated from P^{32}-labeled AMV possessed properties identical with those previously reported for P^{32}-labeled RSV + RAV RNA.[5] Figure 2 shows the results of sucrose gradient centrifugation of a mixture of the nucleic acids from AMV labeled with P^{32} and RSV + RAV labeled with H^3-UR. Tobacco mosaic virus RNA used as carrier is represented by the A_{260} tracing. The nucleic acid from each virus consists of two distinct components. In 0.10 *M* salt solution (Fig. 2*A*) the faster-sedimenting component from each virus moves at approximately two times the rate of TMV-

RNA. This component is considered to be the intact viral RNA.[5] The slowly sedimenting component moves much slower than TMV-RNA and is considered to be degraded viral RNA.[5]

More than 97 per cent of the P^{32} in both components of P^{32}-labeled AMV nucleic acid is rendered TCA-soluble by digestion with pancreatic ribonuclease in 0.15 *M* NaCl, indicating that each component consists of RNA. Similar results have been obtained with RSV + RAV RNA.[5]

The relative proportion of the two RNA components from AMV varies from preparation to preparation, the fast-sedimenting component usually representing 30–60 per cent of the total TCA-precipitable radioactivity. In the case of RSV + RAV RNA the fast component usually represents 50–80 per cent of the total.[5]

As shown in Figure 2, the fast-sedimenting RNA components from AMV and from RSV + RAV move at identical rates during centrifugation in solutions with high (Fig. 2*A*) and with low (Fig. 2*B*) concentrations of salt, and the sedimentation rate of both is markedly dependent on salt concentration.

In order to characterize further the slow-sedimenting RNA component from P^{32}-labeled RSV + RAV, it was centrifuged in a sucrose gradient in the presence of the "4-S" component of chick cell RNA isolated as recently described.[5] Figure 3 shows that the viral RNA has a broader and more irregular profile than the cellular RNA, suggesting that it is more heterogeneous with respect to molecular size. Experiments with the slow-sedimenting component of AMV-RNA showed similar heterogeneity.

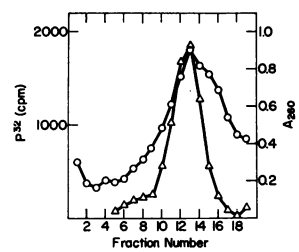

FIG. 3.—Sucrose gradient centrifugation of the slow component of RSV + RAV RNA labeled with P^{32} and the "4-S" component of chick cell RNA. The "4-S" component was isolated from total chick cell RNA by sucrose gradient centrifugation as previously described,[5] and the slow-sedimenting component of RSV + RAV RNA labeled with P^{32} was obtained after fractionation of viral RNA as shown in Fig. 2. A solution containing the slow component of P^{32}-labeled viral RNA (18,000 cpm) and chick cell "4-S" RNA (80 μg) in 0.2 ml was layered over a sucrose gradient as described in Fig. 2, and centrifuged for 18 hr at 36,000 rpm and 4°C in the Spinco SW39 rotor. Fractions were from the bottom of the tube and each was analyzed for absorbancy at 260 mμ (open triangles) and TCA-precipitable radioactivity (open circles).

RNA base compositions: The RNA base compositions are shown in Table 1. The values for the fast-sedimenting RNA components from AMV and RSV + RAV are in close agreement, again suggesting that the two RNA's are very similar. The difference between the two viruses in the values for cytidylic acid and for

TABLE 1

BASE COMPOSITION OF AMV AND RSV + RAV RNA

	Fast-sedimenting Component		Slow-sedimenting Component	
	AMV-RNA	RSV + RAV RNA	AMV-RNA	RSV + RAV RNA
C	23.0 ± 0.2	24.2 ± 0.2	28.5 ± 1.5	26.6 ± 0.3
A	25.3 ± 0.2	25.1 ± 0.3	19.3 ± 0.6	21.3 ± 0.5
G	28.7 ± 0.2	28.3 ± 0.5	34.4 ± 1.3	32.8 ± 0.4
U	23.0 ± 0.2	22.4 ± 0.4	17.6 ± 1.0	19.3 ± 0.3

The base composition of AMV-RNA was determined using P^{32}-labeled RNA isolated from purified virus after growth of leukemic myeloblasts in low phosphate medium containing P^{32} (see *Methods*) for at least 72 hr. The P^{32}-labeled RNA was fractionated by sucrose gradient centrifugation as described in Fig. 2, and the base composition of each RNA component was determined by alkaline hydrolysis and paper electrophoresis as previously described.[5, 19] Each value represents the average and standard error of the base compositions determined for the RNA of 4 separate preparations of P^{32}-labeled AMV. The values for RSV + RAV RNA were taken from Robinson *et al.*[5]

uridylic acid may not be significant. The base compositions for the slow-sedimenting components do not agree as closely. In addition the nucleotide values for the slow components are less reproducible than the values for the fast component, suggesting that the slow component of each preparation consists of a different mixture of RNA fragments. In no case are the previously reported values for base composition of total RNA from AMV[11-13] and RSV[20-22] in close agreement with values reported here. However, in several studies[11, 12, 20, 21] when individual nucleotide values differ from the present values for intact viral RNA, they differ in the direction of the values reported here for degraded RNA. This suggests that the previous analyses were done, using mixtures composed of different proportions of intact and degraded RNA. Other values reported previously for AMV-RNA[13] and RSV-RNA[22] do not resemble in any way the present values, nor do they resemble various components of chick cell RNA.[5]

Sedimentation in the analytical ultracentrifuge: Both RNA components from AMV and from RSV + RAV were examined in the analytical ultracentrifuge using UV optics. The RNA's were isolated from purified virus as described in *Methods*. The two components of each RNA were separated by sucrose gradient centrifugation (as described in Fig. 2), precipitated with alcohol,[5] and then redissolved in the appropriate buffer for analysis in the analytical ultracentrifuge. Avian myeloblastosis virus purified from two liters of tissue culture medium yielded 200 μg of viral RNA (determined by measuring absorbancy at 260 mμ of an RNA solution with a 1-cm light path and assuming a specific absorption of 25.0 per mg RNA per ml), and the fast-sedimenting component represented 46 per cent of the total. Rous sarcoma virus + RAV purified from 30 liters of tissue culture medium yielded 950 μg RNA and the fast-sedimenting component represented 54 per cent of the total RNA. Figure 4*A* shows densitometer tracings from a sedimentation experiment using the fast-sedimenting RNA component from RSV + RAV in a buffer containing Tris·HCl pH 7.4 0.01 *M*, NaCl 0.10 *M*, and EDTA 0.001 *M*. About 80 per cent of the material sediments with a sharp boundary. Most of the remaining material leads the sharp portion of the boundary and probably represents RNA aggregation. Avian myeloblastosis virus RNA demonstrated a similar UV pattern. The sedimentation constants calculated using the sharp portion of the boundary were 79 $S_{20,w}$ for the fast-sedimenting component of RSV + RAV RNA in Tris·HCl (pH 7.4) 0.01 *M*, NaCl 0.20 *M*, EDTA 0.001 *M*; 71 $S_{20,w}$ in Tris·HCl (pH 7.4) 0.01 *M*, NaCl 0.10 *M*, EDTA 0.001 *M*, and 27 $S_{20,w}$ in Tris·HCl (pH 7.4) 0.0005 *M*, EDTA 0.0005 *M*. These values were obtained in experiments with rotor temperatures near 5°C. The values for AMV-

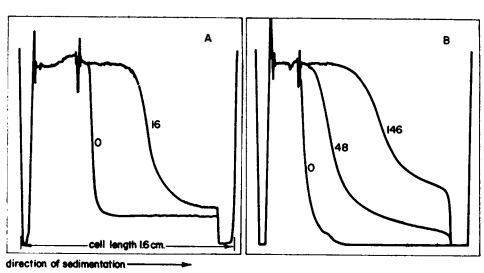

FIG. 4.—Ultraviolet patterns of the two components of RSV + RAV RNA in Tris·HCl (pH 7.4) 0.01 *M*, NaCl 0.10 *M*, EDTA 0.001 *M* sedimenting in the Spinco model E ultracentrifuge. (*A*) Patterns of the fast-sedimenting RNA component at 39,460 rpm and 1.4°C. The line designated 0 is the tracing of the first picture taken at full speed, and 16 is the UV pattern 16 min later. (*B*) Patterns of the slow-sedimenting component at 59,780 rpm and 3.7°C. The line designated 0 is the tracing of the first picture taken at full speed, and 48 and 146 are the UV patterns taken 48 and 146 min later.

RNA were identical. Upon increasing the salt concentration from 0.001 M to 0.10 M, the $S_{20,w}$ of RSV + RAV RNA increased only to 67S. Further increase in salt concentration to 0.20 M resulted in an increase in $S_{20,w}$ to 72S. Thus the change in sedimentation behavior resulting from exposure of the RNA to a low salt concentration is not completely reversible.

Figure 4B shows the results of an experiment with the slow-sedimenting component of RSV + RAV RNA. The RNA appears less homogeneous than the fast-sedimenting component, and a large fraction moves much faster than the material at the sharp portion of the boundary. The slow-sedimenting component of avian myeloblastosis virus RNA showed a similar UV pattern. The sedimentation constant calculated using the sharp portion of the boundary was 4.0 $S_{20,w}$ for RSV + RAV in Tris·HCl (pH 7.4) 0.01 M, NaCl 0.10 M, EDTA 0.001 M. The value for the slow component of AMV-RNA was 4.6S.

Discussion.—The RNA's isolated from AMV and RSV + RAV are identical in several properties. Both consist of two distinct components. The faster-sedimenting RNA's of both have identical sedimentation rates and demonstrate the same change in sedimentation rate with change in salt concentration. In addition, the change on exposure of these RNA's to solutions of low salt concentration is not completely reversible. Such dependence of sedimentation behavior on ionic strength has been shown to be a property of single-stranded RNA and is considered due to the change from a compact tightly coiled configuration at moderate ionic strength to a more expanded configuration at low ionic strength; and this expansion is not completely reversible.[23-25] Such change in secondary and tertiary structure with change in salt concentration appears to be characteristic for each RNA.[23, 25] Thus both RNA's in this study are undoubtedly single-stranded and very similar in size and secondary structure. Other factors which suggest that both RNA's are single-stranded are their susceptibility to digestion by ribonuclease and the base compositions which are not consistent with complimentary base pairing expected for double-stranded RNA. The close similarity in size and structure of these RNA's strengthens the concept that AMV and RSV + RAV are closely related viruses.

Assuming that the fast-sedimenting RNA components from the two viruses are single-stranded, an estimate of molecular weight can be made using the sedimentation constant of 71 $S_{20,w}$ and the empirical relation of Spirin[26] determined for single-stranded RNA in 0.10 M NaCl, 0.01 M EDTA. A value of 12×10^6 is obtained. This value is only an estimate because the relationship of sedimentation constant and molecular weight may differ considerably for different single-stranded RNA's.[23] The present value exceeds the previously reported one,[5] because the more accurate sedimentation constant reported here is somewhat higher than the one used previously. A single molecule with molecular weight in the order of 12×10^6 per virus particle would account for all of the RNA in RSV[22] and AMV[11] determined by chemical analyses of whole virus preparations.

The slow-sedimenting component of RNA is thought to contain viral RNA degraded by ribonuclease in the virus preparation before isolation of the RNA.[5] This is supported by the observation that its fraction of the total RNA is variable and usually represents 100 per cent of the RNA recovered after prolonged storage of virus at 4°C or after freezing and thawing purified virus more than one time.

In addition, this component is heterogeneous with respect to molecular size as shown by its sedimentation profile (Figs. 3 and 4*B*). Finally, the poor reproducibility of the base composition of this component indicates that each preparation consists of a slightly different mixture of RNA fragments. This RNA component is heterogeneous, and although it is likely that it consists mostly of degraded viral RNA, the presence of RNA from other sources cannot be excluded. It is of interest that the RNA from Newcastle disease virus has a slow-sedimenting component with similar properties.[19]

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