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# Isolation of the Ends of La Crosse Virus Small RNA as a Double-Stranded Structure

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The genome of La Crosse virus, a member of the Bunyaviridae, is made up of three molecules. Circular nucleocapsid structures, in three size classes, have been isolated from La Crosse virus (Obijeski et al. J. Virol. **20**:664–675, 1976). Recently, Obijeski et al. (Nucleic Acids Res. **8**:2431–2438) have found that the 5′ and 3′ ends of each segment are complementary in sequence. We determined that a 5′ and 3′ end complementary structure, predicted by the rules of Tinoco et al. (Nature [London] **230**:362–367), can and will anneal under certain conditions. This structure is resistant to RNase in high-salt medium and can be isolated in a reasonably high yield.

Much information has been acquired by using electron microscope techniques to determine the structures of viruses. In the cases of La Crosse virus (LAC) and Uukuniemi virus, members of the *Bunyavirus* and *Uukuniemi* genera, respectively (1), all three classes of the helical nucleocapsids of these viruses have been shown by Obijeski et al. (12) and Petterson and von Bonsdanff (16), respectively, to be circular structures. Using electron microscopic techniques, Hewlett et al. (7) have demonstrated that the three size classes of genome RNAs of Uukuniemi virus are also circular under mildly denaturing conditions but are linearized after incubation with 99% formamide at 60°C. In addition, the circular molecules are destroyed by treatment with RNase A but are unaffected by DNase or proteinase K treatment. Since Obijeski et al. (12) had previously shown that LAC RNAs contain free 5′ and 3′ ends, Hewlett et al. concluded that the Uukuniemi virus genome RNAs are linear molecules which are probably maintained in a circular form by base pairing between complementary sequences at the 3′ and 5′ ends of the RNAs. Circular nucleocapsid structures have also been isolated from detergent-treated preparations of Lumbo virus (a member of the Bunyaviridae) (17) and arenaviruses (15).

More recently, Obijeski et al. (14) and Clerx-van Haaster and Bishop (3) have determined the nucleotide sequences at the ends of the LAC genome RNAs by the use of end-labeling techniques. These authors found that the ends of the genome RNAs are highly conserved and that the 5′ and 3′ ends of each genome segment are complementary in sequence. The most stable structure as predicted by the rules of Tinoco et al. (18) from the sequence data on the ends of the small genome segment is shown in Fig. 1. This structure is 27-mer containing 26 base pairs, with only one G · U pair, one mismatch (A · A), and one looped-out base (A) from the 3′ end. By the rules of Tinoco et al. this structure has a $\Delta G$ of approximately −32 kcal/mol (ca. −134 kJ/mol) and can clearly account for the circular genome RNAs seen by Hewlett et al. (7). We were interested in determining whether this structure would also be sufficiently stable to RNase digestion in high-salt medium to allow its isolation as a double-stranded molecule in a reasonable yield. This isolation appeared to be feasible since a structure of similar stability ($\Delta G$ = −33 kcal [ca. −138 kJ]/mol), a perfectly matched 20-base-pair long-stem vesicular stomatitis virus (New Jersey serotype), can be recovered after RNase digestion (8).

To carry out this experiment, the LAC small RNA was isolated from purified virus and radiolabeled at its 3′ end. This end-labeled RNA was first self-annealed and then digested with RNase A in high-salt medium as described in the legend to Fig. 2. After digestion, the RNase itself was digested with proteinase K, and the sample was electrophoresed on a nondenaturing gel. A single strong band which migrated at approximately 30 base pairs relative to DNA restriction fragments and represented 60 to 70% of the starting radioactivity was routinely obtained (Fig. 2). To further characterize this material, the RNA from this band was recovered, digested with increas-

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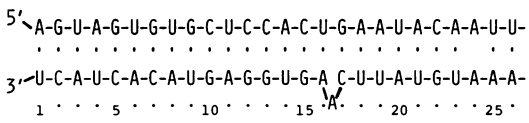


FIG. 1. Predicted structure at the ends of the LAC small RNA. This structure, as predicted by the rules of Tinoco et al. (18), is estimated to have a $\Delta G$ of $-32$ kcal (ca. $-134$ kJ)/mol.

ing concentrations of RNase T1, and then electrophoresed on a 12.5% sequencing gel. The result (Fig. 3) clearly shows the G's expected at positions 10, 12, 13, 15, and 23 from the 3′ end of the LAC small RNA. The length of the untreated

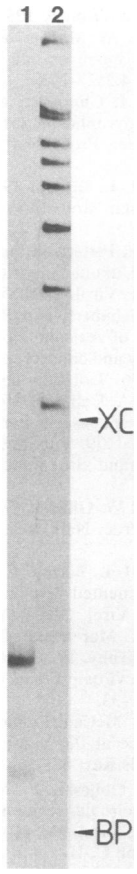


FIG. 2. Isolation of the stem RNA of the LAC small genome. Confluent roller-bottle monolayer cultures of BHK-21 cells were infected with LAC, and the virus was purified as previously described (13). Nucleocapsids were isolated from detergent-treated virus on 6-ml CsCl gradients (20 to 40%) by spinning for 16 h in a Beckman SW41 rotor at 12°C as previously described (9). The nucleocapsid RNA, which was radiolabeled with [$^3$H]uridine, was isolated by phenol extraction and centrifuged immediately on a 5 to 23% (wt/vol) linear sucrose gradient for 90 min at 59,000 rpm in a Beckman SW60 rotor at 12°C. Fractions were collected, and samples were counted for radioactivity to locate the position of the small RNA (12S). LAC minus-strand small RNA was then radiolabeled at its 3′-OH end by the addition of cytidine 3′,5′-[5′-$^{32}$P]biphosphate as described previously (5) and as modified by Leppert et al. (10). The $^{32}$P-labeled, single-stranded ligated genome was annealed in the presence of 20 μg of carrier tRNA. Before being annealed, the total mixture was denatured with 50% dimethyl sulfoxide and heat (60°C, 2 min) and ethanol precipitated. The annealing was performed in a sealed Eppendorf at 65°C for 30 min in a total volume of 30 μl of 2.5 × buffer A (1× buffer A is 0.375 M NaCl, 24 mM Tris, 2.5 mM EDTA [pH 7.4]) containing 0.1% sodium dodecyl sulfate. After annealing, the volume was raised to 100 μl of 2.5× buffer A, pancreatic RNase was added to 20 μg/ml, and the reaction mixtures were incubated for 20 min at 25°C. These reactions were adjusted to contain 0.2% sodium dodecyl sulfate and 500 μg of proteinase K per ml and then further incubated for 20 min at 25°C. After ethanol precipitation, the remaining RNAs were dissolved in 10 μl of TNE buffer containing 12.5% glycerol and dyes and electrophoresed on a 12.5% nondenaturing polyacrylamide gel at 5 mA overnight (lane 1). Lane 2, *Hin*F digests of pBR322. XC, Xylene cyanol; BP, bromophenol blue.

end-labeled strand was measured as 27 nucleotides with reference to a ladder of the starting material. Also note that the stem RNA as isolated (Fig. 3, lane 1) showed no evidence of hidden breaks as expected from the structure shown in Fig. 1, since the mismatched and looped-out bases in this structure are all A's and are not cut by RNase A under these conditions. These results therefore demonstrate that the most stable double-stranded structure predicted by the rules of Tinoco et al. does indeed exist and is sufficiently stable to RNase A digestion in high-salt medium to be recovered in a high yield.

More recently, Bishop et al. (2) have reported the entire nucleotide sequence for the small RNA of snowshoe hare virus (SSH), another member of *Bunyavirus* genus. The nucleotide sequences at the ends of this RNA are remarkably similar to those of the LAC small RNA, such that almost identical structures for the ends of the SSH small RNA can be drawn. The only differences between these two structures are that the single looped-out base at position 17 from the 3′ end is a G in SSH rather than the A in LAC and that the A · U base pair at position 24 in LAC is missing from SSH. Bishop et al. (2) have proposed two possible structures for the ends of SSH small RNA. In light of our results, their second structure (Fig. 3; see reference 2) is by far the most likely.

A strong body of evidence now exists to demonstrate that the ends of *Bunyavirus* genome RNAs can and do self-anneal to form circular

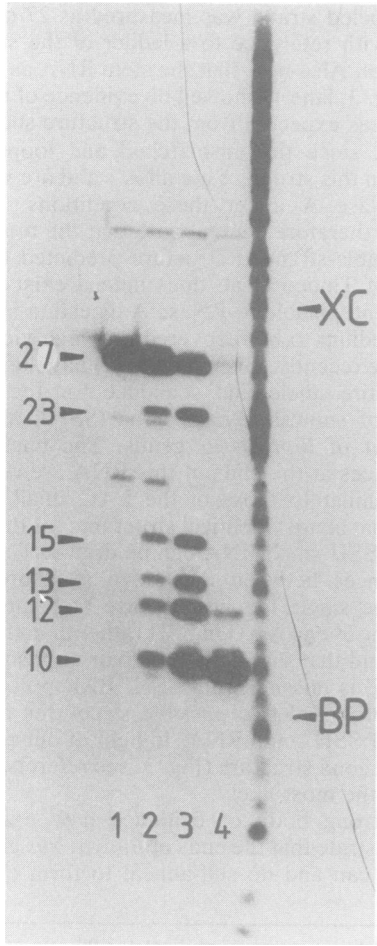


FIG. 3. RNase T1 digestion of stem RNA. The stem RNA (Fig. 2, lane 1) was extracted from the polyacrylamide gel (11), and samples of the extracted band were digested with 0.0001 (lane 2), 0.001 (lane 3), or 0.01 (lane 4) U of RNase T1 per ml in 20 mM sodium citrate (pH 5.0)–7 M urea–1 mM EDTA–0.25 mg of tRNA per ml containing dyes for 10 min at 50°C (3). After digestion, the RNA was placed on a 7 M urea–12.5% polyacrylamide gel and electrophoresed at 23 mA. Lane 1, undigested band; lane 5, ladder formed by digestion on small RNA ligate in 50 mM sodium carbonate at 50°C for 90 min. XC, Xylene cyanol; BP, bromophenol blue. Numbers represent positions from the 3′ end of the LAC small DNA.

molecules. However, it is not yet clear whether the base pairing between the ends of the genome RNAs is in fact also responsible for the circularity of the genome nucleocapsids. There is little information to date on whether the ends of the genome RNA within these nucleocapsid structures are in fact free to associate by base pairing. Further experiments to answer this question are now in progress.

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