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Characterization of Ribonucleic Acid from Visna Virus

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A single-stranded ribonucleic acid(s) has been isolated from purified virions of visna virus. It consists of two major components, namely 63S and "4S," under the conditions employed for ribonucleic acid (RNA) extraction. The 63S component can be converted to subunits by heat and dimethylsulfoxide treatments. Analyses by base composition indicate that the "4S" RNA isolated from visna virus is not a random breakdown product of the 63S component as a result of extraction, nor is it randomly derived from cellular RNA.

Visna virus causes a slow infection of the central nervous system (CNS) of sheep. It is a ribonucleic acid (RNA) virus (9, 11), but its proliferation in cell cultures requires deoxyribonucleic acid (DNA) synthesis early in the growth cycle (21) and the virions contain RNA- and DNA-dependent DNA polymerases (12, 17). Visna virus is, therefore, apparently similar to the RNA tumor viruses (1, 14, 15, 17, 18, 20) in its biochemical properties and also with respect to a number of other characteristics (22).

In addition to the CNS disease, visna virus causes a lymphoproliferative disease in the lungs of sheep (9), but it is not known to be tumorigenic in animals or to cause transformation of cells in vitro. On the other hand, the virus produces a pronounced cytopathic effect in tissue cultures, leading to cell death.

The present study deals with the isolation and characterization of visna virus RNA. The results of this study show that the properties of visna virus RNA are quite similar to those of avian and murine tumor virus RNA.

MATERIALS AND METHODS

Chemicals. Uridine-5-^3H, carrier free ^{32}P-phosphoric acid, and Liquifluor or Omnifluor were purchased from New England Nuclear Corp., Boston, Mass. Cesium sulfate and sodium lauryl sulfate (SDS) were obtained from Fisher Chemical Co. The 23S ribosomal RNA of *Escherichia coli* was the product of Miles Laboratories, Kankakee, Ill. This RNA was repurified by sucrose gradient centrifugation before use. Dimethylsulfoxide (DMSO) and polyvinyl sulfate were provided by Eastman Organic Chemical Co., Rochester, N.Y. The electrophoretically purified bovine pancreatic deoxyribonuclease was purchased from Worthington Biochemical Corp., Freehold, N.J., and ribonuclease was purchased from Calbiochem, Los Angeles, Calif.

Standard buffer solutions. TN buffer (*p*H 7.4) contained 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride and 0.1 M NaCl. TNE buffer (*p*H 7.4) contained 0.01 M Tris-hydrochloride, 0.1 M NaCl, and 0.001 M ethylenediaminetetraacetate (EDTA). TE buffer was composed of 0.01 M Tris-hydrochloride and 0.001 M EDTA (*p*H 7.4). TM buffer was composed of 0.01 M Tris-hydrochloride and 0.005 M $MgCl_2$ (*p*H 7.4).

Cell cultures and media. Cultures of sheep choroid plexus (SCP) cells were used for production of virus. They were grown in 250-ml Falcon plastic flasks and maintained in Eagle's basal medium with 0.2% bovine serum albumin, as described previously (11).

Infection of cells and preparation of radioactive virus. The method of producing visna virus in SCP cell cultures has been described previously (11). For labeling of virus with ^{32}P, the infected cell cultures were incubated with maintenance medium containing 10 μCi of carrier free ^{32}P-phosphoric acid per ml.

Labeling of cells with ^{32}P-phosphoric acid. Uninfected cultures of SCP cells were incubated with the maintenance medium containing 10 μCi of carrier free ^{32}P-phosphoric acid for 18 hr at 37 C.

Measurement of radioactivity. The samples were placed in glass counting vials containing 10 ml of Liquifluor or Omnifluor diluted with toluene. The radioactivity was measured in a Picker Nuclear Liquimat 330 scintillation counter in conjunction with a DAC-512 computer.

Purification of virus. Our previously published method (11) was employed for the purification of visna virus with the following modification. The treatment of the viral preparation with ribonuclease and deoxyribonuclease was omitted, and the partially purified virus was centrifuged to equilibrium in a preformed potassium tartrate gradient (10 to 50%) in TNE buffer at 95,000 × *g* for 3 hr with a Beckman SW27 rotor at 4 C. The fractions containing the virus were pooled and diluted with 1 volume of TNE buffer. The diluted viral suspension was centrifuged in an International ultracentrifuge B-60 with a type A 321 rotor at 75,000 × *g* for 1 hr. The supernatant

was discarded, and the pellet was suspended in 2 ml of TNE buffer by sonic treatment with a needle probe of the Bronwill Biosonik III at an intensity of 30 for 30 sec. The procedures of gradient centrifugation and pelleting were repeated once. This purified virus was routinely used for nucleic acid extraction and enzyme study in our laboratory. The radioactivity profiles of the gradient centrifugations of ^{32}P-labeled visna virus are shown in Fig. 1. The radioactivity peak is located at a density of 1.16 g/ml, corresponding to the location of the virus infectivity in this type of gradient (12). The potassium tartrate gradient is clearly superior to the sucrose gradient (11) in separating visna virus from cell debris, and the symmetrical shape of the virus peak indicates a high degree of purity.

Extraction of RNA from the virus. Before extraction, the purified viral suspension was mixed with 2 μg of polyvinyl sulfate per ml and with SDS to a final concentration of 1%. The mixture was shaken for 10 min at room temperature. Then 1 volume of phenol, which was saturated with TNE buffer and contained 0.1% 8-hydroxyquinoline, was added. The mixture was shaken again for 15 min at room temperature with occasional cooling in ice water. After centrifugation at 1,000 $\times$ g in an IEC PR-2 centrifuge for 20 min, the aqueous phase was recovered and the phenol extraction was repeated once. The final aqueous solution was mixed with 2 volumes of absolute ethanol and allowed to stand at -20 C for 18 hr. RNA was recovered by centrifuging in a Sorvall SS-34 rotor at 12,000 $\times$ g for 30 min. The supernatant was discarded, and the pellet was dissolved in a buffer.

Extraction of RNA from uninfected cells. After removal of the maintenance medium, the cells were scraped into buffered saline with a rubber policeman and washed two times with the same saline. The cells were then suspended in TE buffer followed by addition of 2 μg of polyvinyl sulfate per ml and of SDS to 0.5%. The suspension was subjected to three cycles of freezing and thawing, and the RNA was extracted by the hot phenol method (16). The final aqueous solution was made to 0.1 M with respect to NaCl followed by the addition of 2 volumes of absolute ethanol. The mixture was allowed to stand at -20 C for 2 hr. The RNA was recovered by centrifugation and dissolved in TM buffer. The solution was incubated with 20 μg of deoxyribonuclease per ml at room temperature for 30 min. One more cycle of phenol-SDS extraction at room temperature followed. To the aqueous solution, NaCl was then added to 0.1 M and RNA was precipitated by 2 volumes of ethanol. It was recovered by centrifugation and washed two times with 95% ethanol.

Analysis of RNA by sedimentation centrifugation. Unless otherwise specified, the sucrose solution was prepared in TNE buffer. A 5-ml 5 to 20% (w/v) sucrose gradient was formed by a Beckman gradient former. A 0.2-ml amount of RNA solution was layered on the gradient and centrifuged at 257,000 $\times$ g for 45 min at 4 C with a Beckman SW65 rotor. Samples (0.2 ml) were collected from the bottom of the centrifuge tube. Each fraction was diluted to 1 ml with TNE buffer, and the absorbance at 260 nm was measured in a Beckman DU-2 spectrophotometer, if an RNA marker was included in the sample. Thereafter, 0.1 mg of yeast RNA and 1 ml of 10% cold trichloroacetic acid were added to each fraction. The acid-precipitable material was filtered through a B-6 membrane filter (Schleicher & Schuell, Inc., Keene, N.H.) and washed three times with 5 ml of 5% trichloroacetic acid. The filter was dried, and the radioactivity was measured in a liquid scintillation counter. The sedimentation coefficient of the viral RNA was calculated by the method of Martin and Ames (13) with *E. coli* 23*S* ribosomal RNA as a marker.

Treatment of RNA by DMSO. The method of

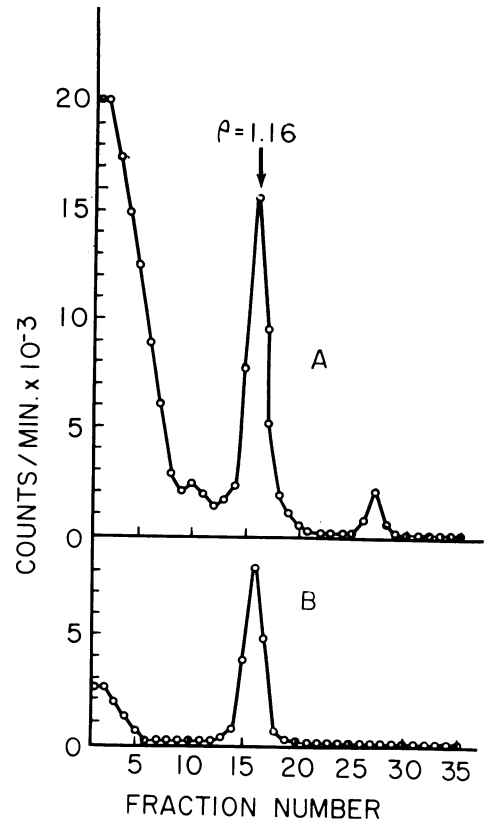

FIG. 1. *Equilibrium centrifugation of ^{32}P-labeled visna virus. (A) Two milliliters of partially purified visna virus labeled with ^{32}P-phosphoric acid was layered on a 33-ml potassium tartrate gradient and centrifuged as described. One-milliliter fractions were collected from the top of the gradient by a ISCO model D density gradient fractionator (Instrumentation Specialties Co., Inc., Lincoln, Nebr.). Samples of 0.05 ml of each fraction were placed on Gelman glass fiber filters in glass scintillation counting vials and were dried. A 10-ml amount of toluene containing Omnifluor was then added, and the radioactivity was counted. (B) Fractions 15 to 19 of A were pooled and recentrifuged. The procedure of A was then repeated.*

Duesberg (6) was employed in this study except that the RNA was dissolved in TE buffer and NaCl was added to the treated solution before ethanol precipitation.

Analysis of base composition. RNA was hydrolyzed in 0.3 M KOH at 37 C for 18 hr. The resultant ribonucleoside 2′-(3′)-monophosphates were separated by paper electrophoresis and run at 1,700 v for 150 min in 0.05 M ammonium formate buffer (*p*H 3.5). The base ratio was calculated from the per cent radioactivity of each nucleotide in the hydrolysate.

RESULTS

Nature of the nucleic acid isolated from purified visna virus. The ^{32}P-labeled nucleic acid which had been isolated from purified virus by phenol-SDS extraction was centrifuged on a sucrose gradient containing 0.1 M NaCl and 0.001 M EDTA as described above. The profile of the acid-precipitable radioactivity is depicted in Fig. 2. It is seen that the radioactive material was separated into two major components. The fast moving component which constituted about 65% of the mixture exhibited a sedimentation coefficient of 63*S* with reference to the 23*S* ribosomal RNA of *E. coli*. The size of the light component was not further determined but will be referred to hereafter as the "4S" unit for the sake of convenience. The small shoulder appearing on the leading edge of the 63*S* peak indicates heterogeneity.

To determine the nature of the visna virus nucleic acid, a sample of a ^{32}P-labeled extract was divided into three equal portions. One of them served as an untreated control, and the other two were incubated with bovine pancreatic ribonuclease and deoxyribonuclease, respectively. The samples were then centrifuged on a sucrose gradient, and the results illustrated in Fig. 3 were obtained. They show that the ^{32}P-labeled nucleic acid was almost completely digested to acid-soluble products by the action of ribonuclease but was resistant to the digestion by deoxyribonuclease. The profile of the control sample was almost identical to the profile of the deoxyribonuclease-treated sample. The data indicate that the viral nucleic acid is an RNA. Figure 4 shows the profile of acid-precipitable radioactivity after centrifugation of ^{3}H-labeled visna RNA through a sucrose gradient containing 0.001 M instead of 0.1 M NaCl. It is seen that the peak of the fast-moving RNA, normally located in fraction 8, shifted to fraction 15, indicating that the rate of sedimentation depends on the ionic strength of the solution. This presents evidence for the single-stranded nature of the 63*S* component (5, 19).

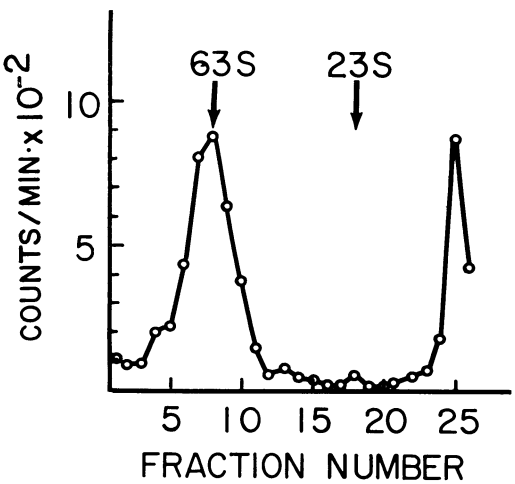

FIG. 2. *Sucrose gradient centrifugation of ^{32}P-labeled extract from purified visna virus. Purified visna virus labeled with ^{32}P-phosphoric acid was subjected to phenol-sodium dodecyl sulfate extraction, and the extract was centrifuged through a sucrose gradient as described in Materials and Methods and the fractions were collected from the bottom of the tube.*

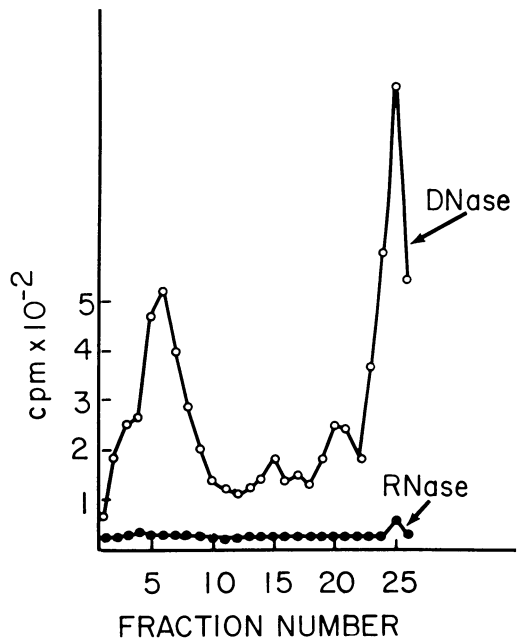

FIG. 3. *Sucrose gradient centrifugation of ^{32}P-labeled extract after treatment with ribonuclease and deoxyribonuclease. The ^{32}P-labeled extract from purified visna virus was dissolved in TM buffer. Two 0.2-ml samples of the solution were each incubated with ribonuclease (50 μg/ml) and deoxyribonuclease (50 μg/ml) at room temperature for 30 min and then centrifuged through sucrose gradients as detailed in Materials and Methods and in Fig. 2.*

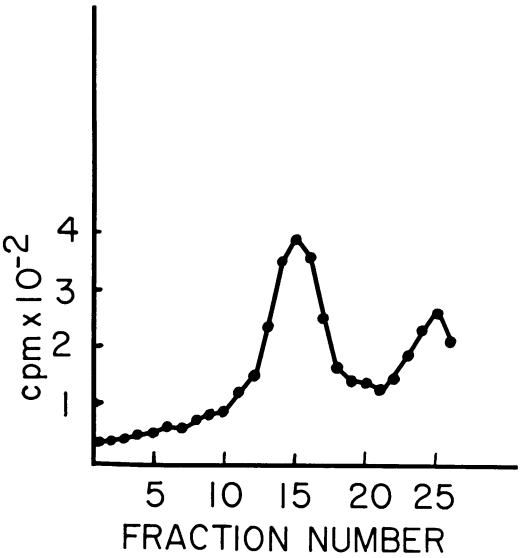

FIG. 4. *Sucrose gradient centrifugation of ^{3}H-labeled visna virus RNA under low ionic strength. The RNA was extracted from purified visna virus labeled with ^{3}H-uridine and centrifuged through a sucrose gradient containing 0.001 M NaCl and 0.001 M EDTA as detailed in Materials and Methods and in Fig. 2.*

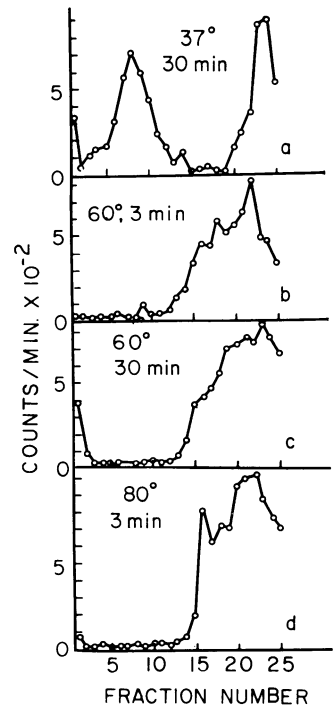

FIG. 5. *Sucrose gradient centrifugation of visna virus RNA after heat treatments. ^{3}H-labeled visna virus RNA was incubated at the indicated temperatures for times indicated. The centrifugation was performed as detailed in Materials and Methods and in Fig. 2. (a) At 37 C for 30 min; (b) at 60 C for 3 min; (c) at 60 C for 30 min; (d) at 80 C for 3 min.*

Effects of heat and DMSO on visna virus RNA. The integrity of the 63*S* visna virus RNA was investigated to determine whether the heavy component contains a single polynucleotide. ^{3}H-uridine-labeled viral RNA was dissolved in TE buffer and heated at various temperatures for specified periods of time. The treated samples were quickly chilled in melting ice and NaCl was added to 0.1 M. Analysis of the RNA samples was performed by sucrose gradient centrifugation, and the results are presented in Fig. 5. The 63*S* RNA remained intact after heat treatment at 37 C for 30 min but was converted to a heterogeneous profile after incubation at 60 C for 3 min. The broad peak extends from 38 to 4*S*. To study the possibility of further conversion of the heterogeneous RNA species to a rather homogeneous subunit, the heat treatment at 60 C was prolonged to 30 min. The result of this experiment was essentially the same as that of the shorter treatment (Fig. 5C). There was no further change in the sucrose gradient profile when the heating of the RNA was performed at 80 C (Fig. 5D).

To investigate further the structural property of the 63*S* RNA, the effect of DMSO was studied. The result of sucrose gradient centrifugation of DMSO-treated RNA is shown in Fig. 6. The heterogeneity of the RNA resulting from DMSO "melting" followed the overall pattern of the heat experiments. However, a broader peak ranging from 49 to 4*S* was obtained. Furthermore, the "melted" RNA seemed to be dominated by a subunit sedimented at 23*S*.

Base composition of viral and 4S cellular RNA. To study the possibility of a random breakdown of the 63*S* RNA to the "4*S*" component, analyses of base composition of the two components were carried out. The two components of viral RNA were isolated from pooled fractions of the sucrose gradient by ethanol precipitation by using yeast RNA as carrier. The ^{32}P-labeled 4*S* cellular RNA was purified by sucrose gradient centrifugation and isolated by the method used for viral RNA. The results of the base composition analysis are given in Table 1. They show that the purine to pyrimidine ratio of the "4*S*" viral RNA is almost three times that of the 63*S* viral RNA. They also show that the guanine plus cytosine (GC) content of these two components is different. The data indicate that the "4*S*" component is not a portion of the 63*S* component which might have been randomly broken down during the extraction. On the other hand, the GC content of the 4*S*

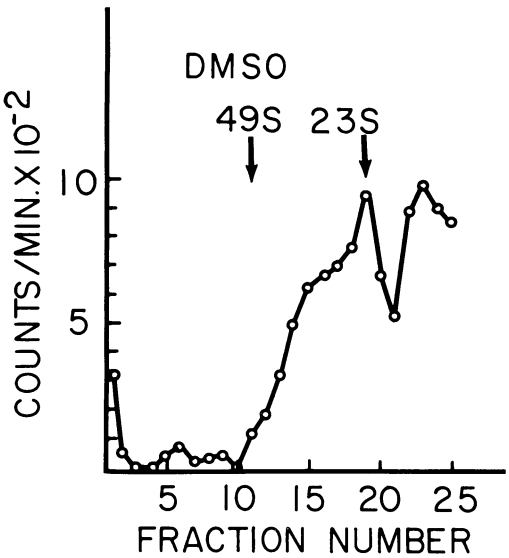

FIG. 6. *Sucrose gradient centrifugation of visna virus ^{3}H-RNA after treatment with dimethylsulfoxide (DMSO). The procedure of DMSO treatment and the processing of sample were described in Materials and Methods and in Fig. 2.*

TABLE 1. *Base composition of viral and 4S cellular RNA*

Nucleotides[a]	63S viral[b]	4S viral[c]	4S cellular
C	28	18	27 ± 0.70
A	23	32	21 ± 0.60
G	23	37	37 ± 1.00
U	26	13	15 ± 0.80
G + C	51	55	64
P/Py	0.80	2.10	1.40

[a] C = cytosine 2′(3′)-monophosphate; A = adenosine 2′(3′)-monophosphate; G = guanosine 2′-(3′)-monophosphate; U = uridine 2′-(3′)-monophosphate; P = purine; Py = pyrimidine.
[b] Mean of two determinations.
[c] Mean of three determinations.

cellular RNA is significantly higher than that of the "4S" viral RNA. A great difference in the purine to pyrimidine ratio between these two 4S RNA species is also seen, suggesting that the "4S" viral RNA is probably not a 4S cellular RNA which might be randomly incorporated into the viral particle.

DISCUSSION

The present modification of our previously published method (11) for the purification of visna virus provides a useful procedure to obtain highly purified virus. This is essential for studies of viral nucleic acid and studies of the activities of DNA polymerase which has been found in visna virions (12, 17).

Evidence has been presented to demonstrate that visna virus RNA is a single-stranded RNA. A small amount of ^{32}P radioactivity resistant to the digestion by ribonuclease (Fig. 3) may represent a double-stranded structure of a portion of the viral RNA. It is also possible that the ^{32}P-labeled material represents a DNA molecule present in the visna virions as was found in Rous sarcoma virus (10) and in avian myeloblastosis virus (15). Since visna virus contains RNA-dependent DNA polymerase (12, 17), the possibility that an RNA-DNA hybrid is present in the virions is not ruled out. A more detailed study of this ribonuclease-resistant material is necessary.

Visna virus RNA consists of two major components, namely a 63S and a "4S" molecule. The 63S RNA is an aggregate. Under similar conditions, the 63S component of visna RNA seems to be more heterogeneous than the counterpart of tumor viruses (4, 6). A comparative study of this component would be of interest because a difference in composition of the RNA aggregate between these two types of virus may shed some light on the pathogenesis of the diseases caused by visna virus and by RNA tumor viruses.

The exact size of the "4S" visna RNA has not yet been determined but is presumed to be in the range of 4 to 7S as has been reported by Bishop et al. (2, 3) and by Erikson (7) for the light RNA component of tumor viruses. The data obtained from base composition analysis do not suggest that the "4S" component is a random breakdown product of the 63S RNA. However, the possibility that this "4S" RNA is a split off product at a specific point of the 63S molecule is not ruled out. The base composition of the "4S" viral RNA does not approximate that of 4S cellular RNA. If this "4S" molecule isolated from the purified virions is cellular in origin, then it probably represents a specific type of 4S RNA of the host cell.

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