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### Authors
Kuznetsov, Yu G
McPherson, Alexander



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# Atomic force microscopy investigation of Turnip Yellow Mosaic Virus capsid disruption and RNA extrusion

Yu. G. Kuznetsov, Alexander McPherson *

*University of California, Irvine, Department of Molecular Biology and Biochemistry, Room 560, Steinhaus Hall Irvine, CA 92697-3900, USA*

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**Abstract**

Turnip Yellow Mosaic Virus (TYMV) was subjected to a variety of procedures which disrupted the protein capsids and produced exposure of the ssRNA genome. The results of the treatments were visualized by atomic force microscopy (AFM). Both in situ and ex situ freeze–thawing produced RNA emission, though at low efficiency. The RNA lost from such particles was evident, in some cases in the process of exiting the virions. More severe disruption of TYMV and extrusion of intact RNA onto the substrate were produced by drying the virus and rehydrating with neutral buffer. Similar products were also obtained by heating TYMV to 70–75 °C and by exposure to alkaline pH. Experiments showed the nucleic acid to have an elaborate secondary structure distributed linearly along its length.


*Keywords:* Decapsidation; Structure; Nucleic acid; Crystals; Conformation

## Introduction

Turnip Yellow Mosaic Virus (TYMV) is among the most thoroughly studied of all of the small, spherical plant viruses (Hirth and Givord, 1988; Markham and Smith, 1949; Matthews, 1991). It is a $T = 3$ icosahedral virus composed of 180 identical protein subunits of Mr = 20,133 (Peter et al., 1972), which assume three slightly different conformations in the capsid. The monopartite genome is composed of 6318 nucleotides (Morch et al., 1988). A subgenomic fragment of 694 nucleotides occasionally accompanies it. TYMV exhibits a number of unusual features that have been the objects of much analysis and an equal amount of speculation. For examples, its genome has a cytidine content of nearly 40% (Bouley et al., 1975; Morch et al., 1988), the capsid is maintained chiefly by strong protein–protein interactions (Bouley et al., 1975; Jonard et al., 1976; Kaper, 1975), it contains a high concentration of polyamines (Beer and Kosuge, 1970), and its structure is unique due to the protrusion of its prominent capsomeres (Canady et al., 1996; Finch and Klug, 1966; Mellema and Amos, 1972).

Recently, a comparative analysis using X-ray diffraction of crystals of native particles and crystals of empty capsids of TYMV, which appear naturally or can be produced artificially by several means (Givord et al., 1972; Kaper and Siberg, 1969a, b; Keeling and Matthews, 1982; Matthews, 1960; Michels et al., 1999), revealed a good deal concerning the organization of the nucleic acid within the virion. The study showed, in particular, two noteworthy features: loops of single-stranded RNA that invest the pentameric and hexameric capsomeres and segments of RNA double helices organized about icosahedral twofold and fivefold axes. In both cases, the RNA structural elements are at least approximately consistent with the icosahedral symmetry of the capsid (Larson et al., 2005).

Previous studies using X-ray crystallography (Larson et al., 2005) suggest that the structure of the RNA inside the virus conforms to a linear series of clusters of double helical stems and loops joined by short linking segments. This kind of arrangement is also consistent with the structures of single-stranded RNA genomes extracted from a number of icosahedral plant and animal viruses as visualized by AFM (Kuznetsov et al., 2005). In the AFM studies, the RNA structures were also interpreted as linear sequences of secondary structural elements, like that proposed for TYMV based on its X-ray analyses, and

* Corresponding author.
*E-mail address:* amcphers@uci.edu (A. McPherson).

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structure prediction from the nucleotide sequence (Hellendoorn et al., 1996).

In this report, we describe AFM studies on the extraction of RNA from TYMV, the appearance of the disrupted particles, and that of the RNA released into the medium. The RNA is seen to be consistent in structure with motifs discussed above, and the manner in which the extruded RNA is elaborated also gives some insight into its packaging within the virion.

## Results

As seen in Fig. 1a, capsomeres on the surfaces of native 30 nm diameter icosahedral particles are evident, and the overall appearance is consistent with expectations from electron microscopy (Adrian et al., 1992; Finch and Klug, 1966; Mellema and Amos, 1972), previous AFM of crystal surfaces (Malkin et al., 1999), and X-ray diffraction analysis (Canady et al., 1996). Particles having one or more capsomeres absent, as we commonly found in old virus samples, are easily distinguished from native virions, as illustrated in Fig. 1b.

Virions lacking capsomeres are almost certainly lacking any RNA core as well. These are called "artificial top component" or ATC, though empty capsids like these have also been known to occur naturally and with high frequency for more than 45 years (Matthews, 1960). Previous studies have shown that encapsidated RNA may escape the interior through the holes left by departed capsomeres (Adrian et al., 1992; Katouzian-Safadi and Berthet-Colominas, 1983; Keeling and Matthews, 1982). Indeed, evidence we provide below further demonstrates this to be true. It is interesting that the crystals, which diffract to high resolution, contain both types of particles, which indicates that the exterior surfaces and overall dimensions are sufficiently close that both may form the same lattice interactions.

We examined many particles lacking capsomeres in trying to determine whether the missing capsomeres were consistently hexamers, pentamers, or a mixture of the two. Although we could not resolve the protein subunits making up capsomeres, we could determine the type of a missing capsomere by the number of capsomeres surrounding the vacancy. In no case could we find only five capsomeres surrounding an absence, it was consistently six. Thus, we conclude that, for those cases where only a single capsomere was lost in allowing expulsion of the RNA, it was almost certainly a hexameric capsomere.

The results from all of the experiments designed to disrupt TYMV particles are summarized in Table 1. Experiments using urea to disrupt TYMV (Kaper, 1975) were successful, but yielded poor AFM images, in large part due to the high concentrations of urea present. Even with extensive washing, urea produced drying artifacts that obscured details. The results of urea treatment were very similar otherwise to what we obtained with heating (see below). The capsids were essentially destroyed by urea treatment, leaving RNA behind.

The most straightforward way to release the RNA from the virions was to simply dry the particles on the AFM substrate and then rehydrate them with water or neutral buffer. When this procedure was applied to particles adhering to mica, the capsids appeared to disgorge the RNA, but the nucleic acid was left in place as a still somewhat condensed mass of thick threads, what we refer to as RNA spiders. Examples are shown in Figs. 2a–c. The RNA is, clearly, no longer in its encapsidated conformation but is transitioning into a more extended state. The ease with which this occurs suggests that the tertiary interactions between various elements within the RNA are weak. Also produced by this procedure were vast numbers of capsid which were missing capsomeres, again similar to ATC. These are shown in Fig. 2d. This entire process of RNA emission from still largely intact capsids further indicates that protein–nucleic acid interactions in TYMV must be rather weak. This has, however, long been understood from earlier biochemical and biophysical work (Kaper, 1975, Mitra and Kaesberg, 1965).

Freeze–thawing has been shown to produce loss of six to ten protein subunits from TYMV virions, presumably one or two capsomeres, with subsequent release of RNA to the exterior (Katouzian-Safadi and Berthet-Colominas, 1983). This was, in fact, the procedure used to prepare ATC for crystallization and

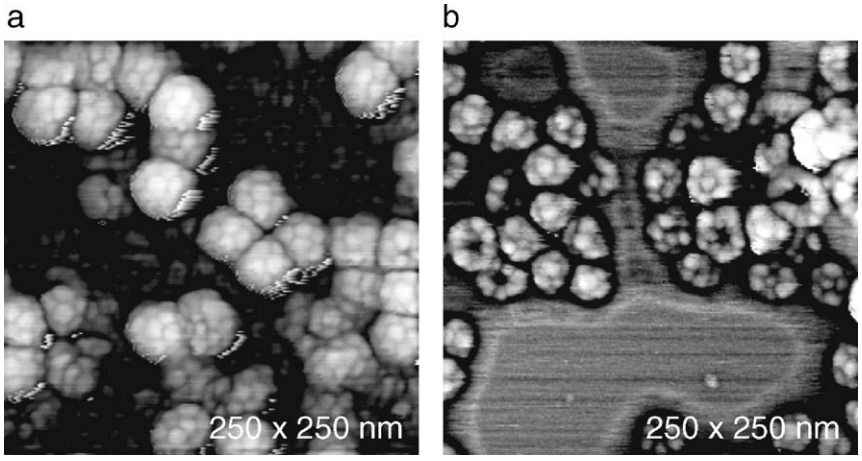


Fig. 1. In panel a is an AFM image of freshly prepared and crystallized TYMV particles. These are fully intact and display capsomeric units on their surfaces consistent with the known $T = 3$ icosahedral symmetry. In panel b is a corresponding AFM image of particles recovered from old crystals. Many exhibit capsid damage, and a large number, though largely intact, are missing one or two capsomeres. These are undoubtedly lacking RNA as well.

Table 1

| Procedure | RNA release summary | Figure | References |
|---|---|---|---|
| Urea | Capsids completely destroyed, RNA emission similar to heating, urea produced drying artifacts obscuring AFM observation | – | (Jonard, 1972), (Kaper and Steere, 1959) |
| Drying–Rehydrating | Capsids partially intact, RNA in place as condensed masses of thick threads containing secondary structural domains transitioning to extended states | 2 | – |
| Freeze–Thaw | Missing capsomeres from capsids are apparent, RNA surrounds capsids as spider-like arrangements of thick threads containing secondary structural domains | 3 | (Kaper and Siberg, 1969a,b), (Adrian et al., 1992), (Katouzian-Safadi and Berthet-Colominas, 1983) |
| Elevated temperature | Capsids in various states of disruption, RNA free and present on substrate, maintaining much of its secondary structure, entire genomes appear as intact masses | 3 | (Lyttleton and Matthews, 1958), (Kaper and Steere, 1959), (Michels et al., 1999), (Hitchborn, 1968), (Mutombo et al., 1993) |
| Elevated temperature in the presence of EDTA | Capsids fragmented into two or several sections connected by thick cords of RNA, suggesting maintenance of secondary and some tertiary structure | 4 | |
| Elevated pH 8.5–9.5 | Extrusion similar to heating, with capsids in various states of disruption, free RNA in abundance that maintains much of its secondary structure | 5 | (Kaper, 1964), (Kaper, 1971a,b), (Kaper, 1975), (Kaper and Helperin, 1965) |
| Elevated pH 9.5–11.0 | Almost all capsids completely destroyed, RNA appears as thin extended threads with loss of secondary structure, degradation of RNA beginning | 5 | (Bosch et al., 1967), (Pley et al., 1969; Pley, 1973), (Keeling and Matthews, 1982), (Halperin and Kaper, 1967; Pleij et al., 1977) |

X-ray diffraction analysis (Larson et al., 2005; van Roon et al., 2004). Exposure of TYMV to freeze–thawing (Kaper and Siberg, 1969a, 1969b) indeed yielded images of excluded RNA, but also those of virions in the process of losing their nucleic acid. This is illustrated in Fig. 3. It is clear from height measurements that the particles in Figs. 3a and b, for example, have retained the dimensions of intact particles, even while their nucleic acid complement is extruded on the substrate surface around them.

Heating was also shown to be an effective means for disrupting the virions, though exposure to elevated temperatures tended to damage capsids severely. A series of temperatures and exposure times were explored, and eventually it was found that heating the virus, in buffer, to 70–75 °C for 1 min was just sufficient. Even then, some virions remained intact, while others completely lost integrity. This was a common observation throughout most of our experiments. Some particles are evidently more robust than their companions and withstand a treatment, whatever its nature, with more resistance than others. There are strong particles and weak particles. The heating procedure, though destroying most capsids, was useful because it tended to drive the free RNA into a more extended conformation and thereby display the sequential array of secondary structural features. This is illustrated by the examples shown in Figs. 3c and d. It should be noted that not only are long, extended strands of RNA present, but short fragments of RNA as well. It may be that these short fragments were produced only after extrusion due to the high temperature, but it is at least equally likely that many virions contain fragmented, cleaved nucleic acid molecules.

Unique images of TYMV particles losing their RNA were obtained when TYMV was heated to 70 °C in the presence of high concentrations of EDTA. In Fig. 4 are examples. The unusual feature here is that the capsid breaks up into large pieces, not simply losing a capsomere or two, but the pieces remain joined by the thick cord of nucleic acid. The RNA is somewhat extended but maintains its secondary structure. The suggestion here is that there may be some point or points within the capsid that establish interactions with the RNA that are sufficient to anchor the two ends. The image here emphasizes the linear arrangement of the secondary structural elements of the RNA along a linear course.

The appearance assumed by RNA molecules following separation from their capsid depends to some extent on the nature of the AFM substrate employed in the experiment. Generally, poly-L-lysine-treated mica was used. Figs. 5a and b, however, represent heating experiments in which the RNA was released on to a mica substrate treated with nickel chloride. Comparison of Figs. 5a and b with c shows that on a nickel chloride-treated substrate the RNA remains more condensed. Presumably, this is due to the influence of the divalent cations of the substrate on the conformation of the RNA.

The final approach to affecting the release of TYMV RNA from the virions was the use of elevated pH (Halperin and Kaper, 1967), and these experiments are illustrated by the AFM images in Figs. 5d–f. The experiments were carried out both in buffer at modest ionic strength and in the presence of 1 M KCl and 3 M KCl. Essentially, no discernable difference was observed in the results whether the salt concentration was high or not. Thus, the results discussed here are those obtained in buffer. Exposure to pH ranging from 8.5 to 12 was investigated as disruption of particles was initiated at 8.5. In general, exposure times of 1 min were employed, though longer periods were also explored. At pH 8.5, many particles remained intact, while weaker particles broke open, not by losing a capsomere, but more extensively and dramatically. Some relatively intact

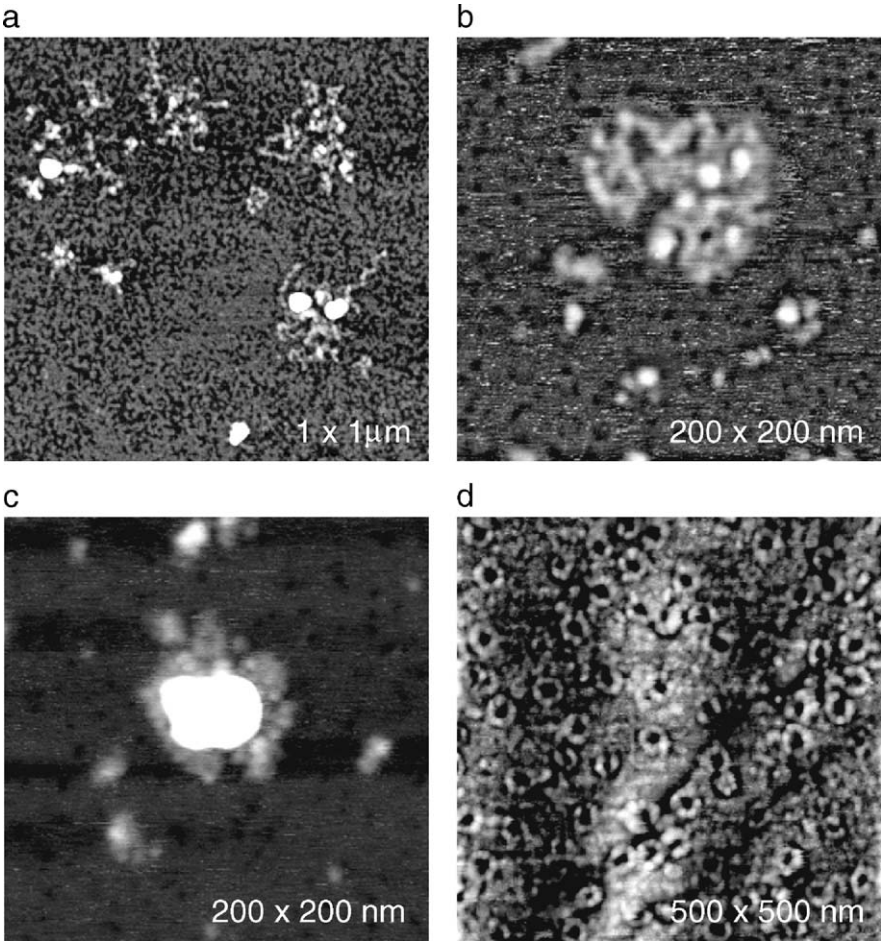


Fig. 2. Samples of TYMV which were dried on the mica substrate and rehydrated by addition of buffer. In panel a, numerous almost fully intact virions can be seen releasing their RNA, which appear as thin, spider-like strands on the substrate. The threads, however, as seen in panel b are considerably thicker than extended strands of RNA and exist in a condensed state indicative of the persistence of secondary structure. In panel c, loss of RNA has just begun, and measurement of the height of the particle releasing the RNA shows it to be consistent with an intact particle. Presumably, it has lost no more than one or two capsomeres. In panel d are particles produced by dehydration–rehydration that have clearly lost only one, or a few capsomeres, as well as their RNA.

particles, nonetheless, remain visible, even as they can be seen to be losing their RNA complement. Examples are seen in Fig. 5d. It should be noted that at high pH the RNA becomes considerably more extended than in previous experiments, indicating that not only have tertiary interactions been disrupted but that secondary structure is likely melting out as well. As Fig. 5d shows, after 7 min at pH 8.5, many intact particles remain even as the extruded RNA from neighbors is transitioning to more extended conformation.

As the pH was raised in 0.5 increments, the number of remaining intact particles decreased, and the number that had burst increased. More extruded RNA was apparent on the substrate. By pH 11, virtually, the only thing seen on the substrate was extended RNA. At pH 12, these molecules exposed lengthy stretches of threadlike, unfolded single-stranded nucleic acid. The latter is clearly unstable and susceptible to degradation. The most prominent feature of the RNA in the experiments involving elevated pH is the relatively extended nature of the RNA and the implied loss of tertiary and secondary structure. This was not so apparent with the other techniques or with RNA extracted from icosahedral viruses using the classical phenol approach (Kuznetsov et al., 2005).

## Discussion

As the images show, AFM can identify particles which are missing one or more capsomeres, so long as the defect is accessible to the cantilever tip. It is also capable of recording more seriously damaged virions. Inspection of populations, probably mixtures of native and naturally occurring empty capsids, or top component, demonstrates that the two can be discriminated. In addition, it shows that there is a significant fraction of the particles lacking nucleic acid and that both can exist in the same crystals with, apparently, equal ease. Given that the generally sensitive interparticle interactions in the crystal are not seriously altered by loss of the RNA, we can conclude that the RNA has little influence, if any at all, on the disposition of amino acid residues on the surface of the capsids or the overall shape of the protein shell.

The images above also show that extrusion or emission of RNA from TYMV can be produced by a number of approaches,

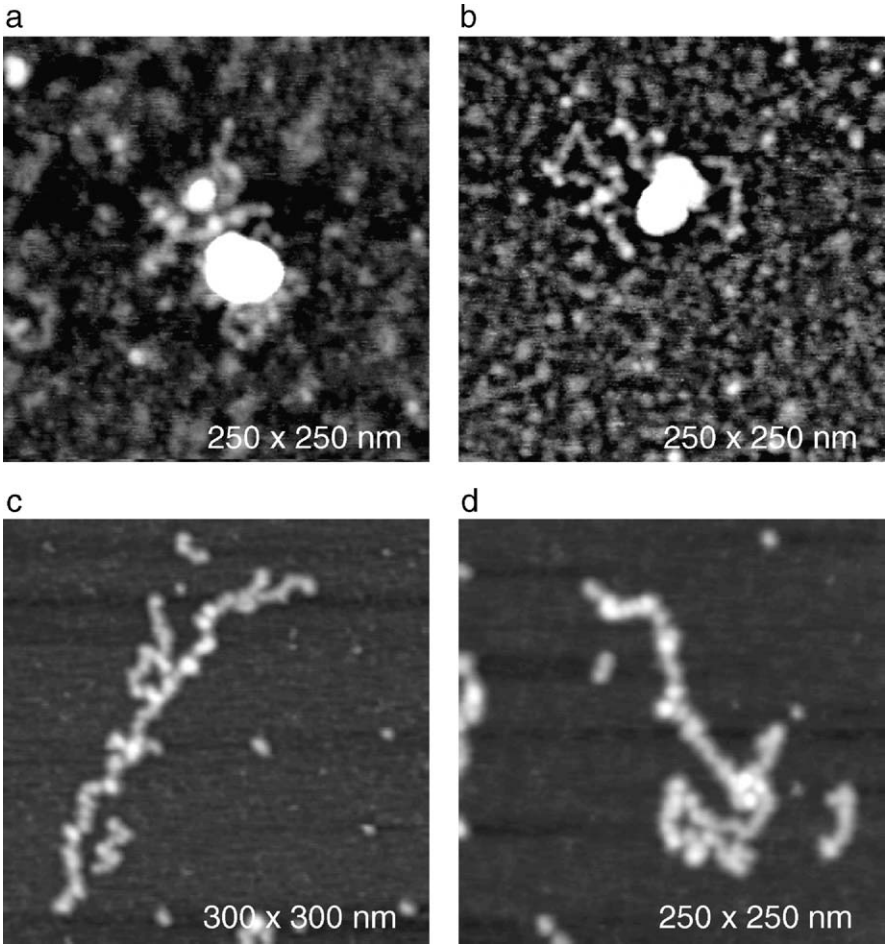


Fig. 3. In panels a and b are two virions which are releasing their RNA as a consequence of freezing and thawing. The appearance of the particles and the liberated RNA closely resemble what was seen for particles subjected to dehydration and rehydration. The heights of the particles are again consistent with intact virions, indicating loss of no more than a few capsomeres. The RNA persists as a condensed, spider-like mass of thick strands beneath the virion. TYMV that have been heated in buffer to 70 °C or greater release their RNA on the poly-L-lysine-coated mica substrate in much the same way as was seen using drying or freezing. These are seen in panels c and d. With heating, tertiary structure of the nucleic acid is more rapidly disrupted following release, the RNA begins to extend and exhibit its linear character. The thickness of the strands, however, indicates that these are extended arrays of secondary structural elements, and the RNA is still highly condensed. The images of the RNA seen here are consistent with those of single-stranded RNA encapsidated by other small icosahedral viruses (Kuznetsov et al., 2005).

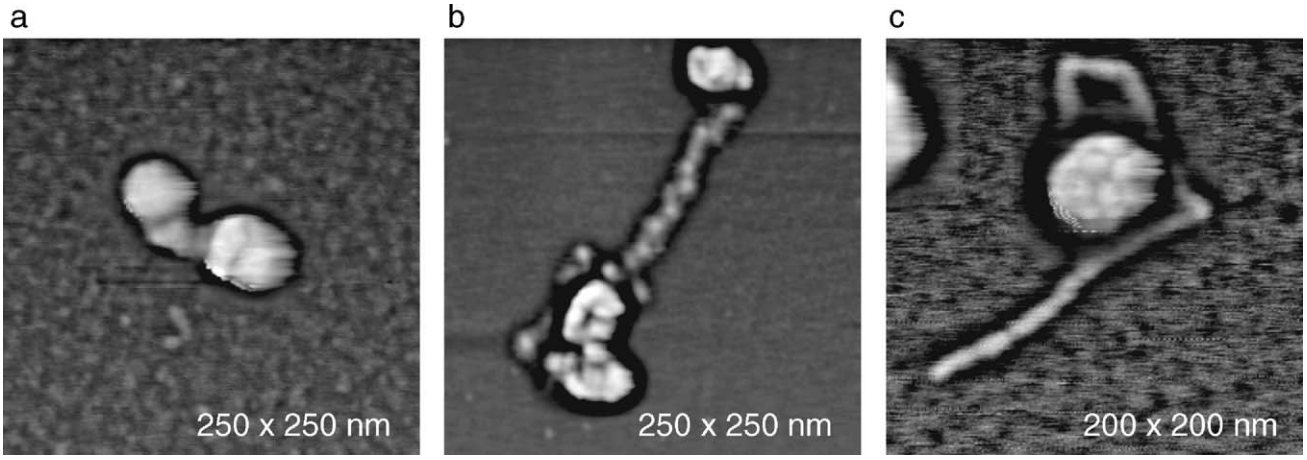


Fig. 4. When TYMV were heated in the presence of elevated concentrations of EDTA, the particles occasionally broke up into large sections held together by thick cords of RNA as shown here in panels a and b. In other instances (c), the particles appeared intact but extruded the nucleic acid as thick cords.

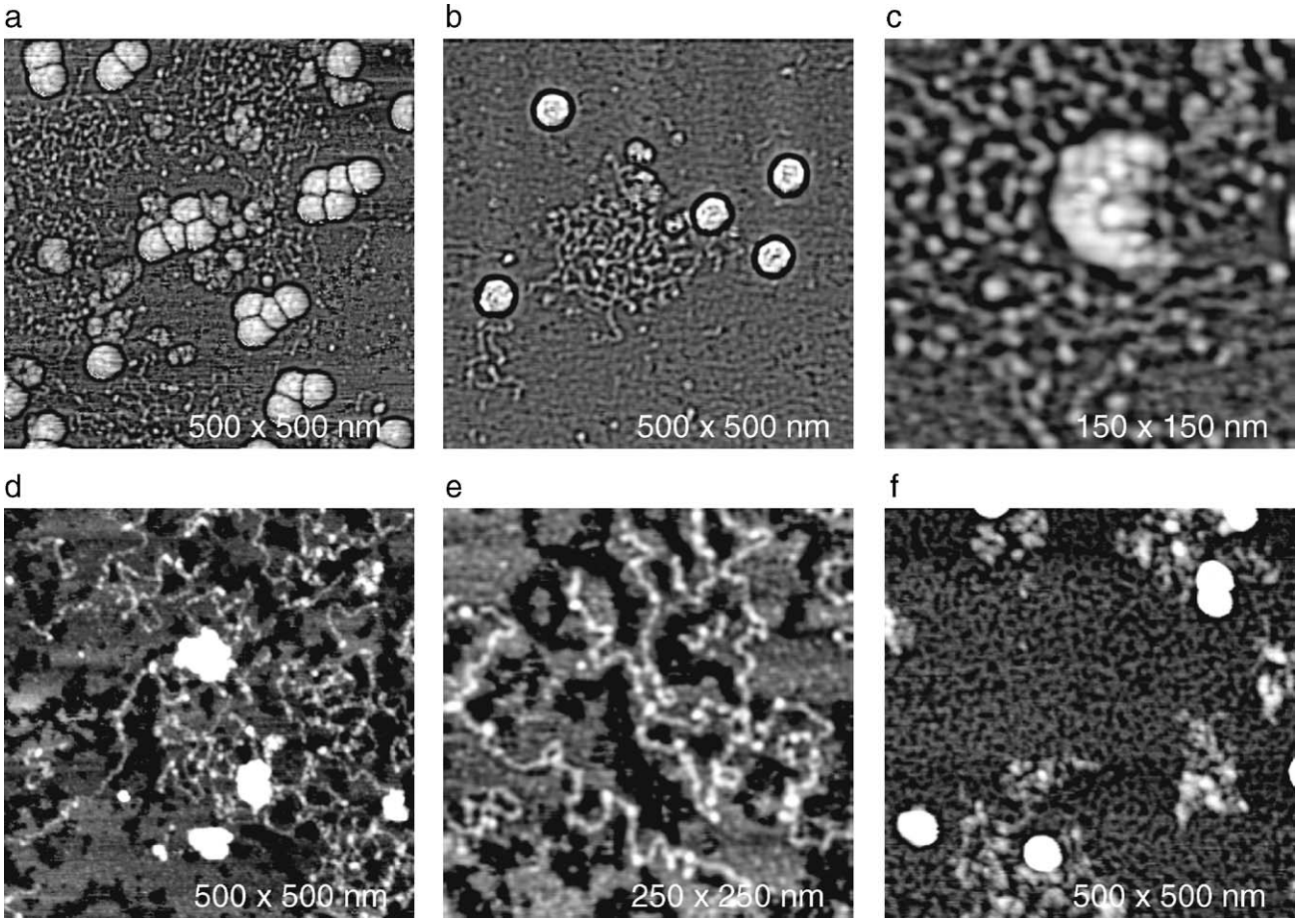


Fig. 5. TYMV particles disrupted by heating to 70–75 °C for 1 min are shown in panels a and b. The substrate here, however, was coated with nickel chloride rather than poly-L-lysine. Because of interactions between the negatively charged nucleic acid and the positively charged ions of the substrate, the RNA remains in a more condensed state after emission from the particles. Note that, even after heating vigorously enough to produce release of RNA, some particles remain on the substrate and exhibit little or no disruption while others, like that in panel c, are severely damaged. TYMV exposed to pH between 8.5 and 12 and spread on poly-L-lysine-treated mica substrates are shown in panels d–f. The unique property of the extruded RNA produced by this treatment is that it more quickly assumes an extended conformation with loss of tertiary structure and some initial loss of secondary structure as well. In panel e, high points along the length of the strands indicate the presence of secondary structural domains. At the highest pH, 12, the strands of RNA from many viruses form chaotic tangles. Even at high pH, however, it can be seen in panel f that some nearly intact virions persist even while in panel d others are totally fragmented.

not a novel observation by any means, and that the extrusion process and its consequences can be visualized and recorded by AFM. In many cases, we were able to record largely intact particles extruding their RNA through portals created by loss of protein capsomeres, where part of the nucleic acid is still within the virion. In addition, it was possible to visualize the extruded,

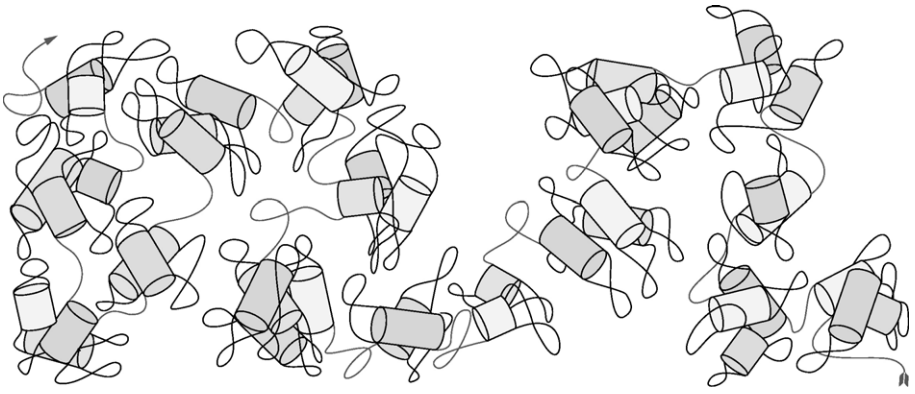

Fig. 6. The AFM observations, as well as X-ray evidence, are most consistent with an RNA molecule that exists as a linear sequence of secondary structural domains, like that illustrated in this drawing. Each domain is a small ensemble of double helical stems and loops. The domains are connected by single-stranded linking regions.

extraviral RNA displayed on the substrate and some of its conformational properties.

Drying followed by rehydration, elevated temperature, freeze–thawing, and high concentrations of EDTA, all produce loss of RNA with varying degrees of disruption to the protein shell. One minute of exposure to 70° to 75 °C produces observable ATC, but extensive damage to some less robust virions is also evident. Damage increases with lengthened exposure times or higher temperatures. With elevated temperature, some virions simply fracture and disintegrate, even in the presence of other particles which remain intact.

Heating in the presence of EDTA was unique in that ATC seemed not to be produced, but the virions broke into large sections. The sections, dispersed on the substrate, were joined by thick cords of nucleic acid. The implication of these experiments was that the RNA could maintain cohesive contact with the capsid, even though split into pieces. These images also showed that the RNA, upon loss of the tertiary interactions that maintain the encapsidated conformation, could be extended as a single, linear, though still condensed molecule.

AFM observations on TYMV that were frozen and then thawed were consistent with previous reports and descriptions of the process. Capsids were indeed observed with capsomeres missing and RNA extruded onto the substrate. The results were similar to those obtained with dehydration and with heating. We did, however, find that some virions underwent more severe disruption and exhibited broken shells. We could not determine if the end of the RNA which exited the virion first was still attached to a capsomere or not. The RNA, which was displayed on the substrate, appeared the same in general conformation as when expelled from the virus using heat or dehydration.

The most reliable and specific means for inducing the extrusion of RNA was by exposure of the TYMV to elevated pH. At moderately alkaline pH, 8.5, most virions suffered little disruption, but some lost capsomeres, extruded their RNA, and became empty. At higher pH, the proportion disgorging RNA increased, and more particles suffering extensive disruption were observed. At high pH, 10 to 11, even brief exposure resulted in almost completely degraded virions and complete emission and display of the RNA on the substrate. Even at high pH, however, some particularly robust virions persisted.

The RNA, which had been expelled from the virions, arranged itself on the substrate as linear sequences of topographically varied elements that, we presume, indicates a series of stem loop substructures along the molecule. This seems the only explanation for the lengths of the extruded RNA chains which are only about one tenth or less of the length of a fully extended TYMV RNA molecule, which is composed of 6318 nucleotides. Observations of TYMV RNA, and its degree of condensation, are consistent with what was observed for poliovirus RNA, satellite tobacco mosaic virus RNA, and previous AFM analyses of TYMV RNA (Kuznetsov et al., 2005). They are further consistent with the conclusions drawn from X-ray diffraction comparison of crystals of native, intact particles and crystals of empty capsids, or ATC (Larson et al., 2005), as well as structure predictions from nucleotide sequence (Hellendoorn et al., 1996). In those investigations, it was also concluded that the RNA was arranged inside the virus as a linear sequence of stem loop secondary structural elements, like that shown schematically in Fig. 6.

It should be noted that the degree of condensation of the RNA observed in the experiments was to some extent a function of the substrate on which it was displayed. Generally, we employed mica coated with poly-L-lysine. In those experiments, the RNA tended to be more extended and to exhibit fewer tertiary and secondary interactions. In some experiments, however, we used nickel-chloride-treated mica as the substrate, and in those cases, the RNA appeared more condensed. This, we presume to be a consequence of the interaction of the divalent nickel ions with the negatively charged phosphate groups of the nucleic acid.

Throughout all of our experiments with TYMV, we consistently observed that, under certain conditions of pH, heat, ionic conditions, etc., some fractions of the TYMV particles were disrupted or degraded, while others exhibited only minor damage, or none at all. Thus, we conclude that not all virus particles are identical but differ in individual character and stability. This is somewhat difficult to explain since the capsids are quite uniform and, one would expect, experience the same interactions between protein subunits. It is possible that differences in stability derive from different conformations of the RNA inside the virions, the dynamics of the nucleic acid, and the influence it exerts on the overall stability of the virions.

A major question that remains is what drives the extrusion of the RNA from the virus particle, in many cases through a relatively narrow opening provided by the loss of one or two capsomeres? Here, we can only speculate. The protein capsid is quite rigid. We know this from AFM and X-ray diffraction results. There is, as far as we know, no active mechanism mediated by the protein to "pump" the nucleic acid from the inside to the outside. Hence, we conclude that the RNA must force itself from the opened capsid by virtue of a pressure that it creates itself. This is not an original hypothesis as others, based on a variety of experimental results, have proposed it as well (Adrian et al., 1992; Kaper, 1975; Keeling and Matthews, 1982; Mutombo et al., 1993). The obvious mechanism for this is electrostatic repulsion between negatively charged phosphate groups as a result of some charge imbalance.

The curious thing is that, in forcing itself through the capsomere lesion, the RNA always seems to exit end first and be extruded in a linear manner. We cannot say if the first end out is the 3′ or the 5′ end, but it seems always to be an end. Hairpins are not observed. The linear end to end emission of the RNA would seem to suggest that the RNA might be attached, perhaps firmly, but not covalently, to some one susceptible capsomere. It is this capsomere that breaks free of the capsid and leads the RNA to freedom. This idea is consistent as well with the experiments with EDTA. There, the capsid broke into segments, but the RNA appeared to be fixed to some point on a distal fragment of the virus. It may well be then that the RNA does enjoy at least some unique, particularly strong interaction between one of its two termini and a chosen capsomere in the

virus capsid. Perhaps it is because of the association that one particular capsomere becomes susceptible to displacement.

The observation that the RNA always exits the capsid "end first" is also consistent with a co-translational disassembly mechanism for plant virus RNA release. Previous investigations have provided evidence that the 5′ end of the RNA is released first, allowing attachment of ribosomes with associated "stripping" of the RNA from the particle as it is translated (Roenhorst et al., 1989). Such a mechanism might further explain the removal or unraveling of secondary structural domains from the RNA as it leaves the particle interior.

## Materials and methods

TYMV was prepared from infected Chinese cabbage as described previously and was the same strain used for earlier crystallographic analyses (Canady et al., 1995; Canady et al., 1996). The virus was crystallized overnight by direct addition of ammonium phosphate immediately after purification. The crystals were collected by centrifugation and redissolved for immediate use in experiments. As related below, older crystals tended to develop empty capsids by loss of RNA, either over time or upon dissolution, but newly grown crystals, when redissolved, did not contain significant numbers of empty capsids.

AFM samples were prepared by addition of 5 μl of about 5 mg/ml virus stock solution to the surfaces of cleaved mica with subsequent fixation, in solution, by addition of 1 μl of 25% glutaraldehyde. The mica was pretreated with either poly-L-lysine or nickel chloride to insure adherence of the virus and the nucleic acid. The samples were then scanned either under buffer, or the samples were air-dried, the substrate washed with water, and dried again. TYMV, disrupted virions, and emergent RNA were scanned at 26 °C using oxide-sharpened silicon nitride tips in a 75 μl fluid cell containing buffer at pH 4.0 or in air. For scanning in air, silicon tips were employed. The images were collected in tapping mode (Hansma et al., 1994; Hansma and Hoh, 1994) with an oscillation frequency of 9.2 kHz in fluid and 300 kHz in air, with a scan frequency of 1 Hz. Procedures were fundamentally the same as described for previous investigations of viruses (Kuznetsov et al., 2001, 2002, 2003; Malkin et al., 2004). In the AFM images presented here, height above substrate is indicated by increasingly lighter color. Thus, points very close to the substrate are dark and those well above the substrate white.

For drying and rehydration experiments, virus was applied to lysine-coated mica and washed with buffer. It was then allowed to dry thoroughly for 15 min and then rehydrated by the layering of buffer atop the substrate. Thus, the entire procedure leading to release of RNA was carried out in situ. For freeze–thawing, urea exposure, and experiments involving heating, the virus was treated in bulk in a small Eppendorf tube and a 5 μl aliquot drawn and placed on the substrate and fixed with glutaraldehyde for AFM imaging as described above. Freezing was affected by plunging the samples, in a small test tube or on the AFM substrate itself, into liquid nitrogen for 1 min. Heating experiments utilized a temperature-controlled water bath.

Because the AFM image of any object is the convolution of the object with the shape of the tip, lateral dimensions are distorted from their true values. Vertical dimensions are, however, accurate to less than a nanometer. Thus, any measurements of particle size or nucleic acid thickness used strictly height above background. Because of the short working distance of the AFM at high magnifications, a problem arises in simultaneously visualizing both high objects (such as virions) and objects lying very close to the substrate (RNA molecules). Thus, in some images, where the nucleic acid was the object of interest, the intact virions, or large pieces of virions, may appear entirely white. In some other images, in order to visualize the details of the capsids, any RNA present on the substrate may appear almost invisible or barely indicated.

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