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# Circular Forms of Uukuniemi Virion RNA: an Electron Microscopic Study

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Because the ribonucleoprotein forms of the segments of the Uukuniemi virus genome have previously been characterized as circular, we examined the isolated RNAs by electron microscopy under conditions of increasing denaturation. After spreading under moderately denaturing conditions (50 or 60% formamide), 50 to 70% of the molecules were circular. Increasing the formamide concentration to 70 and 85% decreased the number of circular forms, and only linear forms were observed after incubation of the RNA at 60°C for 15 min in 99% formamide. When spread from 4 M urea-80% formamide—another condition known to denature RNA—only 5 to 30% circular molecules were observed. Pretreatment of the RNA with 0.5 M glyoxal at 37°C for 15 min prior to spreading from 50% formamide gave less than 5% circular forms. Length measurement of the molecules showed that they were not significantly degraded by any of the methods employed. The circular molecules were destroyed by treatment with pancreatic RNase, but were unaffected by DNase or proteinase K treatment. After complete denaturation of the RNA, the circles could be reformed under reannealing conditions. We conclude that the three size classes of RNA that comprise the Uukuniemi virus genome are circular molecules probably maintained in that form by base pairing between inverted complementary sequences at the 3′ and 5′ ends of linear molecules.

Uukuniemi virus is a tick-borne arbovirus (9), which has been proposed as a member of the newly defined large bunyavirus family (14, 15). The bunyaviruses have a lipoprotein envelope and a ribonucleoprotein core (11, 12, 17, 22). Uukuniemi virus ribonucleoproteins contain three species of single-stranded RNA, called L (29*S*), M (22*S*), and S (17*S*) (10, 12). Since an RNA polymerase is present in purified virions (16), it appears that Uukuniemi virus is a negative-strand virus (1). Associated with the RNAs is (at least) one species of a polypeptide (N), with an apparent molecular weight of about 25,000 (11).

The three ribonucleoproteins of Uukuniemi virus, which probably have a helical symmetry (23), are circular as revealed by electron microscopy (12). Similar circular ribonucleoproteins have also been reported to exist in Lumbo virus (18) and La Crosse virus (8), both members of the bunyavirus family (15). These findings raised the questions whether the RNAs of the bunyaviruses freed from the N protein would also be circular and, if so, whether they would be closed covalently, by hydrogen bonding of the 3′ to the 5′ end or by a linking protein. To study these questions we examined the RNAs of Uukuniemi virus by electron microscopy under conditions of increasing denaturation. Our results suggest that the RNA species can be isolated in a circular form and that the circularization probably is due to hydrogen bonding between inverted complementary sequences at the 3′ and 5′ ends of the molecules.

(Part of this work was presented at the ICN-UCLA meeting, March 1976 [13].)

## MATERIALS AND METHODS

**Cells.** Preparation of chicken embryo cells was as described by Sandelin and Estola (20). HeLa cells in suspension were cultivated in Joklik modified minimal essential medium supplemented with 7% horse serum as described previously (2).

**Virus.** Stock virus of Uukuniemi virus was prepared at low multiplicity from the prototype strain S23 after five clonings in primary cultures of chicken embryo cells (10). The origin and cultivation of polio stock virus was as described (2, 21).

Uukuniemi virus was grown in petri dishes in secondary cultures of chicken embryo cells in the presence of Eagle minimum essential medium supplemented with 2% calf serum (10). The virus was

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harvested, concentrated, and purified as described previously (16). Poliovirus was cultivated in HeLa cells in suspension and purified as described (21).

**Extraction of RNA.** Uukuniemi virus RNA was extracted by sodium dodecyl sulfate (SDS) and phenol. Purified virus was treated with 1.0% SDS and extracted three times with an equal volume of water-saturated phenol-chloroform-isoamyl alcohol (25:24:1) followed by three extractions with chloroform-isoamyl alcohol (24:1) at room temperature. The RNA was then precipitated in the presence of 0.4 M sodium acetate and 1 mM EDTA with 2.5 volumes of 95% ethanol at −20°C. The RNA was collected by centrifugation and reprecipitated once. Immediately before spreading for electron microscopy, the RNA was collected by centrifugation, washed once with 95% ethanol, dried in a lyophilizer, and dissolved in distilled water. All buffers and solutions were treated when possible with 0.01% diethylpyrocarbonate and briefly autoclaved prior to use. At all steps extreme care was taken to avoid contamination with RNases.

Poliovirus RNA was extracted as described previously by Granboulan and Girard (5).

**Electron microscopy.** Uukuniemi virus and poliovirus RNAs were prepared for electron microscopy as described above and resuspended in RNase-free water at a concentration of about 50 to 200 μg/ml. RNA was treated and spread for electron microscopy at a final concentration of 1 to 3 μg/ml, using one of the following procedures.

**(i) FA method.** The RNA was spread by a modified Kleinschmidt technique (4) from solutions containing formamide (FA) at concentrations ranging from 30 to 85%, 0.1 M Tris (pH 8.2), 0.01 M EDTA, and 100 μg of cytochrome *c* (Calbiochem) per ml. The hypophase contained a 30% lower concentration of FA and 1/10 of the electrolyte concentration. The FA used was deionized overnight with a mixed bed resin (Bio-Rad Laboratories).

**(ii) Urea-FA method.** The RNA was spread from a solution of freshly prepared urea (4 M) and FA (80%, final concentrations) (24) and the same buffer as above and spread onto a hypophase of 50% FA with 1/10 of the electrolyte concentration.

**(iii) Glyoxal method.** The RNA was treated with 0.5 M glyoxal (Eastman Kodak Co.) at 37°C for 15 min in a buffer containing 0.01 M phosphate (pH 7.0) and 1 mM EDTA as described by Hsu et al. (6). The RNA sample was then diluted 1:50 or 1:100 in 50% FA and, after the addition of cytochrome *c*, immediately spread onto a hypophase of 20% FA.

The protein film containing the RNA was picked up on Parlodion-coated grids, stained with uranyl acetate ($5 \times 10^{-5}$ M in 90% ethanol), washed in 90% ethanol, and rotary shadowed with platinum-palladium at an angle of about 10°. The specimens were examined in a Philips EM201 electron microscope. The magnifications of micrographs were calibrated with the aid of a carbon grating replica (54,864 lines/inch [ca. 2.54 cm]). The lengths of the RNA molecules were measured with an electronic graphics calculator (Numonics Corp.). For both Uukuniemi virus and poliovirus RNAs several hundreds of molecules were measured per method used.

**Enzyme treatment of Uukuniemi virus RNA.** Purified Uukuniemi virus RNA was treated with either pancreatic RNase, DNase, or proteinase K. Treatment with pancreatic RNase (EC 3.1.4.22; Worthington Biochemicals Corp.) was carried out at a concentration of 50 μg/ml at 37°C for 45 min in the presence of 0.1 M Tris, pH 7.2. Beef pancreas DNase (EC 3.1.4.5; Worthington Biochemicals Corp.) (50 μg/ml) treatment was at 37°C for 10 min in the presence of 10 mM Tris (pH 7.2) and 10 mM magnesium acetate. Proteinase K treatment (100 μg/ml) was at 37°C for 45 min in the presence of 0.01 M Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 0.5% SDS. The reactions were stopped by transferring the tubes onto ice. After adjustment to 0.4 M sodium acetate, 0.01 M EDTA, and 1% SDS, the samples were extracted twice with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol as described above. The RNA was then precipitated with ethanol at −20°C. Immediately prior to spreading for electron microscopy the precipitated material was collected by centrifugation, washed once with cold ethanol, and dissolved in RNase-free water. The RNA was spread from 60% FA as described above.

## RESULTS

In this study we used Uukuniemi virus RNA extracted from purified virions by SDS and phenol. We chose not to fractionate the three RNA species present in the virions to minimize manipulation and thus also degradation (generation of "hidden breaks") of the RNA. In all preparations examined under an electron microscope, the L, M, and S RNAs were therefore present as a mixture. The studies described below were carried out with RNA from two different virus batches with essentially similar results.

**RNA spread from increasing concentration of FA.** A preparation of total Uukuniemi virion RNA was spread from solutions containing increasing concentrations of FA and examined by electron microscopy. When the spreading solution contained 40% FA, most molecules appeared collapsed and showed extensive secondary structure, as expected for single-stranded RNA (Fig. 1A). Although some of the molecules appeared to be circular, no definite statement regarding circularity could be made from these images.

Many of the molecules spread from a solution containing 50 or 60% FA were extended, and 50 to 70% of them appeared to be circles (Fig. 1B and C). Molecules spread from 70% FA were smoother and more extended, and 40 to 50% of them were scored as circular (Fig. 1D). When the FA concentration was increased to 85%, the fraction of circular molecules dropped to about 30% (Fig. 1E). Finally, when Uukuniemi virus

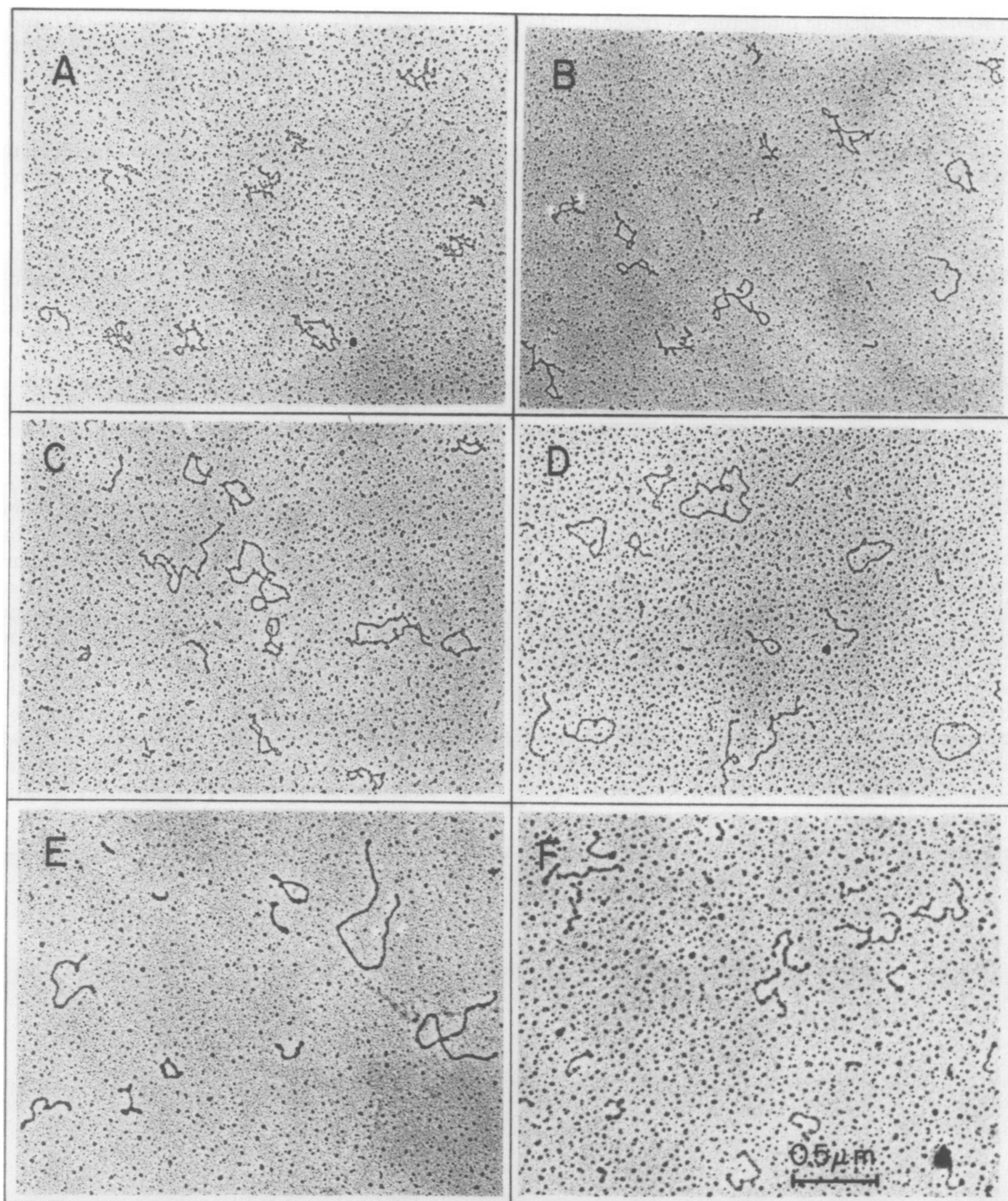


FIG. 1. *Electron micrographs of Uukuniemi virus RNA spread from increasing concentrations of FA. The RNA was spread from 40% (A), 50% (B), 60% (C), 70% (D), and 85% (E) onto a hypophase containing 30% less FA and 1/10 of the electrolyte concentration in the spreading solution. RNA was also heated at 60°C for 15 min in 99% FA prior to spreading from 60% FA (F).*

RNA was heated at 60°C for 15 min in 99% FA and then spread immediately from 60 or 85% FA at room temperature, no circular structures were seen (Fig. 1F). At FA concentrations above 70%, circular forms of the largest (L) RNA were rarely observed, whereas the circular forms of the medium-sized (M) and small (S) RNAs seemed to be more resistant to denaturation by FA. When single-stranded poliovirus RNA was spread from any of these FA concen-

trations, no circular molecules were seen (data not shown).

**RNA spread from urea-FA and after treatment with glyoxal.** As another method for studying RNA under moderately denaturing conditions, we spread the molecules from 4 M urea in 80% FA. This method partially denatures HeLa cell rRNAs (24). Uukuniemi virus RNA molecules were well extended and displayed little secondary structure (Fig. 2A). The fraction of circular molecules varied from 5% in one preparation to 30% in another.

Samples of RNA were also incubated in 0.5 M glyoxal, a reagent that adds to adenosine, cytidine, and guanosine and especially blocks the hydrogen-bonding ability of guanosine residues (3, 6). After incubation, the molecules were spread from 50% FA. Under these conditions less than 5% circular molecules were seen (Fig. 2B).

**Types of circular molecules observed.** Figure 3 shows high-resolution images of circular molecules selected from preparations spread at 70% FA. Three size classes of RNAs were readily apparent. The few L RNA circles that were seen appeared to have certain distinct regions of secondary structure ("panhandles"). The L RNA circles shown in Fig. 3A have two such panhandles. We have not observed a large enough number of these molecules to determine the regularity of the spacing between these structures. Molecules of the medium (M) and small (S) size classes can be found both with (Fig. 3B and D) and without (Fig. 3C and E) panhandles. The absence of visible secondary structure in these circles does not rule out the presence of panhandles because the resolution of the rotary-shadowed molecules in the micrographs is on the order of 100 bases.

**Length measurements and molecular weight estimations.** The lengths of Uukuniemi virus and poliovirus RNAs were determined after treatment with glyoxal (Fig. 4) or spreading from urea-FA (Fig. 5). In both cases Uukuniemi virus RNA gave three distinct peaks, representing L, M, and S RNAs, whereas poliovirus RNA gave one dominant peak. Poliovirus RNA was larger than any of the Uukuniemi virus RNA species. Under these two conditions too few circular Uukuniemi virus RNA molecules were observed for statistical comparison of the lengths of circular and linear forms in each length class. Assuming a molecular weight of $2.5 \times 10^6$ for poliovirus RNA as a standard, the molecular weights of Uukuniemi virus L, M, and S RNAs are as presented in Table 1. In the urea-FA method $1 \times 10^6$ daltons of RNA appears equivalent to 0.91 $\mu$m and, using the glyoxal method, $1 \times 10^6$ daltons corresponds to 0.83 $\mu$m; both values are in good agreement with published data (6, 24). By taking the mean value of the two methods, Uuku-

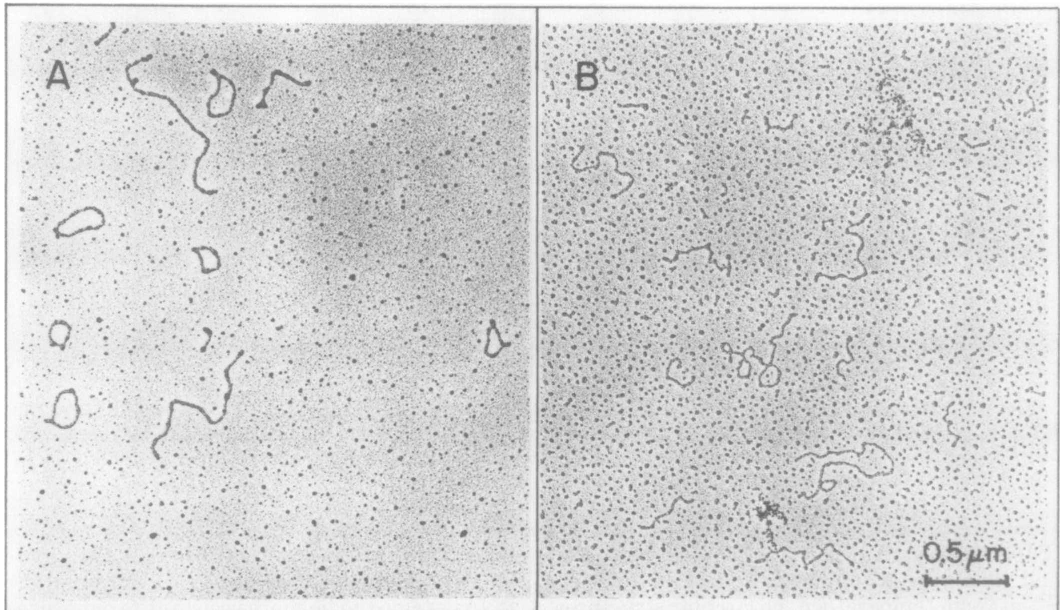


FIG. 2. *Electron micrographs of Uukuniemi virus RNA spread from 4 M urea-80% FA (A) and after treatment with 0.5 M glyoxal at 37°C for 15 min prior to spreading from 50% FA (B).*

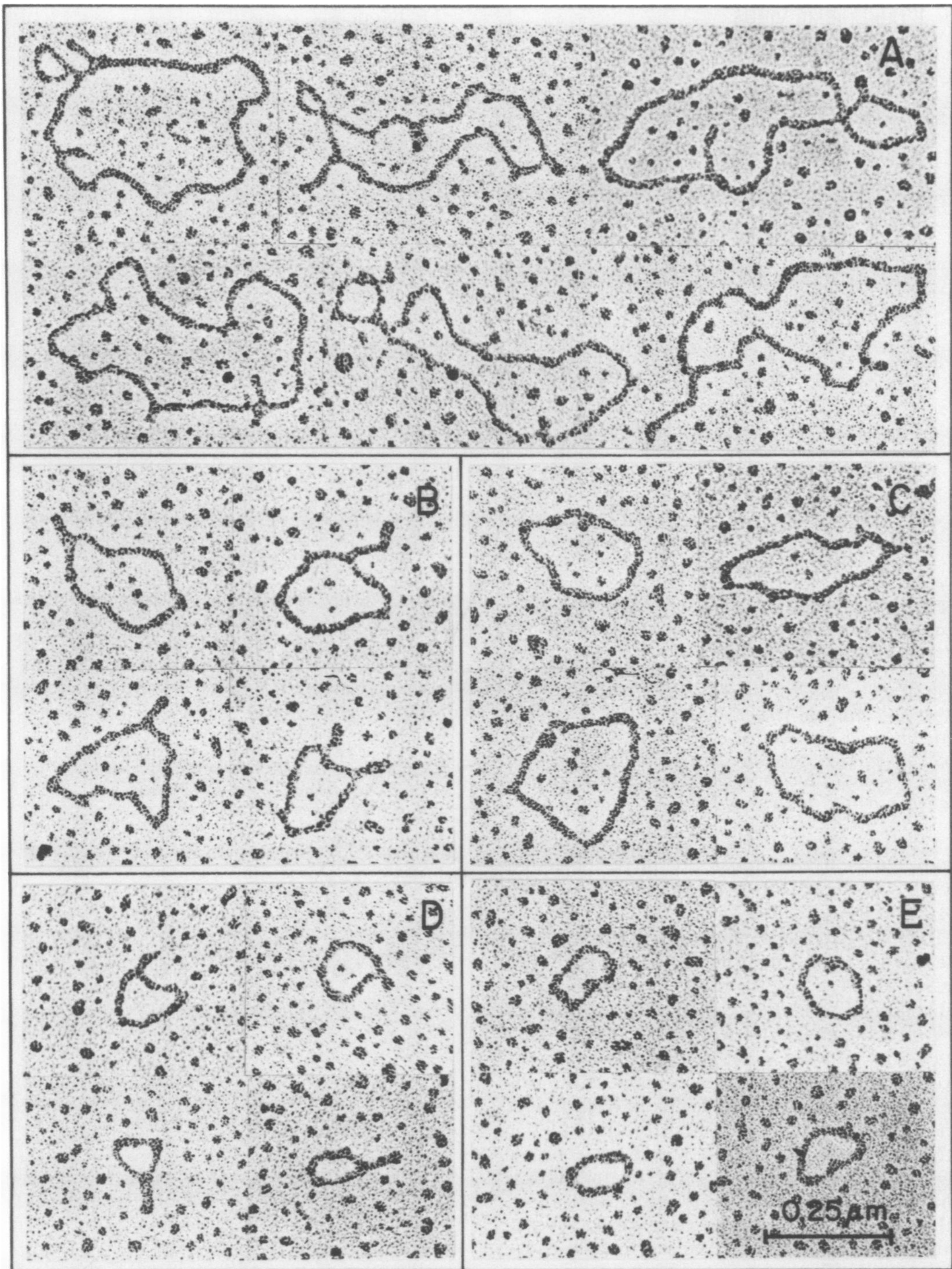


FIG. 3. *Electron micrographs of selected circular Uukuniemi virus RNA molecules. The RNA was spread from 70% FA onto a 40% hypophase. Shown are L (A), M (B), and S (D) RNA molecules with distinct panhandles. Many of the M and S molecules were also seen without any apparent panhandles (C and E).*

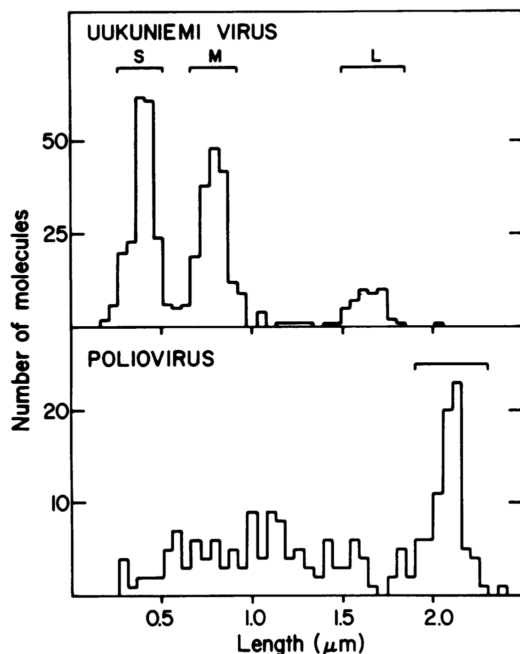


FIG. 4. *Length distribution of Uukuniemi virus and poliovirus RNA molecules spread from 50% FA after treatment with 0.5 M glyoxal at 37°C for 15 min. A total of 441 Uukuniemi virus RNA and 206 poliovirus RNA molecules were measured. In this histogram and the one shown in Fig. 5 all molecules, which were well extended and which did not cross each other, were measured in each field.* ⊢⊣, *Size range of each RNA class selected for determination of the mean length (shown in Table 1).*

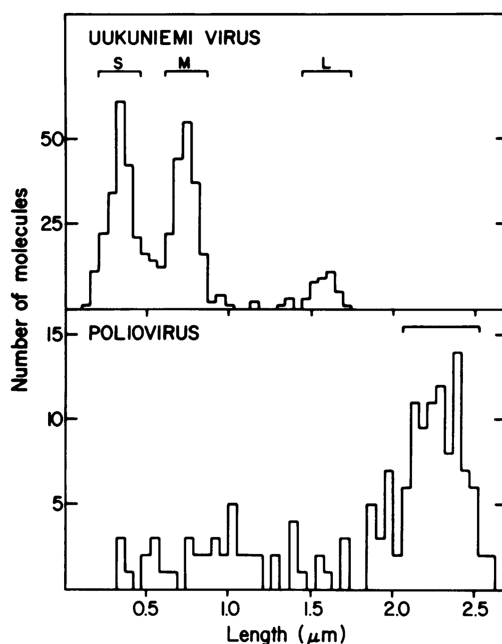


FIG. 5. *Length distribution of Uukuniemi virus and poliovirus RNA molecules spread by the urea-FA method. A total of 458 Uukuniemi virus RNA and 153 poliovirus RNA molecules were measured.* ⊢⊣, *Size range of each RNA class selected for determination of the mean length (shown in Table 1).*

niemi virus RNAs appear to have molecular weights of about $1.9 \times 10^6$ (L), $0.9 \times 10^6$ (M), $0.4 \times 10^6$ (S).

To be able to study more accurately whether the circular and linear molecules in each size class differed in length, we measured 434 molecules spread from 70% FA. In this experiment 40% circular molecules were observed. A histogram (not shown) again showed that both the circular and linear molecules gave three distinct peaks and that both forms in each length class had the same mean length (Table 2).

**Nature of circles.** As shown above, increasing the concentration of FA or use of glyoxal converted all circular forms into linear molecules, suggesting that the circles were not covalently closed. To further characterize the cause of the circularity, we treated Uukuniemi virus RNA with pancreatic RNase, DNase, and proteinase K. Both linear and circular molecules were resistant to DNase treatment, but were completely destroyed by RNase (data not shown). About 40% of the molecules remained circular after 45 min of proteinase K digestion (Fig. 6A) as compared with 50 to 60% circles for untreated molecules spread under the same conditions. This treatment is sufficient to solubilize more than 95% of a preparation of radioactively labeled HeLa cell protein (M. J. Hew-

TABLE 1. *Lengths and molecular weights of Uukuniemi virus RNA species*

| Method and RNA | No. of molecules measured | No. of molecules in peak | Length (μm; mean ± SD[a]) | Mol wt × $10^6$ ± SD[a,b] |
|---|---|---|---|---|
| Urea-FA | | | | |
| L | 458 | 40 | 1.59 ± 0.06 | 1.76 ± 0.07 |
| M | | 179 | 0.74 ± 0.06 | 0.82 ± 0.07 |
| S | | 188 | 0.34 ± 0.07 | 0.34 ± 0.07 |
| Polio | 153 | 84 | 2.27 ± 0.10 | |
| Glyoxal | | | | |
| L | 441 | 41 | 1.65 ± 0.07 | 1.97 ± 0.08 |
| M | | 160 | 0.80 ± 0.05 | 0.95 ± 0.06 |
| S | | 192 | 0.40 ± 0.06 | 0.47 ± 0.07 |
| Polio | 206 | 74 | 2.09 ± 0.07 | |

[a] SD, Standard deviation.
[b] Calculated from the length of the similarly treated and spread poliovirus RNA standard and assuming it has a molecular weight of $2.5 \times 10^6$ (5).

lett and V. Ambros, unpublished data). Thus, the circularity of Uukuniemi virus RNA does not appear to depend on either a DNA or a protein linker.

To determine whether a linear Uukuniemi virus RNA can reform circles, the RNA was completely denatured by incubating a sample at 60°C for 15 min in 99% FA. As shown above, this treatment yields only linear molecules.

TABLE 2. *Comparison of the lengths of circular and linear forms of L, M, and S RNA species*

| Form of molecule | No. of molecules measured | Mean length (μm) ± SD[a] | No. of molecules in peak |
|---|---|---|---|
| Circular | 164 | | |
| L | | 1.56 ± 0.09 | 4 |
| M | | 0.73 ± 0.06 | 123 |
| S | | 0.39 ± 0.05 | 44 |
| Linear | 270 | | |
| L | | 1.55 ± 0.08 | 38 |
| M | | 0.67 ± 0.07 | 65 |
| S | | 0.36 ± 0.07 | 106 |

[a] SD, Standard deviation.

The RNA was then allowed to self-anneal in 50% FA, 0.4 M NaCl, and 0.5% SDS at 37°C for 3 h. After precipitation by ethanol, the RNA was spread from 60% FA. About 40% of the molecules were scored as circles (Fig. 6B), indicating that the circles can indeed be reformed.

## DISCUSSION

It is apparent that all three size classes of virion RNA of Uukuniemi virus can exist in a circular form and that these circles are due to noncovalent interactions, which are most likely base pairing between inverted complementary sequences at the ends of the molecules. The following evidence supports these conclusions. (i) Circular forms of Uukuniemi virus RNA are observed at FA concentrations of between 50 and 85%. At these concentrations no circles were seen with poliovirus single-stranded RNA, which is only 25% longer than the longest Uukuniemi RNA. This rules out the possibility of the circles being artifacts of the method. (ii) More denaturing conditions (urea-FA, treatment with glyoxal) convert most of the molecules to linear forms, whereas heating at 60°C

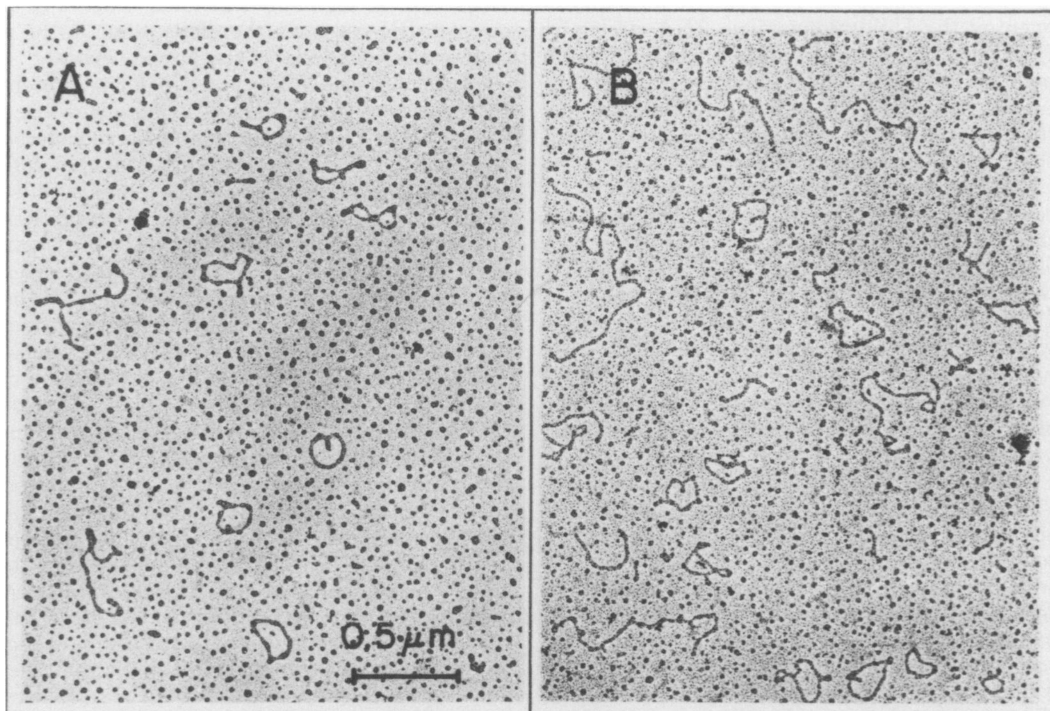


FIG. 6. *Electron micrographs of proteinase K-treated and self-annealed Uukuniemi virus RNA. The RNA was treated with proteinase K as described in the text and spread from 60% FA (A). Another portion was completely denatured in 99% FA at 60°C for 15 min. After raising the salt concentration to 0.4 M NaCl and lowering the FA concentration to 50%, the RNA was allowed to self-anneal at 37°C for 3 h. The molecules were then spread from 60% FA. Many of the circles have reformed (B).*

in 99% FA gives only linear molecules. (iii) Conversion to linear forms is probably not due to nicking and degradation of the RNAs since length measurements showed that both poliovirus and Uukuniemi virus RNAs remain intact during the urea-FA and glyoxal treatments. (iv) Proteinase K and DNase treatment did not affect the stability of the circular forms, making protein or DNA linkers unlikely. (v) Molecules that had been completely denatured were able to reform circles after reannealing. In addition to this, we have also recently found that a 5′-terminal pppAp. . . . occurs on each species of Uukuniemi virus RNA (Pettersson, Baltimore, Hewlett, and Coffin, submitted for publication), confirming that the observed circles are not covalently closed.

The RNA of Sindbis virus, a member of the togaviruses, has also been reported to form circles (6). A comparison of Sindbis virus and Uukuniemi virus RNA circles suggests a difference in the stability of the closure. Sindbis virion RNA is completely converted to linear molecules by about 55% FA – a much lower concentration than is required to open Uukuniemi virus RNA – but is less efficiently opened up by glyoxal treatment (6). The relative difficulty of opening the Uukuniemi virus RNA circles by FA as compared with Sindbis virus RNA could be due to either: (i) the shorter length of Uukuniemi virus RNAs relative to Sindbis virus RNA, (ii) a longer base-paired region joining the ends of Uukuniemi virus RNAs together, or (iii) a higher guanosine plus cytosine (G + C) content of the base-paired region. The fact that the panhandles in Sindbis virus RNA are clearly visible in electron micrographs suggests that they are actually longer than those of Uukuniemi virus RNA circles. That glyoxal, which is thought to selectively block G + C hydrogen bonding (3, 6), opens Uukuniemi virus RNA so easily suggests that a high content of G + C pairs may stabilize the Uukuniemi virus RNA circles.

Many of the circular molecules of Uukuniemi virus RNA are seen to have distinct panhandles of variable length. It is possible that they represent the base-paired regions at the 3′ and 5′ ends of the molecules. However, we could also see circular molecules with no apparent panhandles and linear molecules with regions of secondary structure. In addition, the technique would not resolve a secondary structure feature of less than 100 base pairs. We cannot, therefore, at the present time specify whether the panhandles represent the site of circularization or merely regions of internal base pairing.

The ratio of L, M, and S RNA molecules shown in the histograms (Fig. 4 and 5) is different from that observed when virion RNAs are fractionated on polyacrylamide gels or sucrose gradients (10; data not shown). Thus, in the histogram the number of M and, in particular, L molecules is reduced relative to that of the S molecules. This is probably due to the fact that many of the longer molecules were not measurable because they crossed each other or were frequently cut off at an edge of a field. It is also possible that some of the longer molecules may have been nicked to yield shorter molecules.

The molecular weights determined for the Uukuniemi virus RNA species here are significantly lower than previous estimates (10). Earlier estimates were not, however, done under denaturing conditions, and the sedimentation and electrophoretic behavior of circular single-stranded RNA molecules may be anomalous. We therefore believe that our present length values are more dependable.

Three species of circular RNA have recently been found also in Lumbo virus (19), another bunyavirus belonging to the California encephalitis group (15). In addition, circular ribonucleoproteins have been observed in La Crosse virus (8), a virus serologically related to Lumbo virus (15). A segmented genome consisting of circular RNA species therefore seems to be a characteristic feature of the bunyaviruses.

Since both the RNA and ribonucleoprotein (12) species of Uukuniemi virus have now been shown to be circular, it should be considered whether the circular forms might have a specific role in the life cycle of this virus. Because Sindbis virus RNA (6) and the RNAs from Sendai virus defective-interfering particles (7) can also form circles, the occurrence of inverted complementary sequences at the ends of the RNA molecules may represent a common feature of certain viral single-stranded genomes. The replication of RNA viruses, irrespective of whether a positive- or negative-strand virus (1), must involve the synthesis of a complementary RNA, which then serves as template for the synthesis of new virion RNAs. An inverted complementary sequence at the ends of an RNA molecule implies that RNA of the opposite polarity will also have the same structure at its ends. Thus, the 3′ end of both the virion RNA and the complementary RNA would have the same primary sequence, which could be a recognition site for a replicase. If this is the case, only one species of replicase would be necessary to copy both the positive and negative strands. It is, however, also possible that a double-stranded panhandle could be required for the replicase to correctly initiate. If so, the circular forms could be essential for replication of the RNA.

## ACKNOWLEDGMENTS

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