Three Unique Viral RNA Species of Snowshoe Hare and La Crosse Bunyaviruses

JON CLEWLEY, JON GENTSCH, AND DAVID H. L. BISHOP*

Department of Microbiology, The Medical Center, University of Alabama in Birmingham, Birmingham, Alabama 35294

Received for publication 6 January 1977

Two-dimensional gel electrophoreses of RNase T₁-derived oligonucleotides of the three individual RNA segments of the bunyavirus snowshoe hare virus indicate that its three RNA segments possess distinct nucleotide sequences. The fingerprints of the RNA species of snowshoe hare virus differ from those of the antigenically closely related La Crosse virus. Three viral RNA species have been identified in preparations of Melao and Trivittatus as well as snowshoe hare, Lumbo, and La Crosse bunyaviruses.

The *Bunyaviridae* are a newly defined family of lipid-enveloped RNA viruses that appear to possess segmented RNA genomes (17). By serological analyses, about 90 bunyaviruses have been divided into 11 groups with some, but not all, members of each group being serologically related to one or more members of another group (1, 4, 8, 9, 17, 26, 27). These 11 groups constitute the *Bunyamvera* supergroup of viruses of different groups are not known. Fifty relationships between viruses of one group and viruses of different groups, are not known. Fifty other bunyaviruses are serologically unrelated to members of the supergroup although they exhibit certain structural features in common with members of the supergroup (1, 16, 17, 19, 21-23).

The California encephalitis viruses, one of the eleven groups of the supergroup, include the viruses La Crosse, snowshoe hare, Lumbo, Bocas, Inkoo, California encephalitis, Trivittatus, Jerry Slough, Tahyna, Jamestown Canyon, Melao, San Angelo, and Keystone. Reports concerning the structural properties of several members of this group have been published (3, 6, 6a, 7, 10-12, 19, 20, 28). For both La Crosse and snowshoe hare viruses two virion envelope glycoproteins (G1, molecular weight, 115×10^3 ; and G2, molecular weight, 38×10^3) and one internal major nucleocapsid protein (N, molecular weight, 23×10^3) have been identified. (6, 6a, 11, 12).

As shown for La Crosse, Lumbo, and Uukuniemi viruses (the latter a bunyavirus unrelated to members of the supergroup), the virion RNA species appear to exist in the form of separate nucleocapsids (12, 15, 24). Three size classes of nucleocapsids have been observed and

shown to each contain one distinct size class of RNA (L, large; M, medium; and S, small). It is not known whether the three RNA species code for different polypeptides or even if they contain unique nucleotide sequences.

Using temperature-sensitive, conditional lethal mutants of snowshoe hare virus, it has been shown that for certain dual mutant virus infections, wild-type recombinants are produced at a high frequency (6). Whereas such evidence is compatible with the virus possessing a segmented genome (with recombinants being formed by segment reassortment), it does not provide direct evidence that segment reassortment in fact occurs, nor does it prove that the segments contain unique nucleotide sequences.

The purpose of this communication is to show that the S, M, and L RNA species of snowshoe hare virus each contain unique oligonucleotides. Also, it will be shown that the oligonucleotide patterns of the three RNA species of La Crosse virus, a virus antigenically closely related to snowshoe hare virus, are quite different from their snowshoe hare counterparts.

MATERIALS AND METHODS

Materials. Acrylamide and bisacrylamide were obtained from Serva Laboratories through Accurate Chemical Corp., Hicksville, N.Y. Sankyo RNase T₁ was obtained through Calbiochem, La Jolla, Calif., and bacterial tRNA came from Boehringer Mannheim Corp., New York. [³²P]sodium orthophosphate came from I.C.N., Irvine, Calif.

Viruses. La Crosse and Lumbo viruses were originally obtained from J. F. Obijeski, Center for Disease Control, Atlanta, Ga. Snowshoe hare, Trivittatus, and Melao viruses were kindly provided by R. Shope of the Yale Arbovirus Research Unit, New

Haven, Conn. Each virus was plaque cloned in BHK-21 cells.

Infection of cells and virus purification. Confluent monolayers of 1×10^7 BHK-21 cells were infected at room temperature for 30 min with a virus stock at an input multiplicity of infection of 0.001 PFU per cell. The cultures were overlaid with 25 ml of minimal essential medium, supplemented with 5% (vol/vol) fetal calf serum, and incubated at 33°C for 20 h. The supernatant fluids were then discarded and replaced with 10 ml of minimal essential medium supplemented with 5% (vol/vol) fetal calf serum and 250 μ Ci of [32 P]sodium orthophosphate per ml. The infected cells were incubated at 33°C for an additional 36 to 48 h, and the supernatant fluids were recovered and clarified by low-speed centrifugation at 4°C for 30 min at $8,000 \times g$ in a Sorvall RC-5 centrifuge to remove cell debris. Virus was purified from these fluids by polyethylene glycol-NaCl precipitation followed by centrifugation first in a combination potassium tartrate-glycerol gradient and then in a sucrose gradient as described previously (11, 13). Virus bands recovered from the sucrose gradients were diluted fivefold with TSE buffer (0.01 M Tris-0.15 M NaCl-0.002 M EDTA, pH 7.4) and then pelleted through a cushion of 1 ml of 30% (wt/vol) sucrose in TSE buffer by centrifuging at 35,000 rpm for 120 min at 4°C in a Spinco SW41 rotor. Virus pellets were finally suspended in TSE buffer at protein concentrations of about 1 mg/ml, clarified by centrifugation at $5,000 \times g$ for 10 min, and stored at 4°C until required for use.

Purification of labeled viral RNA species. Purified, 32 P-labeled virus was adjusted to 1% (wt/vol) sodium dodecyl sulfate (SDS) and immediately layered on preformed linear gradients of 30 to 15% (wt/vol) sucrose containing 0.1% SDS, 0.02 M Tris-hydrochloride, 0.1 M NaCl, and 0.002 M EDTA, pH 7.4. The gradients were centrifuged at 40,000 rpm in a Spinco SW41 rotor at 20°C for 5.5 h. Fractions (0.3 ml) were collected into siliconized, sterile, glass scintillation vials by dripping out from the bottom of the gradients. The distribution of radioactivity was then determined, and the fractions containing the large, medium, or small RNA species were pooled separately. Carrier, unlabeled, chicken embryo RNA (100 μ g) was added to the three RNA samples which were then phenol extracted to recover the RNA species. After two successive alcohol precipitations to remove all traces of SDS, the RNA samples were freeze-dried, dissolved in 15 μ l of 0.02 M Tris-hydrochloride-0.002 M EDTA, pH 7.4, and stored at -20°C.

RNA gel electrophoresis and base-ratio determinations. RNA species were resolved by electrophoresis in 2.4% (wt/vol) polyacrylamide gels as described previously (2). Base-ratio analyses of purified 32 P-labeled RNA species were performed as described elsewhere (25).

Electrophoresis of RNA using slab gels of polyacrylamide. RNA samples were electrophoresed using 2.7% (wt/vol) polyacrylamide gels and a Hoefer slab gel apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). The gels were prepared by combining 4.5 ml of 30% (wt/vol) acrylamide and 1.5% (wt/vol) bisacrylamide with 16.66 ml of 3E

buffer (0.12 M Tris-0.06 M sodium acetate-0.003 M EDTA, pH 7.2) and diluting the mixture with deionized water to give a final volume of 50 ml. The mixture was degassed for 30 s, 40 μ l of *N,N,N',N'*-tetramethylethylenediamine (TEMED) and 0.4 ml of fresh, 10% (wt/vol) ammonium persulfate were added, and the mixture was poured into the slab gel holder over a preformed 0.75-cm-deep, 7.5% (wt/vol) polyacrylamide base gel. This base gel was prepared by combining 2.5 ml of 30% (wt/vol) acrylamide-1.5% (wt/vol) bisacrylamide with 3.33 ml of 3E buffer and diluting it with deionized water to give a final volume of 10 ml. The base gel was poured after addition of 7 μ l of TEMED and 67 μ l of fresh, 10% (wt/vol) ammonium persulfate. After the upper gel had polymerized over the base gel, the gel was pre-electrophoresed for 1 h at 50 V and 50 mA with a constant voltage setting and electrophoresis buffer E containing 0.1% SDS. RNA samples were electrophoresed for 5 h at 50 V and 50 mA and, after removing one of the two glass plates, radioautographed using Kodak XR-2 X-ray film. Cooling water was circulated through the apparatus during the electrophoresis. The base gel was included not only to catch low-molecular-weight RNA species but also to stabilize the gel during the radioautography manipulations.

Nuclease digestion of RNA and separation of oligonucleotides by two-dimensional gel electrophoresis. The two-dimensional electrophoresis and RNA digestion conditions employed in this study were based on the procedures described by De Wachter and Fiers (5) with the modifications described below. RNA samples were mixed with 10 U of RNase T₁ at 37°C for 30 min. Each digest was mixed with solid urea to give a 6 M urea final concentration and then with 8 μ l of a solution containing 6 M urea, 50% (wt/vol) sucrose, 0.2% xylene cyanol FF, 0.2% bromophenol blue, and 15 mg of bacterial tRNA per ml. The mixture was layered into the load wells of the 10% (wt/vol) first-dimension polyacrylamide gel. This gel (35 by 13 by 0.35 cm) was formed by mixing 62.5 ml of 40% (wt/vol) acrylamide-1.3% (wt/vol) bisacrylamide with 90 g of urea-6.25 ml of 1 M citric acid and diluting with deionized water to give a final volume of 250 ml. To this mixture were added (in order) 0.9 ml of 10% (wt/vol) ascorbic acid, 0.9 ml of 0.25% ferrous sulfate, and 0.09 ml of 30% (wt/vol) hydrogen peroxide. The mixture was immediately poured into the gel holder which consisted of two glass plates (40 by 20 by 0.5 cm) that were held apart by silicone rubber spacers on the two sides (spacer size, 40 by 3 by 0.35 cm) and bottom (spacer size, 14 by 3 by 0.35 cm). A liquid-tight seal was maintained by means of a thin film of silicone stopcock grease (Dow Corning Corp., Midland, Mich.) placed on the spacers and adjacent glass surfaces and by a contiguous row of binder clips clamping the plates and spacers in position. Before polymerization occurred, three wedge-shaped silicone rubber spacers were positioned in the top of the gel holder to give the three loading wells.

After the gel polymerized, the bottom spacer and the three wedges were removed and, where they had contracted away from the side spacers, the edges of the gel were sealed with 1% (wt/vol) agarose. The

gel was positioned vertically with its base in the anode buffer chamber, and electrophoresis buffer was added to the top of the gel so that the wells and any remaining space were filled with liquid. A paper wick saturated with electrophoresis buffer and wrapped in Saran Wrap (Dow Chem. Co., Indianapolis, Ind.) was inserted between the plates just above the top of the gel, and its other end was placed in the cathode buffer chamber. The liquid level in the top cathode chamber was approximately 0.5 cm higher than the top of the gel plates to prevent removal of buffer from the top of the gel by capillary action. The gel was preelectrophoresed for 1 h at 6°C. The electrophoresis buffers (4 liters in each chamber) consisted of 0.025 M citric acid, pH 3.5, containing 6 M urea. After the three samples were loaded, the gel was electrophoresed at 900 V and 25 to 50 mA using a Buchler power supply set for constant voltage.

When the faster-moving bromophenol blue dye (green at pH 3.5) had migrated 19 cm (usually after 5 to 8 h), the electrophoresis was terminated and the two silicone side spacers and one of the two glass plates were carefully removed. On the basis of the distribution of radioactivity (as determined by monitoring with a hand geiger counter), a strip of gel (25 by 1 by 0.35 cm) was cut out for each of the three samples. The green dye was usually about 12 cm from the top end of each strip. Each strip of the first-dimension electropherogram was then positioned parallel to, but 6 cm away from, the bottom 30-cm side of a glass plate (38 by 30 by 0.5 cm). A second glass plate was placed on top of the strip, and two silicone rubber spacers (38 by 1.5 by 0.35 cm) were placed along the 38-cm sides of the plates. A bottom silicone rubber spacer (27 by 1.5 by 0.35 cm) was added, and the plates, spacers, and gel were clamped with binder clips such that a liquid-tight enclosure was formed with an air space surrounding the enclosed strip.

The second-dimension, 20% (wt/vol), polyacrylamide gel was then cast so that it polymerized around this strip. The second-dimension gel was formed by mixing (for each gel) 175 ml of 40% (wt/vol) acrylamide-1.3% (wt/vol) bisacrylamide with 35 ml of 1 M Tris-borate, 0.025 M EDTA, pH 8.3, 138.6 ml of deionized water, 1.2 ml of fresh 10% (wt/vol) ammonium persulfate, and 0.12 ml of TEMED. After polymerization, the bottom silicone rubber spacer was removed, and the binder clips were replaced by 10 large O-rings (19-cm inside diameter, 0.5 cm of thick rubber) to hold the plates together under liquid (see below). The sides of the gel were sealed with 1% agarose containing 0.1 M Tris-borate-0.0025 M EDTA, pH 8.3. The top and bottom surfaces of the gel were also sealed with a 2-mm-thick layer of the same agarose-buffer mixture.

The whole apparatus was placed vertically in a 60-liter rectangular aquarium such that the top of the gel apparatus was 3 cm above the level of the 55 liters of aquarium buffer. A paper wick, covered in Saran Wrap, was inserted between the top of the glass plates and passed to the anode buffer chamber. The aquarium, which served as the cathode chamber, contained 55 liters of 0.1 M Tris-borate-0.0025 M EDTA, pH 8.3. The anode buffer chamber, containing 15 liters of the same buffer, was positioned

such that the level of its buffer was about 0.5 cm above the top of the glass plates. Electrophoresis in the second dimension was performed for 24 to 36 h at 6°C at 200 V and 50 mA per gel, with a constant voltage setting on the Buchler supply. Electrophoresis was terminated for each gel when its original green dye (which turned blue at the alkaline pH) had migrated 21 cm towards the anode. Up to three gel slabs together can be conveniently electrophoresed with this arrangement. Upon termination of the electrophoresis, the gels were carefully removed from the plates, and the surface liquid was removed from them by blotting with filter paper. The gels were then sealed first in Saran Wrap and then in a polyethylene bag. Gels were autoradiographed in Wolf X-ray film cassettes (Wolf X-ray Corp., New York) using Kodak XR-2 X-ray film (14 by 17 inches [ca. 35.5 by 43.1 cm]). Films were placed on both sides of the gel and developed 1 to 3 days later, depending on the amount of radioactivity originally applied.

DEAE-cellulose column chromatography of combined pancreatic and RNase T₁ digests of ³²P-labeled viral RNA species. The procedures used to determine the genome complexities of viral RNA species by DEAE-cellulose column chromatography of nuclease digests of RNA have been described (18).

RESULTS

RNA species of Melao, Lumbo, Trivittatus, and La Crosse bunyaviruses. Using gel electrophoresis, minor differences in the electrophoretic mobilities of the RNA species of La Crosse, snowshoe hare, Bunyamwera, and Main Drain viruses have been observed (6a). The separations obtained by slab gel electrophoresis of the three major RNA species of Melao, La Crosse, Lumbo, and Trivittatus viruses are presented in Fig. 1. Although for some of the virus preparations only small amounts of material were available, it is evident that each virus possesses three RNA species (L, M, and S) which vary, if at all, to only minor extents in their electrophoretic mobilities.

Base-ratio analyses of La Crosse and snowshoe hare virion RNA species. Purified preparations of ³²P-labeled La Crosse or snowshoe hare viruses were mixed with SDS (final concentration, 1%), and the RNA species were resolved on gradients of sucrose containing 0.1% SDS. Three major peaks of radioactivity were observed in addition to the solubilized phospholipid components (see Fig. 2A for the results obtained for snowshoe hare virus; similar results were obtained for La Crosse virus [data not shown]). Fractions of the gradient were pooled to obtain each RNA species as free as possible from the other RNA species (Fig. 2A). For each virus, the three pools of material were extracted for RNA, and portions were sub-

FIG. 1. Polyacrylamide slab gel electrophoresis of *La Crosse*, *Lumbo*, *Melao*, and *Trivittatus* viral RNA species. Purified ^{32}P -labeled virus preparations of *Lumbo* (LUM), *Trivittatus* (TVT), *Melao* (MEL), and *La Crosse* (LAC) viruses were treated with SDS (1% final concentration) and then loaded on and electrophoresed in a slab gel of 2.7% polyacrylamide (see text). The RNA species were identified by radioautography. Although in some cases the RNA bands were faint, in each case three RNA species (L, M, and S) were resolved.

jected to electrophoresis in 2.4% polyacrylamide gels to determine the distribution of radioactivity between the L, M, and S RNA species (see Fig. 2B through D for the snowshoe hare results). Some contamination of L by M (and vice versa) was observed for both viruses.

Base-ratio analyses of the various RNA species were performed, and the results are presented in Table 1. It is noteworthy that particularly for the L RNA species, uridine 5'-monophosphate was the most abundant ribonucleotide.

Two-dimensional gel electrophoresis of the oligonucleotides obtained from the individual RNA species of snowshoe hare virus. The three ^{32}P -labeled RNA species isolated from snowshoe hare virus were digested with RNase T_1 , and the digests were resolved by two-dimensional gel electrophoresis and then radioautographed (Fig. 3). On each radioautogram are

FIG. 2. Resolution of the L, M, and S RNA species of snowshoe hare virus. Purified ^{32}P -labeled snowshoe hare virus was dissociated by 1% (wt/vol) SDS and (A) resolved by centrifugation in a gradient of sucrose (see text). The indicated fractions were pooled to harvest the L, M, and S RNA species, and portions (B, L RNA; C, M RNA; D, S RNA) were resolved by discontinuous gel electrophoresis employing 2.4% (wt/vol) polyacrylamide gels.

TABLE 1. Base ratios of snowshoe hare and *La Crosse* viral RNA species

RNA species	Base ratio ^a		
	S	M	L
Snowshoe hare			
CMP	24.1 ± 0.2	20.7 ± 0.2	19.4 ± 0.2
AMP	25.6 ± 0.4	27.1 ± 0.2	27.3 ± 0.5
GMP	19.2 ± 0.2	18.0 ± 0.2	16.3 ± 0.2
UMP	31.1 ± 0.2	34.2 ± 0.2	36.9 ± 0.4
<i>La Crosse</i>			
CMP	22.6 ± 0.3	20.3 ± 0.1	19.4 ± 0.2
AMP	26.6 ± 0.2	27.1 ± 0.3	27.7 ± 0.3
GMP	18.4 ± 0.2	17.7 ± 0.2	16.1 ± 0.2
UMP	32.7 ± 0.2	34.9 ± 0.5	36.8 ± 0.4

^a The base-ratios of the ^{32}P -labeled RNA species (S, M, and L) of snowshoe hare and *La Crosse* viruses, obtained as described in the legend to Fig. 2 and in the text, were determined as described elsewhere (24).

FIG. 3. Two-dimensional oligonucleotide fingerprints of snowshoe hare L, M, and S viral RNA species. Purified ^{32}P -labeled snowshoe hare L (A), M (B) or S (C) RNA species, obtained as described in the legend to Fig. 2 and the text, were digested with RNase T_1 , and the digests were resolved by two-dimensional gel electrophoresis in polyacrylamide gels (see text) and radioautographed. The position of two dye markers are indicated by ×. By aligning those faint contaminant L-type spots on the M radioautogram with the well-pronounced spots of the L radioautogram, the unique M oligonucleotides, not present as major spots in the L radioautogram, were identified. These are indicated in (B) by arrows. Likewise, those S spots not present as major spots in the M radioautogram are identified with arrows (C). The S spots in (C) not present as major spots in the L radioautogram are indicated by *. Although several of the M and S oligonucleotides are not identified by symbols, this does not necessarily mean that they are identical to the oligonucleotides present in the other radioautograms.

indicated the positions of the two dye markers; in the upper center is the dark blue bromophenol blue dye, and lower down is the light blue xylene cyanol dye.

The RNase T₁-derived oligonucleotides of snowshoe hare L RNA were evidently more numerous than those obtained from the M or S RNA, indicating the greater nucleotide complexity of the L RNA. This result was not unexpected since gel analyses have indicated an apparent molecular weight of about 3×10^6 for snowshoe hare L RNA and apparent molecular weights of 1.9×10^6 and 0.45×10^6 for the M and S RNA species, respectively (6a).

The large oligonucleotides derived by RNase T₁ digestion of RNA migrate slowly in the second-dimension electrophoresis (5). We expect that not only will the larger RNase T₁-derived oligonucleotides of one RNA species differ from those of an unrelated RNA, but also that their number will reflect to some degree the sequence complexity of their progenitors. Obviously, the base-ratio and actual sequence of an RNA will affect that relationship so that no direct conclusion can be made of the size of an RNA from the number of large oligonucleotides recovered. It is, however, noteworthy that there are, respectively, 36, 15, and 7 large oligonucleotides that fall on and below a horizontal line drawn halfway between the two dye markers for the snowshoe hare L, M, and S electropherograms shown in Fig. 3.

Close examination of Fig. 3 reveals that the radioautograms of each electropherogram have two types of spots, faint and barely discernible ones and well-pronounced ones. The faint spots present in the M RNA pattern mimic the well-pronounced spots in the L radioautogram (cf. Fig. 3A and B). We conclude that the faint spots in the M pattern are derived from contaminant L RNA species in the M RNA preparation (and vice versa). This result is not totally unexpected due to the similar sedimentation coefficients and incomplete resolution obtained for the M and L RNA species (Fig. 2A). Whether faint S RNA-derived oligonucleotides are present in the M or L fingerprints is difficult to judge; however, the reverse condition (faint M and L oligonucleotide spots in the S pattern) was observed (Fig. 3C). By comparing the counts per minute per gel plug of the major nucleotides in the S digest fingerprint with those of the faint spots, values of 3 to 6% were obtained for the relative representation of the contaminant nucleotides. The fact that L and M oligonucleotides were present in the S RNA digest can presumably be attributed to the presence of L and M RNA fragments in the S RNA preparation.

The questions for which these experiments were designed to provide answers were, for example, whether the M RNA is identical in sequence to part of the L RNA or whether it contains distinguishable nucleotide sequences (and oligonucleotides). Thus, if the M RNA were a deletion derivative of the L RNA, then one would expect to find all of the M RNA oligonucleotides in the L oligonucleotide fingerprint. However, if the M RNA were a unique RNA containing separate genetic information, then one would expect to find few identical large oligonucleotides in the two patterns. Likewise, if the S RNA were a deletion derivative of the M (or L) RNA, then one would expect to find all of the large oligonucleotides of the S RNA in the M (or L) fingerprints.

Since the faint spots on the M radioautogram were clearly derived from the L RNA species, conclusions concerning whether the M oligonucleotides were unique could be made simply by positional comparisons of the two radioautograms.

Many of the oligonucleotides present as major spots in the autoradiogram of the M RNA digest were clearly absent as major spots in the autoradiogram of the L RNA. Some of these unique M oligonucleotides are indicated by arrows in Fig. 3B. Whether other large-size oligonucleotides in the lower part of the M electropherogram, which appear to have similar electrophoretic mobilities to some L digest oligonucleotides, are in fact identical to the latter will have to await comparative sequence analyses of the individual oligonucleotides. As expected, in the top sections of the two electropherograms, many of the L and M spots were identical; this is the region of the electropherogram where non-unique small oligonucleotides were recovered (5).

It can be seen by comparing Fig. 3B with C that many of the large oligonucleotides, nucleotide sequences by comparison with the L RNA species. These analyses do not tell us if any part of the M RNA is homologous to the L RNA sequence. To answer that question, hybridization and more specific sequences analyses will be needed.

It can be seen by comparing Fig. 3B with Fig. 3C that many of the large oligonucleotides, which are present as major spots in the radioautogram of the snowshoe hare S RNA digest, have no counterpart major spots in the radioautogram of the M RNA digest (see the spots indicated by arrows in Fig. 3C). Careful comparison of Fig. 3A and C also indicates that several of the S oligonucleotides (indicated by * in Fig. 3C) are absent from the L digest. Whether others are in fact identical or differ-

FIG. 4. Genome complexity analyses of snowshoe hare L, M, and S RNA species. Combined pancreatic RNase and RNase T_1 digests of ^{32}P -labeled snowshoe hare L, M, or S RNA species were resolved by DEAE-cellulose column chromatography (18). In the left panel (A), the resolution obtained for the L RNA digest is shown, and the principal nucleotide isopleths, mono ① through heptanucleotides ⑦, are labeled. The two mononucleotide isopleths represent the separation obtained between the pyrimidine nucleotides and GMP. Alkali analyses were used to confirm the composition of the various oligonucleotide isopleths (25). The hexanucleotides were recovered from each chromatogram and separated into their component fractions (A_3C , A_3G , and A_3U) by gel electrophoresis at pH 3.5 employing the 10% polyacrylamide system described in the text. The distributions of label were determined (B) and indicated that the S hexanucleotides contain only A_3C . The ratio of label between A_3C , A_3G , and A_3U for the M hexanucleotides was 2.0 to 1.2 to 0.8, respectively. For the L hexanucleotides, the ratio of label between the A_3C and A_3U plus A_3G (which were poorly resolved) was 2.0 to 3.1. It was concluded that there were minimally one hexanucleotide in the S RNA combined nuclease digest, four hexanucleotides in the M RNA digest, and probably five hexanucleotides in the L RNA digest (see Table 2).

TABLE 2. Oligonucleotides obtained by combined pancreatic RNase and RNase T_1 digestion of snowshoe hare L, M, and S RNA species^a

Oligonucleotide	Mol wt of RNA species		
	L	M	S
1 + 1!	4,475 $\pm$ 140	2,452 $\pm$ 48	663 $\pm$ 25
2	1,108 $\pm$ 30	704 $\pm$ 12	161 $\pm$ 12
3	333 $\pm$ 5	190 $\pm$ 4	48.8 $\pm$ 2
4	117 $\pm$ 10	50.4 $\pm$ 2	12.1 $\pm$ 1
5	27.5 $\pm$ 2	15.8 $\pm$ 0.2	5.3 $\pm$ 0.2
6	5.0	4.0	1.0
7	1.0 $\pm$ 0.1	0	0
Total nucleotides	8,335 $\pm$ 268	4,734 $\pm$ 93	1,218 $\pm$ 59
Molecular weight	2.8 $\pm$ 0.1 $\times 10^6$	1.6 $\pm$ 0.03 $\times 10^6$	0.41 $\pm$ 0.02 $\times 10^6$

^a Preparations of ^{32}P -labeled snowshoe hare L, M, and S RNA species, obtained as described in the legend to Fig. 2, were digested by pancreatic RNase and RNase T_1 , the digests were resolved by DEAE-cellulose column chromatography (18), and the recovery of label in the various nucleotide size classes (mono [1+1!], di [2], tri [3], etc.; see the legend to Fig. 4A) was determined. From the number of hexanucleotides resolved by electrophoresis at pH 3.5 (Fig. 4B), the minimum numbers of hexanucleotides were determined. These values were used to compute the total number of nucleotides in each nucleotide size class recovered from each column and, consequently, the molecular weights of the RNA species, assuming that the average nucleotide molecular weight was 337 (see Table 1). The analyses represent the average of two separate determinations.

FIG. 5. Two-dimensional oligonucleotide fingerprints of LaCrosse L, M, and S viral RNA species. Purified ^{32}P -labeled LaCrosse L (A), M (B), or S (C) viral RNA species, obtained as described for snowshoe hare virus (see Fig. 2 and text), were digested with RNase T_1 , and the digests were resolved by two-dimensional gel electrophoresis in polyacrylamide gels and then radioautographed (see text). The clearly unique M and S oligonucleotides are indicated by arrows and asterisks, using the procedure and convention described in the legend to Fig. 3.

ent, but possess similar electrophoretic mobilities, will also have to await oligonucleotide sequence analyses.

These results indicate that the S RNA does not possess an identical sequence to part of the M or L RNA species.

Genome complexity analyses of the L, M, and S RNA species of snowshoe hare virus. Combined pancreatic and RNase T₁ digests of ³²P-labeled snowshoe hare L, M, or S RNA species were resolved by DEAE-cellulose column chromatography (Fig. 4A), as described previously (18), and hexanucleotides were recovered. To determine the number of these hexanucleotides (and thereafter, the ³²P counts per minute per mononucleotide), the recovered nucleotides were resolved by gel electrophoresis at pH 3.5 by the first-dimensional system described above (Fig. 4B) to separate A₅C from A₅G and A₅U. By achieving this, a determination of the minimum number of hexanucleotides and, therefore, the mononucleotide specific activity (see legend to Fig. 4) could be made. From the mononucleotide specific activity and the computations of the label recovered from the DEAE-cellulose columns, the numbers of nucleotides in the L, M, and S RNA species were determined (Table 2). It was concluded from these results that the molecular weights of the L, M, and S RNA species of snowshoe hare were $2.8 \pm 0.1 \times 10^6$, $1.6 \pm 0.03 \times 10^6$, and $0.41 \pm 0.02 \times 10^6$, respectively.

Two-dimensional gel electrophoresis of the oligonucleotides of the individual RNA species of La Crosse virus. The three RNA species isolated from ³²P-labeled La Crosse virus were digested with RNase T₁, and the digests were resolved by two-dimensional gel electrophoresis (Fig. 5). The objectives of the analyses were similar to those described in the preceding section, with the additional goal of determining if the oligonucleotide fingerprints of the La Crosse RNA species are similar to their snowshoe hare counterparts.

The results obtained for La Crosse RNA species indicate that its S RNA is not identical to part of the M RNA species, nor is the S or M RNA species identical to part of the L RNA. Those M oligonucleotides which, by comparison with the L oligonucleotides, are clearly unique are pinpointed by arrows (Fig. 5B), as are those S oligonucleotides which are unique by comparison with the M oligonucleotides (Fig. 5C). The S oligonucleotides, which are not found in the L RNA digest, are indicated by a * in Fig. 5C.

The L, M, and S La Crosse RNA digests bear few, if any, similarities to the digests of their

snowshoe hare counterparts (i.e., compare Fig. 3A with Fig. 5A, etc.).

DISCUSSION

Multiple RNA species have now been identified in virion preparations of the bunyaviruses Lumbo, Uukuniemi, La Crosse, snowshoe hare, Main Drain, Bunyamwera, Trivittatus, and Melao (3, 6, 6a, 7, 12, 14). Most sucrose gradient or gel electrophoresis analyses indicate that these viruses possess three RNA species with molecular weights of 3×10^6 , 1.9×10^6 , and 0.5×10^6 , although somewhat higher values have been described for Uukuniemi virus. Both 5' end to total nucleotide analyses for La Crosse or snowshoe hare and the genome complexity analyses reported here concur with these conclusions (6a, 12). Base-ratio analyses have been et al., in press). Base-ratio analyses have been described for the RNA species of Uukuniemi virus with results quite different for those reported here for snowshoe hare and La Crosse viruses.

The fact that the three RNA segments of snowshoe hare and La Crosse viruses can be distinguished by oligonucleotide fingerprinting provides further evidence of segmented genomes for these viruses. Whereas definitive proof has yet to be obtained that each segment codes for different proteins or that segment reassortment leads to recombinant formation, the results obtained here support the hypothesis that the genomes of bunyaviruses may indeed be segmented.

ACKNOWLEDGMENTS

We thank Reginald Anderson for excellent technical assistance.

This study was supported by National Science Foundation grant PCM76-22218 to D. H. L. Bishop.

LITERATURE CITED

1. Berge, T. O. 1975. International catalogue of arboviruses. U.S. Department of Health, Education, and Welfare Publ. (CDC) 75-8301, 2nd ed. Center for Disease Control, Atlanta.
2. Bishop, D. H. L., and P. Roy. 1971. Properties of the product synthesized by vesicular stomatitis virus particles. *J. Mol. Biol.* 58:799-814.
3. Bouloy, M. S., F. H. Krams-Ozden, and C. Hannoun. 1973/74. Three-segment RNA genome of Lumbo virus (Bunyavirus). *Intervirology* 2:173-180.
4. Calisher, C. H., and K. S. C. Maness. 1970. Arbovirus identification by an agar-gel diffusion technique. *Appl. Microbiol.* 19:557-564.
5. De Wachter, R., and W. Fiers. 1972. Preparative two-dimensional polyacrylamide gel electrophoresis of ³²P-labeled RNA. *Anal. Biochem.* 49:184-197.
6. Gentsch, J., and D. H. L. Bishop. 1976. Recombination and complementation between temperature-sensitive mutants of a bunyavirus, snowshoe hare virus. *J. Virol.* 20:351-354.

- 6a. Gentsch, J., D. H. L. Bishop, and J. F. Obijeski. 1977. The virus particle nucleic acids of four bunyaviruses. *J. Gen. Virol.* 34:257-268.
7. McLerran, C. J., and R. B. Arlinghaus. 1973. Structural components of a virus of the California encephalitis-complex: La Crosse virus. *Virology* 53:247-257.
8. Murphy, F. A., and P. H. Coleman. 1967. California group arboviruses: immunodiffusion studies. *J. Immunol.* 99:276-284.
9. Murphy, F. A., A. K. Harrison, and S. G. Whitfield. 1973. Bunyaviridae: morphologic and morphogenetic similarities of Bunyamwera serologic supergroup viruses and several other arthropod-borne viruses. *Intervirology* 1:297-316.
10. Murphy, F. A., S. G. Whitfield, P. H. Coleman, E. R. Rabin, A. B. Jenson, J. L. Melnick, M. R. Edwards, and E. Whitney. California group arboviruses: electron microscopic studies. *Exp. Mol. Pathol.* 9:44-56.
11. Obijeski, J. F., D. H. L. Bishop, F. A. Murphy, and E. L. Palmer. 1976. Structural proteins of La Crosse virus. *J. Virol.* 19:985-997.
12. Obijeski, J. F., D. H. L. Bishop, E. L. Palmer, and F. A. Murphy. 1976. Segmented genome and nucleocapsid of La Crosse virus. *J. Virol.* 20:664-676.
13. Obijeski, J. F., A. T. Marchenko, D. H. L. Bishop, B. W. Cann, and F. A. Murphy. 1974. Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. *J. Gen. Virol.* 22:21-33.
14. Pettersson, R., and L. Kaariainen. 1973. The ribonucleic acids of Uukuniemi virus, a noncubical tick-borne arbovirus. *Virology* 56:608-619.
15. Pettersson, R. F., and C.-H. von Bonsdorff. 1975. Ribonucleoproteins of Uukuniemi virus are circular. *J. Virol.* 15:386-392.
16. Pettersson, R. F., L. Kaariainen, C.-H. von Bonsdorff, and N. Oker-Blom. 1971. Structural components of Uukuniemi virus, a non-cubical tick-borne arbovirus. *Virology* 46:721-729.
17. Porterfield, J. S., J. Casals, M. P. Chumakov, S. Y. Gaidamorich, C. Hannoun, I. H. Holmes, M. C. Horzinek, M. Mussgay, N. Oker-Blom, and P. K. Russell. 1976. Bunyaviruses and Bunyaviridae. *Intervirology* 6:13-24.
18. Repik, P., and D. H. L. Bishop. 1973. Determination of the molecular weight of animal RNA viral genomes by nuclease digestions. I. Vesicular stomatitis virus and its defective T particles. *J. Virol.* 12:969-983.
19. Rosato, R. R., J. M. Dalrymple, W. E. Brandt, R. D. Cardiff, and P. K. Russell. 1974. Biophysical separation of major arbovirus serogroups. *Acta Virol.* 18:25-30.
20. Rosato, R. R., M. L. Robbins, and G. A. Eddy. 1974. Structural components of Oriboca virus. *J. Virol.* 13:780-787.
21. Saikku, P., and C.-H. von Bonsdorff. 1968. Electron microscopy of the Uukuniemi virus, an ungrouped arbovirus. *Virology* 34:804-806.
22. Saikku, P., C.-H. von Bonsdorff, M. Brummer-Korvenkontio, and A. Vaheri. 1971. Isolation of non-cubical ribonucleoprotein from Inkoo virus, a Bunyamwera supergroup arbovirus. *J. Gen. Virol.* 13:335-337.
23. Saikku, P., C.-H. von Bonsdorff, and N. Oker-Blom. 1970. The structure of Uukuniemi virus. *Acta Virol.* 14:103-107.
24. Samso, A., M. Bouloy, and C. Hannoun. 1975. Presence de ribonucleo-proteins circulaires dans le virus Lumbo (Bunyavirus). *C.R. Acad. Sci.* 280:213-215.
25. Sanger, F., G. G. Brownlee, and B. G. Barrell. 1965. A two dimensional fractionation procedure for radioactive nucleotides. *J. Mol. Biol.* 13:373-398.
26. Sather, G. E., and W. McD. Hammon. 1967. Antigenic patterns within the California-encephalitis-virus group. *Am. J. Trop. Med. Hyg.* 16:548-557.
27. Wellings, F. M., G. E. Sather, and W. McD. Hammon. 1970. Immunoelectrophoretic studies of the California encephalitis virus group. *J. Immunol.* 107:252.
28. White, A. B. 1975. Structural polypeptides of California encephalitis virus: BFS-283. *Arch. Virol.* 48:281-290.