Multipartite Nature of Potato Virus X

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Potato virus X (PVX) was among the first viruses to be purified. Nonetheless, properties of the purified virus remain contentious. The literature has been heavily influenced by the concept of a virus as a monopartite entity. Despite the fact that electron micrographs invariably show large proportions of shorter virus particles, the latter are universally ignored. Seven distinct classes of particle lengths were detected. Seven RNA species of approximate sizes 6.4, 3.6, 3.0, 2.1, 1.8, 1.4, and 0.9 kb were extracted from these purified virus preparations. This study shows clearly that shorter PVX particles are not breakage products and indicates that they may reflect fundamental properties of the genome strategy. Furthermore, other potexviruses have been found to contain many shorter particles, and the level of these particles is stable during purification. PVX is generally believed to consist of particles of single length even though the literature does not confirm this conclusion. The notion of a single particle length appears to reflect historical concepts of what a virus should be rather than what PVX is. This report considers whether shorter rods present in virus preparations of PVX are distinctive products of infection. The problem addressed is significant because it affects conclusions concerning the mechanisms of PVX biosynthesis and replication.

Potato virus X (PVX), the type member of the potexviruses, is a single, flexuous rod 515 nm in length (7). The viral genome consists of a single-stranded, plus-sense RNA of 6.4 kb that is capped at the 5' terminus and polyadenylated at the 3' terminus (27, 37). During infection, PVX synthesizes subgenomic RNAs to express internal cistrons, resulting in six subgenomic RNAs in extracts from PVX-infected leaf tissue (13). Only four internal open reading frames are predicted within PVX genomic RNA (19). Recently, the PVX particle description was extended to include another rod length population containing the coat protein mRNA (18).

Early studies to determine the size and shape of PVX involved physicochemical techniques such as viscosity, X-ray crystallography, X-ray diffraction, ultracentrifugation, fluorescence, and/or diffusion (2–5, 8, 22–24, 33, 40, 42). These methods involve chemical or photometric detection which gives weight average distributions emphasizing larger particles. The polydisperse nature of PVX virus solutions was noted in these studies and in electron microscope studies but was not investigated (3, 10, 21, 25, 41).

The smaller particles in a heterogeneous population could be due to preparative breakage products of the 515-nm rod, defective interfering (DI) particles, and/or satellites, characteristic products of a particular virus strain, or natural products of the infection process. Since the number of virus particles that we detected coincided with the number of viral RNAs reported in extracts from PVX-infected tobacco plants in earlier literature, experiments were designed to determine the origin of the shorter rods.

Results. Drops of purified virus preparations (22) were examined under an electron microscope. The preparation was heterogeneous with respect to rod length. In addition to the full-length particle, there were a substantial proportion of particles shorter than 515 nm (12). Six classes predominated: 290, 240, 170, 140, 110, and 75 nm (Fig. 1). The frequency rod length distribution is shown in Fig. 2A.

Two experiments addressed the possibility of particle shearing during purification (38). Rod length frequency distributions of a standard purified virus preparation were compared with those of the original unpurified extract. It is

FIG. 1. Electron microscopic examination of a quick dip of partially purified American Type Culture Collection potato virus X particles at a magnification of $\times 5,000$.

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FIG. 2. Rod length frequency distributions of PVX observed after purification by the procedure described in the text (A) and the original extract after grinding infected leaf tissue with a mortar and pestle (B).

FIG. 3. Frequency distributions of rod lengths from PVX virus strains C (A), G (B), and HB (C).

apparent that some of the variable rod lengths were the result of purification (Fig. 2). However, there was a common clustering of particle sizes in both.

Experiments were then done to determine the extent of rod length variation resulting from purification. Full-length virus particles were separated from the smaller particles by sucrose gradient fractionation and repurified. The population of shorter rods increased 5 to 7%, while the majority of full-length particles remained intact. However, the rod lengths did not correspond in size to the six predominant shorter rods.

Particle shearing could also result from specimen preparation for electron microscopy. Specimen staining and drying on a filmed grid, which are prerequisites for electron microscopic examination, can shrink and break rod-like virus particles (35). Four percent of rods showed hairline breaks between adjoining rods that together equal 515 nm. These breaks probably arose from sample preparation. In particle measurements, rods displaying breaks of this kind were measured as 515 nm.

To determine whether the different particle sizes were unique to this American Type Culture Collection PVX isolate, crude extracts and partially purified preparations of three other PVX strains, C, G, and HB, were examined. Each strain exhibited non-Gaussian rod length distributions, indicating that this is a general feature of PVX (Fig. 3).

The presence of shorter rods in virus preparations could also indicate an impure preparation. Shorter rods attached specifically to grids coated with PVX antiserum (11), indicating that they were not contaminants.

Three alternative explanations for the presence of shorter particles include DI particles, satellites, and encapsidated subgenomic RNAs. Two strains of PVX, Robert's and V349, were obtained from laboratories that had reported that PVX was a single-length rod (32, 36). These strains served as controls. Each isolate was clonally purified on the local lesion host *Gomphrena globosa* and then inoculated onto a systemic host. Isolated virus exhibited seven discrete particle lengths, suggesting that the six shorter rods are natural products of infection (i.e., encapsidated subgenomic RNAs) excluding satellites and/or DI particles.

To determine whether rod length variation was a feature unique to PVX, other potexviruses, including papaya mosaic virus, narcissus mosaic virus, white clover mosaic virus, and cassava common mosaic virus, were examined. In each case, multiple particle sizes were detected (Fig. 4).

Until now, a single RNA species, 6.4 kb, has been detected in purified virus preparations. In my hands, smaller

FIG. 4. Rod length frequency distributions of narcissus mosaic virus (A), papaya mosaic virus (B), white clover mosaic virus (C), and cassava common mosaic virus (D).

RNA species were detected from purified virions; however, these RNAs were smeared. Modification of a virus purification method developed for a potyvirus (14) allowed reproducible isolation of intact virus particles and viral RNAs. An RNA preparation was analyzed in an agarose gel (Fig. 5). Brome mosaic virus RNAs were loaded in lane 1 as molecular weight markers. Healthy tobacco leaf tissue was processed identically to PVX-infected tobacco leaf tissue as a control (lane 2). Seven discrete PVX RNA species, 6.4, 3.6, 3.0, 2.1, 1.8, 1.4, and 0.9 kb, were detected in lane 3.

To locate the six smaller RNA species within the genomic RNA, ^{32}P -labeled DNA fragments extending from nucleotides 29 to 733 at the 5' end and from nucleotides 3452 to 6302 at the 3' end were incubated with Northern (RNA) blots of PVX viral RNAs from mock-inoculated and PVX-infected tobacco leaf tissue. One major RNA species, PVX genomic RNA, was detected with the 5' probe. With the 3' probe, however, six smaller RNA species were detected in addition to PVX genomic RNA (Fig. 6).

Discussion. Shorter rods are universally present in unpurified infected leaf extracts and standard purified PVX virus preparations. Repurification of full-length particles suggests that only 8 to 10% of shorter rods result from preparative methods. Furthermore, the shorter rods are not distinctive products of a particular strain, satellites, or DI particles. The experiments performed establish the origin of shorter rods as natural products of PVX infection.

Journal articles from 1946 (39) and throughout the 1960s (3, 10, 21, 25, 40, 41) contain electron micrographs of PVX which show at least four different particle sizes, but the authors appear to have regarded them as fragmentation artifacts. I measured the rod lengths of PVX particles in electron micrographs published by Levin (25) and Venekamp and Mosch (41). Clusters occur at 515, 298, 245, 177, 145, 115, and 70 nm in ratios of 7:5:6:3:7:2:5 and 3:5:3:3:10:9:2, respectively. The lengths of these particles are in close agreement with the data presented in this study. The implication of these orderly patterns is that PVX is multipartite.

In PVX-infected tobacco leaf tissue, six subgenomic RNAs were found in total RNA preparations (13). If subgenomic RNAs as well as the full-length genomic RNA direct protein expression as recent experiments suggest, then there could be six corresponding particle sizes. In this investigation, electron micrographs show six predominant rod lengths, which is consistent with the number of RNAs reported. However, the presence of seven viral RNAs is inconsistent with the genomic organization of PVX RNA, which is reported to contain five open reading frames (19). The discrepancy of two additional virus particle sizes, and the two RNAs that they are presumed to contain, might also mean that more than five proteins are encoded in the virus genome. With the extraction of seven RNAs from purified virus preparations, I propose that PVX synthesizes and

FIG. 5. Analysis of PVX viral RNAs isolated from a purified virus preparation. Lanes: 1, brome mosaic virus RNAs (1.0 μ g); 2, healthy control (7.0 μ g); 3, PVX RNAs (7.0 μ g). Preparation of purified PVX included a previously described procedure (14), with several modifications. Fresh deribbed leaf tissue was ground up in extraction buffer in a 1:10 mass of tissue/buffer volume ratio. After low-speed centrifugation, 0.1% Triton X-100, 4% polyethylene glycol, and 0.1 M sodium chloride were added to the supernatant. After stirring for 1 h, the virus was recovered by centrifugation at 8,000 rpm for 10 min.

encapsidates subgenomic RNAs not necessary for infection. This is not a novel phenomenon, as the cowpea strain of tobacco mosaic virus (TMV) is such an example (28).

This report of the multipartite length distribution of PVX and the possible role of the particles in replication parallels the study of TMV replication. Viruses have been historically viewed as monopartite, which impeded recognition of divided genomes. For years, the significance of multiple TMV particle sizes present in purified virus preparations was debated. Initially, many concluded that TMV had a uniform length of 300 nm (17, 26, 35). In this same era, PVX was described as a flexible rod of single length (1, 20, 34).

By convention, virus identification reflects properties of a class of particles responsible for infectivity. Since the

shorter rods of TMV and PVX were not infectious, many researchers regarded them as purification artifacts. Years later, TMV strains were shown to contain particles with subgenomic RNAs (6, 9, 15, 16, 29, 30, 31, 38). However, the smaller particles of PVX were never examined.

This is the first report of the detection of six distinct nucleoprotein components in association with the full-length PVX particle, suggesting that the particle morphology description of PVX may be incomplete. In addition, this is the first report of the extraction of six discrete, reproducible RNA species of approximate sizes 3.6, 3.0, 2.1, 1.8, 1.4, and 0.9 kb along with genomic RNA (6.4 kb) from purified virus preparations. The data presented suggest that the shorter rods are natural products of infection. The presence of six RNAs in addition to genomic RNA and their correlation in number and size with RNAs reported as subgenomic RNAs in earlier literature suggest that these products are also subgenomic. In addition, Northern blot hybridization results demonstrate that the genetic information at the 3' terminus of the genomic RNA is duplicated in the six smaller RNA species whereas the information at the 5' terminus is not. These observations suggest that the origin site of assembly exists within the coat protein gene. Two additional open reading frames corresponding to the 3.6- and 3.0-kb RNAs have yet to be located.

Although resolution and characterization of the shorter particle components and their RNAs will be a formidable task, this report indicates that such efforts will be rewarding.

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FIG. 6. Northern blot analysis of PVX RNAs from purified virus preparations. Shown is an autoradiogram prepared after electrophoresis under denaturing conditions. Hybridization was carried out by using 32 P-labeled randomly primed cDNA of the 3' end of PVX genomic RNA containing nucleotides 3452 to 6302 (A) and the 5' end of PVX genomic RNA containing nucleotides 29 to 733 (B). Lanes: a, PVX RNAs isolated from virions; a₁, healthy RNA sample isolated from mock-inoculated tobacco leaf tissue; c in panel A and b in panel B, the 3' cDNA fragment of PVX genomic RNA used as a probe in panel A; b in panel A and c in panel B, the 5' cDNA fragment of PVX genomic RNA used as a probe in panel B.

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